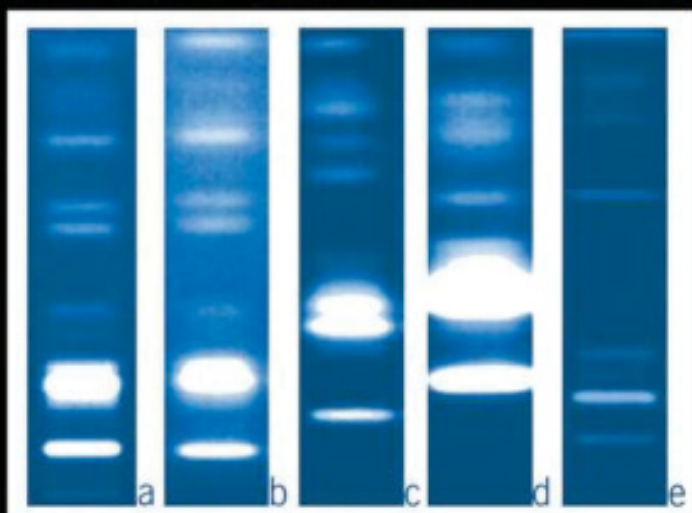


Handbook of Thin-Layer Chromatography

Third Edition, Revised and Expanded



edited by
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Handbook of Thin-Layer Chromatography

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To President Arthur J. Rothkopf and Provost June Schlueter in appreciation of the
continuing support of Lafayette College for our research and publication activities as
emeritus professors

Preface to the Third Edition

Contributing authors in the third edition of the *Handbook of Thin-Layer Chromatography* were asked by the editors to cover new advances in their fields and delete old technologies and obsolete information. The authors expanded chapters when necessary to cover topics adequately. The result is chapters that describe the state-of-the-art of each subject, with updated references.

The same overall organization of the second edition was adopted. Part I contains chapters on the theory, principles, practice, and instrumentation of thin-layer chromatography (TLC). Part II chapters cover applications of TLC to a variety of compound classes. A subject index, an expanded glossary of important terms, and a list of sources of supplies and equipment are included. Within the two parts of the book, some changes in topics have occurred, and some contributors have been replaced.

In Part I, new contributing authors wrote Chapter 3 ("Optimization" by Claudia Cimpoiu), Chapter 4 ("Sorbents and Precoated Layers in Thin-Layer Chromatography" by Fredric M. Rabel), Chapter 5 ("Instrumental Thin-Layer Chromatography" by Eike Reich), and Chapter 12 ("Thin-Layer Radiochromatography" by István Hazai and Imre Klebovich). Automation and robotics were covered in Chapter 14 of the second edition, but a chapter on this topic is not included in this edition because of a lack of sufficient new information.

Part II contains chapters on two new compound classes: hydrocarbons (Chapter 19 by Vicente Cebolla and Luis Membrado) and herbals (Chapter 18 by Eike Reich and Anne Blatter). The following are new authors of chapters in Part II: Irena Choma ("Antibiotics," Chapter 15), Mark D. Maloney ("Carbohydrates," Chapter 16), Fumio Watanabe and Emi Miyamoto ("Hydrophilic Vitamins," Chapter 20), Alina Pyka ("Lipophilic Vitamins," Chapter 23), Marija Kastelan-Macan and Sandra Babić ("Pesticides," Chapter 27), Joseph Sherma ("Steroids," Chapter 30), and W. M. Indrasena ("Toxins [Natural]," Chapter 32). No topics were eliminated from Part II.

Throughout the book, practical aspects are emphasized in order to help those in university, government, industrial, and independent testing laboratories understand the principles of TLC and apply it to their analyses. This book is a useful reference volume for chemists, biochemists, biologists, laboratory technicians, laboratory managers, medical technologists, biotechnologists, forensic scientists, veterinary toxicologists, pharmaceutical analysts, environmental scientists, and attendees of workshops or short courses on TLC. It is also a useful reference for graduate and undergraduate students in chemistry, biochemistry, biology, and related programs, particularly those in quantitative analysis, instrumental analysis, and separation science.

Whenever possible, suggestions by reviewers of the second edition were incorporated in this edition. We would be pleased to receive comments, notification of errors, and suggestions for deletion of topics, new topics, or new authors for the next edition.

Joseph Sherma
Bernard Fried

Preface to the Second Edition

The second edition of the *Handbook of Thin-Layer Chromatography* updates and expands the coverage of the field of TLC and HPTLC in the first edition. The same overall organization of the first edition has been maintained: an initial series of chapters on theory, practice, and instrumentation and a second section of chapters concerned with applications to important compound types. The literature has been updated to as recently as 1995 in most chapters.

A number of changes have occurred in the topics covered, and several of the chapters have been written by new contributing authors: "Optimization" by Qin-Sun Wang (Chapter 3); "Basic Principles of Optical Quantitation in TLC" by Mirko Prosek and Marko Pukl (Chapter 10); "Thin-Layer Radio-chromatography" by Terry Clark and Otto Kelin (Chapter 12); "Natural Pigments" by Øyvind M. Andersen and George W. Francis (Chapter 22); "Pharmaceuticals and Drugs" by Gábor Szepesi and Szabolcs Nyiredy (Chapter 24); "Nucleic Acids and Their Derivatives" by Jacob J. Steinberg, Antonio Cajigas, and Gary W. Oliver, Jr. (Chapter 26); and "Hydrophilic Vitamins" by John C. Linnell (Chapter 30). These changes resulted from either the inability of the original authors to contribute to the second edition or our desire to change the emphasis of coverage of certain topics.

The separate chapter on photographic documentation of thin-layer chromatograms in the first edition (Chapter 9) has been eliminated and the subject is now covered in Chapter 8 ("Detection, Identification, and Documentation" by K.-A. Kovar and Gerda E. Morlock). A new chapter titled "Automation and Robotics in Planar Chromatography" by Eric P. R. Postaire, Pascal Delvordre, and Christian Sarbach (Chapter 14) has been added. A chapter on polymers and oligomers was not included in this edition because of a lack of sufficient new information on this topic.

Suggestions made by reviewers of the first edition have been incorporated into this revision—for example, clear line drawings have replaced photographs in some chapters. As in the past, we welcome comments regarding this edition—notification of errors, suggestions for improvements in the topics covered, new topics, or new authors.

Joseph Sherma
Bernard Fried

Preface to the First Edition

This book has been designed as a practical, comprehensive laboratory handbook on the topic of thin-layer chromatography (TLC). It is divided into two parts, the first of which covers the theories and general practices of TLC (Chapter 1–13), while the second (Chapters 14–31) includes applications based mainly on compound types. The book will be a valuable source of information for scientists with a high degree of expertise in the separation sciences, but because most chapters include considerable introductory and background material, it is also appropriate for the relatively inexperienced chromatographer.

Contributors to the book are recognized experts on the topics they have covered and include many of the best-known and most knowledgeable workers in the field of TLC throughout the world. As much as possible, we attempted to adopt a uniform style for each chapter while still allowing authors the latitude to present their topics in what they considered to be the most effective way. Consequently, in the applications chapters (14–31), most authors have included the following sections: introduction, sample preparation, layers and mobile phases, chromatographic techniques, detection, quantification, and detailed experiments. Authors were encouraged to use many figures and tables and to be as practical as possible except for the chapters devoted to theory (2, 3, and 10). The literature covered by most authors includes mainly the period from 1975 to 1989. Some of the more significant older literature has also been covered, but many authors refer to the earlier comprehensive treatises by Stahl and Kirchner for this material. Authors have been selective in their choice of references and present TLC methods that are most suitable for laboratory work.

It is important to point out that the *Handbook of Thin-Layer Chromatography* has a comprehensive, organized plan and, unlike many recent books in the field, is not a random collection of chapters on “advances” or papers from a symposium. An earlier laboratory handbook on TLC was written by Egon Stahl in 1965. We hope that our handbook may have at least a small fraction of the impact in the near future that this classic work had on the development and growth of TLC during the past 25 years. If the book is well accepted and contributors cooperate, we hope to update coverage of all important aspects of TLC with regular later editions.

Joseph Sherma
Bernard Fried

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1

Basic TLC Techniques, Materials, and Apparatus

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I. INTRODUCTION AND HISTORY

The purpose of this chapter is to present an overview of all important aspects of thin-layer chromatography (TLC). It briefly reviews information and provides updated references on topics covered in the remaining chapters in Part I and refers readers to the specific chapters. It treats topics that are not covered in separate chapters, such as sampling and sample preparation and the more classical procedures of TLC, in more detail. A suggested source of additional information, both basic and advanced, on the practice and applications of TLC is the primer written by Fried and Sherma (1).

A. Introduction to TLC

Thin-layer chromatography and paper chromatography comprise “planar chromatography.” TLC is the simplest of all the widely used chromatographic methods to perform. A suitable closed vessel containing solvent and a coated plate are all that are required to carry out separations and qualitative and semiquantitative analysis. With optimization of techniques and materials and the use of available commercial instruments, highly efficient separations and accurate and precise quantification can be achieved. Planar chromatography can also be used for preparative-scale separations by employing specialized layers, apparatus, and techniques.

Basic TLC is carried out as follows. A small aliquot of sample is placed near one end of the stationary phase, a thin layer of sorbent, to form the initial zone. The sample is then dried. The end of the stationary phase with the initial zone is placed into the mobile phase, usually a mixture of two to four pure solvents, inside a closed chamber. If the layer and mobile phase were chosen correctly, the components of the mixture migrate at different rates during movement of the mobile phase through the stationary phase. This is termed development of the chromatogram. When the mobile phase has moved an appropriate distance, the stationary phase is removed, the mobile phase is rapidly dried,

and the zones are detected in daylight or under ultraviolet (UV) light with or without the application of a suitable visualization reagent.

Differential migration is the result of varying degrees of affinity of the mixture components for the stationary and mobile phases. Various separation mechanisms are involved, the predominant forces depending upon the exact properties of the two phases and the solutes. The interactions involved in determining chromatographic retention and selectivity include hydrogen bonding, electron-pair donor/electron-pair acceptor (charge transfer), ion-ion, ion-dipole, and van der Waals interactions. Among the latter are dipole-dipole (Keesom), dipole-induced dipole (Debye), and instantaneous dipole-induced dipole (London) interactions.

Sample collection, preservation, and purification are problems common to TLC and all other chromatographic methods. For complex samples, the TLC development will usually not completely resolve the analyte from interferences unless a prior purification (cleanup) is carried out. This is most often done by selective extraction and column chromatography. In some cases substances are converted, prior to TLC, to a derivative that is more suitable for separation, detection, and/or quantification than the parent compound. TLC can cope with highly contaminated samples, and the entire chromatogram can be evaluated, reducing the degree of cleanup required and saving time and expense. The presence of strongly adsorbed impurities or even particles is of no concern, because the plate is used only once (2).

Detection is simplest when the compounds of interest are naturally colored or fluorescent or absorb UV light. However, application of a detection reagent by spraying or dipping is required to produce color or fluorescence for most compounds. Absorption of UV light is common for most aromatic and conjugated compounds and some unsaturated compounds. These compounds can be detected simply by inspection under 254 nm UV light on layers impregnated with a fluorescence indicator (fluorescence quench detection).

Compound identification in TLC is based initially on a comparison of R_f values to authentic reference standards. R_f values are generally not exactly reproducible from laboratory to laboratory or even in different runs in the same laboratory, so they should be considered mainly as guides to relative migration distances and sequences. Factors causing R_f values to vary include dimensions and type of chamber, nature and size of the layer, direction of the mobile-phase flow, volume and composition of the mobile phase, equilibration conditions, humidity, and sample preparation methods preceding TLC. See Chapter 11 in Ref. 1 for a discussion of reproducibility in TLC. Confirmation of identification can be obtained by scraping the layer and eluting the analyte followed by infrared (IR) spectrometry, nuclear magnetic resonance (NMR) spectrometry, mass spectrometry (MS), or other spectrometric methods if sufficient compound is available. These methods can also be used to characterize zones directly on the layer (in situ).

B. History of TLC

The history of liquid chromatography, which dates back to the first description of chromatography by Michael Tswett (3) in the early 1900s, was reviewed by Sherma (4). Recent reviews of TLC were written by Ettre and Kalasz (5), Sherma (6), Kreuzig (7), and Berezkin (8). TLC is a relatively new discipline, and chromatography historians

usually date the advent of modern TLC from 1958. A review by Pelick et al. (9) tabulates significant early developments in TLC and provides translations of classical papers by Izmailov and Schraiber and by Stahl.

In 1938, Izmailov and Schraiber separated certain medicinal compounds on unbound alumina or other adsorbents spread on glass plates. Because they applied drops of solvent to the plate containing the sample and sorbent layer, the procedure was termed drop chromatography. Meinhard and Hall in 1949 used binder to adhere alumina to microscope slides, and these layers were used in the separation of certain inorganic ions with the use of drop chromatography; this method was called surface chromatography. In the 1950s, Kirchner and colleagues at the U.S. Department of Agriculture performed TLC as we know it today. They used silica gel held on glass plates with the aid of a binder, and plates were developed with the conventional ascending procedures used in paper chromatography. Kirchner coined the term “chromatostrips” for his layers, which also contained fluorescence indicator for the first time. Stahl introduced the term “thin-layer chromatography” in the late 1950s. His major contributions were the standardization of materials, procedures, and nomenclature and the description of selective solvent systems for resolution of important compound classes. His first laboratory manual (10) popularized TLC, and he obtained the support of commercial companies (Merck, Desaga) in offering standardized materials and apparatus for TLC.

Quantitative TLC was introduced by Kirchner et al. in 1954 when they described an elution method of determination of biphenyl in citrus fruits. Densitometry in TLC was initially reported in the mid-1960s using commercial densitometers such as the Photovolt and Joyce Loeb Chromascan. Plates with uniform, fine-particle layers were produced commercially in the mid-1970s and provided impetus for the improvements in theoretical understanding, practice, and instrumentation that occurred in the late 1970s and 1980s and led to the methods termed high-performance thin-layer chromatography (HPTLC) and instrumental HPTLC. Centrifugally accelerated preparative layer chromatography (PLC) and overpressured layer chromatography (OPLC), which are the major forced-flow planar chromatographic techniques, were introduced in the late 1970s.

These and other high-performance and quantitative methods caused a renaissance in the field of TLC that is reflected in this Handbook. Although the major use of TLC will probably continue to be as a general low-cost and low-technology qualitative and screening method in laboratories worldwide, there is no doubt that TLC will continue to evolve and grow in the new millennium as a highly selective, sensitive, quantitative, rapid, and automated technique for analysis of all varieties of samples and analytes and for preparative separations. To keep abreast of this inevitable progress in TLC, the biennial reviews of advances in theory, practice, and applications by Sherma, the most recent of which was published in 2002 (11), are indispensable.

C. Comparisons of TLC to HPTLC and Column Liquid Chromatography (HPLC)

Detailed comparisons of TLC to other chromatographic methods, especially HPLC, and of TLC to HPTLC are presented in Chapters 1 and 2 of Ref. 1. TLC involves the concurrent processing of multiple samples and standards on an open layer developed by a mobile phase. Development is performed, usually without pressure, in a variety of modes,

including simple one-dimensional, usually in ascending or horizontal mode; multiple; circular (rarely); and multidimensional. Zones are detected statically, with diverse possibilities. Paper chromatography, which was invented by Consden, Gordon, and Martin in 1944, is fundamentally very similar to TLC, differing mainly in the nature of the stationary phase. Paper chromatography has lost favor compared to TLC because the latter is faster and more efficient, allows more versatility in the choice of stationary and mobile phases, and is more suitable for quantitative analysis.

High-performance TLC layers are smaller; contain sorbent with smaller, more uniform particle size; are thinner; and are developed for a shorter distance compared to TLC layers. These factors lead to faster separations, reduced zone diffusion, better separation efficiency, lower detection limits, less solvent consumption, and the ability to apply more samples per plate. However, smaller samples, more exact spotting techniques, and more reproducible development techniques are required to obtain optimal results.

High-performance liquid chromatography involves the elution under pressure of sequential samples in a closed on-line system, with dynamic detection of solutes, usually by UV absorption. The predominant mode of HPLC is reversed phase (RP) on bonded silica columns, whereas for TLC normal phase (NP) on silica gel is most widely used. This makes the two methods complementary for compound separation and identification.

A paper by Sherma (12) offers a detailed review of the relationship of TLC to other chromatographic methods, especially HPLC. TLC is the most versatile and flexible chromatographic method for separation of all types of organic and inorganic molecules that can be dissolved and are not volatile. It is rapid because precoated layers are usually used without preparation. Even though it is not fully automated as is possible for HPLC, TLC has the highest sample throughput because up to 30 individual samples and standards can be applied to a single plate and separated at the same time. The ability to separate samples simultaneously in parallel lanes is important in applications that require high sample throughput, e.g., surveillance programs to detect food containing unacceptable levels of drug residues, to ensure a safe drinking water supply, to control the use of recreational and performance-enhancing drugs, and similar screening applications (13).

Modern computer-controlled scanning instruments and automated sample application and development instruments allow accuracy and precision in quantification that are in many cases equivalent to those obtained with HPLC and gas chromatography (GC). There is a wide choice of layers and developing solvents (acidic, basic, completely aqueous, aqueous-organic). Solvents that can interfere with HPLC UV detection can be used in TLC because the mobile phase is removed from the plate prior to detection. Every sample is separated on a fresh layer, so that problems involved with carryover and cross-contamination of samples and sorbent regeneration procedures are avoided. Mobile-phase consumption is low, minimizing the costs of solvent purchase and disposal. Because layers are normally not reused, sample preparation methods are less demanding, and complex, impure samples can be applied to the layer without concern for the extra (ghost) peaks and noneluting compounds that shorten the life of HPLC columns.

Simultaneous sample cleanup and separation of target compounds are often achieved with TLC (13). The wide choice of development methods and pre- or postchromatographic detection reagents leads to unsurpassed specificity in TLC, and all components in every sample, including irreversibly sorbed substances, can be detected.

There is no need to rely on peaks drawn by a recorder or to worry about sample components possibly remaining uneluted on a column. Because it is an off-line method, the various steps of the procedure are carried out independently. Examples of the advantages of this approach include the ability to apply compatible detection methods in sequence and to scan zones repeatedly with a densitometer using different parameters that are optimum for individual sample components. HPLC can generally provide a higher separation power than TLC, but most HPLC separations do not require high efficiency, so the methods are quite comparable in such applications.

The pyramidal screening approach, in which TLC is used as a screening step followed by HPLC confirmation and quantification of only positive samples, can result in less analytical time and lower cost than when all samples are analyzed by HPLC (13). Abjean (14) showed that 300 meat samples could be analyzed for sulfonamide drugs by a single analyst in 12 days using TLC screening and HPLC analysis of positive samples compared to 50 days for HPLC multiresidue analysis alone. The cost was 80% less, and confirmation of residue identity was more reliable because two independent methods were used. The simultaneous identification of chloramphenicol, nitrofurans, and sulfonamides in pork or beef is an example of TLC multiclass screening (15). The drugs were identified by homogenization and extraction from 1 g of tissue with ethyl acetate, cleanup of the extract on a silica gel solid-phase extraction (SPE) cartridge, and separation by TLC. Spraying with pyridine detected nitrofurans, and subsequently fluorescamine detected chloramphenicol and sulfonamides. Twenty samples could be analyzed per day per analyst for three residue classes by a single method. The determination of antibiotics in milk (16) and of poly cyclic aromatic hydrocarbons (PAHs) in soil (17) are other TLC screening methods that have demonstrated advantages in terms of simplicity, time, and cost compared to HPLC.

D. The Literature on TLC

The literature of TLC has been reviewed biennially by Sherma since 1970 (latest review, Ref. 11). The major journals for papers on TLC are *Journal of Planar Chromatography-Modern TLC*, *Journal of Liquid Chromatography & Related Technologies*, and *Acta Chromatographica*. Other chromatographic journals such as *Chromatographia*, *Journal of Chromatographic Science*, and *Journal of Chromatography, A*, and *B* and general analytical journals such as *Journal of AOAC International*, *Analytical Biochemistry*, *Analytical Chemistry*, and *The Analyst* contain some articles on TLC. The Camag Bibliography Service (CBS) regularly abstracts TLC papers and is available in paper and CD-ROM versions.

Books that have appeared since the publication of the second edition of this Handbook are those by Kaiser et al. (18) (a random collection of chapters on techniques and applications in German), Hahn-Deinstrop (19) (a practical book focused on pharmaceutical analysis), and Fried and Sherma (20) (the only TLC book organized by discipline). Special issues on thin layer chromatography of the *Journal of Liquid Chromatography & Related Technologies*, edited by Sherma and Fried, were published as Issues 1 and 10 of Volume 22/1999 and Issue 10 of Volume 24/ 2001. Book chapters (21, 22) and an encyclopedia article (23) covering TLC, several general review articles (13, 24, 25), and a guide to method development (26) were published within the last

seven years. Cazes' *Encyclopedia of Chromatography* (27) contains 30 articles on methods and applications of TLC. The IUPAC Commission on Analytical Nomenclature published a list of approved terms and definitions for planar Chromatography in 1993 (28).

II. THEORY AND FUNDAMENTALS

The basic parameter used to describe migration in TLC is the R_f value, where

$$R_f = \frac{\text{distance moved by the solute}}{\text{distance moved by mobile phase front}}$$

R_f values vary from 1 to 0, or from 100 to 0 if multiplied by 100 (hR_f).

The capacity factor, k' , is the ratio of the quantities of solute distributed between the mobile and stationary phases, or the ratio of the respective times the substance spends in the two phases,

$$k' = \frac{t_s}{t_m} = \frac{\text{retention time in stationary phase}}{\text{retention time in mobile phase}}$$

The capacity factor and R_f are related by the equation

$$k' = \frac{1 - R_f}{R_f}$$

The classic Van Deemter equation and its modifications have been used to describe zone spreading in GC and HPLC in terms of eddy diffusion, molecular diffusion, and mass transfer. The efficiency of a zone in HPTLC is given by the equation

$$N = 16 \left(\frac{R_f Z_f}{W_b} \right)^2$$

where N is the number of theoretical plates, Z_f is the distance of solvent migration, and W_b is the diameter of the zone (29). In contrast to column chromatography, in which all solutes move the same distance, separated components migrate different distances in TLC, and their zones are broadened to varying degrees. Therefore, N is dependent on the substance migrating as well as on the migration distance, and efficiency must be reported in terms of a compound with a specific R_f value such as 0.5 or 1.0.

Separation efficiency and capacity in TLC were discussed by Poole (13). Efficiency is limited by less than optimal velocity of the mobile phase driven by capillary forces, leading to zone broadening that is largely dominated by molecular diffusion. Mobile-phase velocity decreases approximately quadratically with migration distance, resulting in the migration of zones through regions of varying efficiency and the need to specify plate height for the layer as an average value. For sorbents with narrow particle size range, solvent front velocity is greater for coarse-particle layers than for layers with fine particles (30). It has also been shown that for RP layers with bonded long-chain alkyl groups, mobile phases with larger percentages of water will ascend very slowly, requiring plates to be prepared from particles with a larger diameter (10–13 μm) than those used for

the usual HP layers (5 μm) or from sorbents with a lower degree of surface modification. Polar-bonded sorbents, such as cyano or amino, are wetted by aqueous solvents (30).

Guiochon and coworkers (31–35) showed that for capillary flow TLC on fine-particle (HP) layers, zone broadening is controlled by the size of the sorbent particles for short migration distances and molecular diffusion for long migration distances. For large-particle sorbent layers, the packing and slow mass transfer processes can both contribute to broadened, irregularly shaped zones. High plate numbers can be generated on layers with relatively large particles only with long migration distances, especially for solutes with large diffusion coefficients. HPTLC layers produce the highest efficiency for short migration distances of 5–6 mm, and efficiency eventually is poorer than for TLC as the migration distance increases and molecular diffusion overtakes zone center separation to become the limiting factor. Longer solvent front migration distances require layers with a larger particle size to obtain a reasonable range of mobile-phase velocities and total number of theoretical plates (13, 24). The results of these studies indicate that HPTLC plates can produce more compact zones in a shorter development distance, increasing the speed and detection limits of the zones. About 5000 theoretical plates can be obtained for a 5–7 cm development on HPTLC plates, whereas a development distance of approximately 15 cm is needed to obtain this number of plates for a layer with larger particles (30). The experimental zone capacity for baseline separated peaks in a chromatogram resulting from capillary controlled flow is about 12–14, and this is not strongly dependent on the average particle size of the layer (13). Zone capacity for forced-flow development is 30–40; for capillary controlled flow automated multiple development (AMD), 30–40; and for two-dimensional (2-D) capillary flow, approximately 100.

An equation (36) for resolution (R_s) of two zones in TLC by a single ascending development is

$$R_s = \frac{\sqrt{NR_2}}{4[(k'_1/k'_2) - 1][1 - R_2]}$$

where k'_1 and k'_2 are the capacity factors for the two solutes to be separated and N is the number of theoretical plates. The subscript 2 refers to the zone with the higher R_f value. As in the analogous resolution equation for HPLC, this equation includes terms related to the efficiency of the layer, the selectivity of the TLC system, and the capacity of the system (the zone positions on the layer). Resolution increases with the square root of the layer efficiency (N), which depends linearly on the R_f value. In terms of zone position, studies have shown that maximum resolution is obtained in the R_f range of 0.2–0.5 (30). The most effective means for increasing resolution on a TLC or HPTLC layer with the usual capillary flow, one-dimensional single development is to improve selectivity by variation of the mobile phase, the choice of which is aided by systematic optimization methods such as simplex, PRISMA, and others that have been developed (37) (see Chap. 3). Other approaches for increasing resolution include the use of capillary flow with multiple or two-dimensional development or forced-flow development.

The foregoing discussion applies to capillary flow TLC, in which the migration velocity of the mobile phase through the layer is controlled by capillary forces and decreases as development distance increases (38). The optimum velocity necessary for maximum efficiency is not realized in capillary flow TLC. In forced-flow planar

chromatography, the mobile phase is driven by centrifugal force [rotation planar chromatography (RPC)] or by a pump (OPLC) (see Chap. 7) through a layer enclosed by a polymeric or metal membrane under external pressure. RPC is used mainly for PLC (see Chap. 11), whereas many applications of OPLC for analytical separations have been reported. RPC never reaches an overall mobile-phase velocity that would give the highest separation efficiency, because the radial velocity of solvent migration diminishes from the center to the circumference of the plate (39). In OPLC, mobile-phase velocity can be controlled at a predetermined constant close to optimal value so that solvent front migration is a linear function of time (30). As a result, average plate height is approximately independent of migration distance and is most favorable for HPTLC plates, zone broadening by diffusion is minor even over long migration distances, plate number increases linearly with migration distance, and resolution continues to increase as migration distances increases (30, 38). The time required for the mobile phase to cover the same distance in OPLC is typically five- to tenfold shorter than in TLC, depending on the surface tension, viscosity, and the ability to wet the layer. Separation time is further reduced because the number of theoretical plates needed to achieve a separation is generated in a shorter time because of the near-optimal mobile-phase flow rate (39). Poole (13) showed that for a development distance of 18 cm, forced-flow development can produce 8000 theoretical plates in 9 min. Increased efficiency is obtained by use of longer bed lengths (e.g., serial coupling of stacked, connected layers) over longer times.

Electro-osmotic flow caused by applying an electric field across a wet layer containing both ionized silanol groups and mobile ions is an additional mechanism for moving the mobile phase through the layer. Nurok (39) reported that separation of six pyrimidines on silica gel with acetonitrile mobile phase was 12 times faster than with conventional TLC and that separation in the RP mode is two to three times faster depending on the mobile phase. Only preliminary studies of this approach have been carried out to date, and Poole (13) reports that the mobile-phase velocity declined with migration distance and showed only moderate increase compared to capillary flow, and that the demonstrated improved performance with electro-osmotic flow has been below that predicted by theory.

The classic book by Geiss (40) is recommended as an excellent source of information on the fundamentals of TLC. Although the book is highly theoretical and mathematical, numerous practical summaries and suggestions can be found throughout its chapters to guide anyone working with TLC. Especially useful in better understanding TLC is Chapter 6 in Geiss (40), on the role of the vapor phase. It explains and distinguishes chamber saturation (saturation of the chamber atmosphere), sorptive saturation (preloading of the layer from the atmosphere), and capillary saturation (saturation of the layer through the rising mobile phase) and the results caused by different chamber types and solvent mixtures. It is safe to say that few practitioners of TLC clearly understand these complicated effects that occur during development. The Geiss book also contains a discussion and a decision flow chart for optimization of separations of two closely related substances or a wide polarity range multicomponent mixture with the use of different mobile phases, development approaches, chamber types, and layers.

Readers are directed to Chapter 2 of this Handbook and to Ref. 41 for discussions of the physicochemical theory and mechanism of TLC. Reference 42 covers studies of quantitative structure-retention relationships, one of the more important theoretical fields of TLC.

III. SAMPLING AND SAMPLE PREPARATION

A. Sampling for TLC Analysis

One of the most important steps in analysis is that of obtaining an appropriate sample of the material to be analyzed. If a nonrepresentative sample is taken, the analytical result will be unreliable no matter how excellent the procedure and laboratory work. As an example, the purity of a bottle of 100 analgesic tablets should not be determined by analyzing one tablet, which might be nonrepresentative of the average tablet. A better plan is to grind together 10 tablets to form a homogeneous powder and take a sample weight equivalent to the average weight of one tablet for the analysis. In this way, the composition of the laboratory sample has a much higher probability of accurately representing the average composition of the entire contents of the bottle.

The sample should not change or be lost as a result of storage prior to TLC analysis. The integrity of most samples can be maintained by storage in a freezer. However, with some samples, freezing and thawing or the introduction of the common fixatives formalin or ethanol can affect the results of subsequent analyses (43). The storage container should be airtight to prevent volatilization of the sample or introduction of air, water, or other vapors. The container should be constructed from a material chosen such that impurities are not leached into the sample from the inside surface and analyte cannot be lost by adsorption on the inside surface. Plastic is a common choice for storage of samples to be analyzed for metals, and glass for samples with organic analytes.

A detailed discussion of sampling procedures for different types of gas, liquid, solid, and bulk samples is beyond the scope of this chapter. Chapter 4 in Ref. 1 contains information on obtaining and storing human, warm- and cold-blooded animal, microbial organism, and plant material samples for TLC. Most college textbooks on quantitative analysis and instrumental analysis contain sections or chapters on the theory and practice of sampling (e.g., Ref. 44).

B. Sample Preparation

Sample preparation for TLC is covered in Chapter 4 of Ref. 1 with an emphasis on biological samples. The only chapter on sample preparation specifically for TLC was written by Sherma (45), but because of its date it does not contain modern methods. A review paper on sample preparation for chromatographic analysis of plant material (46) and two reports on instruments for sample preparation (47, 48) contain information on the newest methods. Sections on sample preparation related to specific compound types will be found in most of the applications chapters in Part II of this Handbook.

If the analyte is present in low concentration in a complex sample such as biological or plant material, then extraction, isolation, and concentration procedures must usually precede TLC. Because layers are not reused, it is often possible to spot cruder samples than could be injected into an HPLC column, including samples containing irreversibly sorbed impurities. On the other hand, any impurities that would comigrate with the analyte and adversely affect its detection or cause a distorted or trailing analyte zone must be removed prior to TLC. Isolation and/or preconcentration procedures for TLC are similar to those used for GC and HPLC and include Soxhlet extraction (49), sonication

extraction (50), supercritical fluid extraction (SFE), and SPE. Purification of extracts is accomplished by methods such as solvent partitioning, column chromatography, desalting, and deproteinization.

1. Direct Spotting of Samples

Certain samples can be successfully analyzed by direct spotting without extraction or cleanup. The applied volume must give a detectable zone with a scan area that can be bracketed by the scan areas of a series of standard concentrations if densitometric quantification is desired. Impurities must not retain the compound at the origin, distort its shape (cause tailing), or alter the R_f value of the zone. The quantification of benzoic and sorbic acid preservatives in beverages directly applied onto a plate with a preadsorbent spotting strip is an example (51). The preadsorbent facilitated the analysis because samples could be quickly and easily applied over a large area, the initial zone was automatically concentrated at the layer interface upon development, and the kieselguhr strip retained sample impurities. Unpurified urine and serum samples have also been applied successfully to preadsorbent layers for determination of amino acids, drugs, and lipids.

2. Direct Application of Sample Solutions or Extracts

For determination of macro constituents in relatively pure matrices, samples can be dissolved in an appropriate volume of pure solvent followed by spotting of an aliquot of solution on the layer. This approach has been used for HPTLC assay of active ingredients of many pharmaceutical dosage forms, e.g., cimetidine in acid reduction tablets (52). Natural or synthetic vanilla flavors were determined in chocolate by slurring the sample with 95% ethanol, sonication, filtering to remove solid material, and direct application to the layer (38). Fillers and other inert ingredients in samples such as foods and Pharmaceuticals often remain undissolved. This will cause no problem if the analyte is dissolved completely and the insoluble material is filtered or centrifuged into a pellet or allowed to settle to the bottom of the sample container prior to spotting clear test solution.

Extracts of trace constituents in some types of adequately pure samples can also be spotted directly after concentration of an extract to a suitable volume. Any coextracted impurities must be resolved from the analyte by the TLC separation step or not detected by the visualization method used. To minimize the amount of coextractives, the least polar analyte that will quantitatively extract the analyte should be used, leaving as many polar impurities as possible unextracted. Direct spotting of extracts was used to determine hydrocarbons in wastewater extracted with heptane by means of a microseparator (53) and the pesticide dichlorvos in minced visceral tissue extracted with ethyl acetate (54).

3. Cleanup of Extracts by Solvent Partitioning

Extracts that are too impure for direct spotting can be cleaned up by partitioning with immiscible solvents. The principle of differential partitioning is to leave impurities behind in one solvent layer while extracting the analyte into the other layer. Acids are converted into salts that are soluble in aqueous solutions at high pH but are un-ionized

and extractable into organic solvents at low pH. Basic compounds are extracted into organic solvents at high pH and into water in their salt forms at low pH. In practice, the pH should be at least two units below the pK_a of an acid and two units above the pK_a of a base in order to have a large enough fraction of uncharged molecules to allow efficient extraction into organic solvents. As an example, the mycotoxin patulin was determined in apples, apple concentrate, and apple juice by extraction with ethyl acetate, cleanup by partition with 1.5% sodium carbonate solution, and silica gel TLC-densitometry (55).

Other uses of liquid-liquid extraction in sample preparation are to remove oils, fats, and lipids from samples if these compounds will interfere with subsequent TLC and to concentrate sample solutions prior to spotting.

4. Cleanup of Extracts by Column Chromatography

Chromatography on gel permeation, silica gel, alumina, Florisil, and carbon columns, among others, has been very widely used for cleanup of samples, often after preliminary purification by solvent partitioning. Examples are the TLC determination of uracil herbicides in roots of *Echinacea angustifolia* Moench (Asteraceae) after acetone extraction, partitioning with cyclohexane and then chloroform, and purification on a Florisil R column eluted with dichloromethane–acetone (9:1) (56) and 12 dyes in food extracts after elution from an XAD-2 column with acetone, methanol, and water (57). Column chromatographic cleanup, which usually employs large volumes of solvents to elute fractions of the sample, has been largely replaced by SPE in order to speed up and simplify extraction and cleanup and save on the cost of purchasing and disposing of solvents.

5. Modern Sample Preparation Systems

The field of sample preparation has moved increasingly toward the use of disposable microcolumns and cartridges in order to speed up and simplify extraction and cleanup. These sample preparation systems are of two basic types. Columns packed with diatomaceous earth and designed for efficient liquid–liquid extractions in place of separatory funnels are available with capacities ranging from 0.3 to 300 mL of sample (e.g., Chem Elute Hydromatrix columns from Varian). The packing is either unbuffered or buffered at pH 4.5 and 9.0 for extraction of acidic and basic compounds, respectively. The aqueous sample is poured into the column, and after a 5 min wait, organic extracting solvent is poured into the column. The eluent containing the analyte is collected, evaporated to dryness under nitrogen flow, reconstituted in an appropriate solvent, and spotted for TLC analysis. Extraction columns of this type are used for screening drugs of abuse in urine (e.g., Extube Tox Elute 10 and 20 mL columns from Varian).

The second method, SPE, uses sorbent phases with a variety of mechanisms and formats. The most common formats are microcolumns or cartridges with 100–500 mg of sorbent packed in 1–5 mL syringe barrels. Other SPE formats include pipet tips, disks, fixed 96-well plates, flexible 96-well plates, 384-well plates, and large-volume cartridges and flash Chromatography columns (58). The well plates are compatible with the use of TLC for drug discovery combinatorial chemistry high-throughput applications (59).

The sorbents available from Varian in their Bond Elute columns are illustrative of the products of other SPE product manufacturers. These include the following.

Nonpolar extraction: C₁₈, octadecyl; C₈, octyl; C₂, ethyl; CH, cyclohexyl; PH, phenyl; CNE, end-capped cyanopropyl

Polar extraction: CN, cyanopropyl; 2OH, diol; SI, silica; NH₂, aminopropyl

Cation-exchange extraction: SCX, benzenesulfonic acid (strong); PRS, propylsulfonic acid (strong); CBA, carboxylic acid (weak)

Anion-exchange extraction: SAX, quaternary amine (strong); PSA, primary/secondary amine (pK_a 10.1, 10.9); NH₂, aminopropyl (weak); DEA, diethylaminopropyl (weak)

Varian also supplies a covalent extraction phase (PBA, phenylboronic acid) for nucleotides, nucleosides, carbohydrates, and catecholamines and specialty phases for determination of grease, oils, fats, phenols, PAHs, organic acids, tricyclics, benzodiazepines, Pharmaceuticals, explosives, pesticides, and neutral, basic, and acidic drugs. Bond Elute sorbents are supplied in 50 mg to 10 g weights in cartridges up to 60 mL in volume.

Figure 1 shows a Speedisk (J.T.Baker) Positive Pressure Processor for semiautomated elution of 1, 3, and 6 mL SPE columns in batches of 1–48 samples. Totally automated SPE systems are also available commercially (47).

SPE is used to concentrate solutes from dilute solution, e.g., to collect nonpolar organic constituents on C₁₈ cartridges. The analytes are recovered by elution from the column with a few milliliters of an appropriate solvent and spotted for TLC. The concentration factor obtained for this method, which has been termed “trace enrichment,” is the ratio of the sample volume to the elution volume. SPE can also be used to purify concentrated solvent extracts in place of classical large columns that require up to hundreds of milliliters of elution solvents. A sequence of eluents of increasing strength can be used to elute compounds with different polarities in different frac-

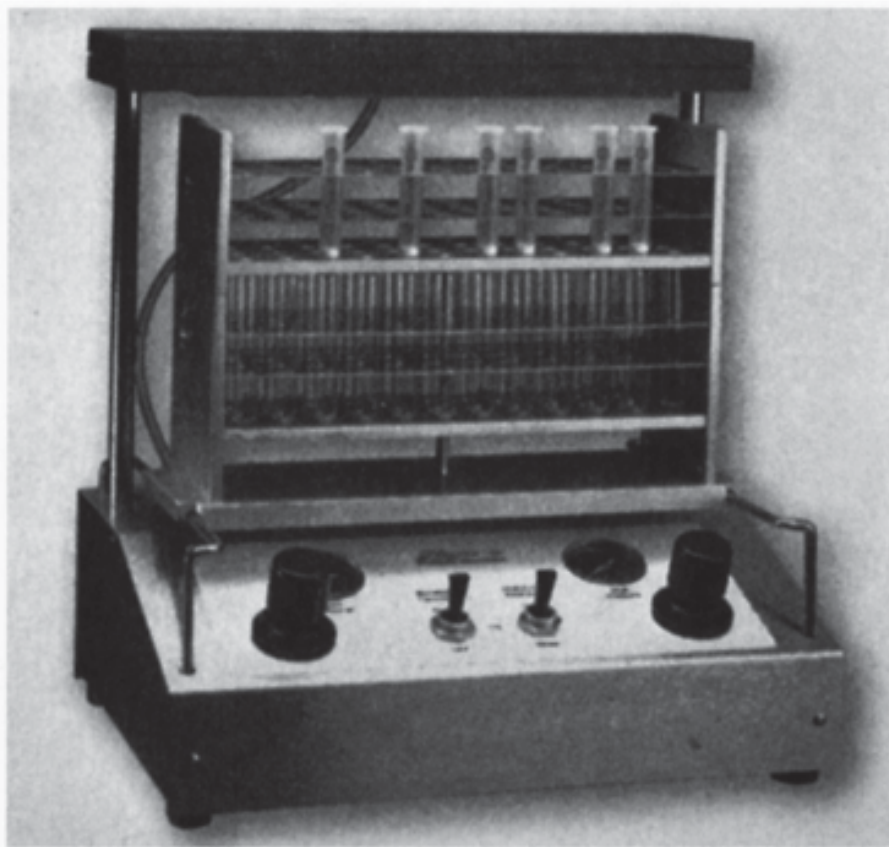


Figure 1 Speedisk 48 Positive Pressure Processor for SPE.
(Photograph supplied by Mallinckrodt Baker Inc.)

tions, and multiple SPE columns can be connected in series for improved cleanup and/or fractionation.

The basic steps of SPE, illustrated for the most commonly used reversed-phase C_{18} cartridge, can be summarized as follows:

Conditioning. The cartridge is prepared for receiving the sample by passing a volume of an appropriate solvent followed by a volume of liquid similar to the sample matrix. For the C_{18} cartridge, methanol is passed through followed by water for extraction of an aqueous sample.

Retention. The sample is applied, and the analyte and other components with attraction for the sorbent are retained. Non- or weakly attracted components will pass through, providing the first stage of cleanup. With the C_{18} cartridge, the most polar interferences will elute first, and retention increases as polarity decreases.

Rinsing. One or more solvents with decreasing polarity are passed through to elute interferences that are more polar than the analyte but keep the analyte on the column.

Elution. A sufficiently nonpolar eluent is passed to remove the analyte. Interferences more nonpolar than the analyte will have a greater attraction for the C₁₈ sorbent and remain uneluted.

The following is an abbreviated guide to the SPE of different classes of sample analytes:

Nonpolar extraction. A polar solution (water, buffers) containing a nonpolar analyte is applied to a C₁₈, C₈, C₂, CNE, CH, PH, or 2OH column that was preconditioned with methanol followed by water or buffer (see listing above for abbreviations). The sample must be buffered, if necessary, to suppress analyte ionization. Polar interferences are removed by washing with water or buffer or a weak organic-aqueous solvent that will not elute the analyte [e.g., water (buffer)-methanol (9:1)]. The analyte is eluted with a nonpolar solvent such as methanol, acetonitrile, tetrahydrofuran (THF), hexane, or methylene chloride.

Polar extraction. A nonpolar solution containing a polar analyte is applied to an SI, CN, 2OH, or NH₂ column that was preconditioned with the nonpolar solvent in which the analyte is dissolved, such as hexane or chloroform. Viscous samples are diluted in a non-polar solvent, and water is removed from the sample, e.g., by filtration through Whatman phase-separating paper. Nonpolar interferences are removed by washing with a nonpolar solvent or a polar-nonpolar mixture that is not strong (polar) enough to elute the analyte. The analyte is recovered by elution with a polar solvent such as methanol or isopropanol.

Anion-exchange extraction. An aqueous, low ionic strength sample (water, plasma, diluted urine) containing inorganic or organic anions is applied to an SAX, NH₂, PSA, or DEA column. Both the chosen column and the analyte must be ionic for exchange to occur. The column is conditioned with methanol followed by a buffer whose pH is 2 units above the pK_a of the analyte and <7.8 for NH₂, PSA, and DEA columns. The sample pH is adjusted as above for conditioning and applied to the column. Interferences are removed by washing with the sample buffer and with an organic solvent such as acetonitrile or methanol, if necessary. The analyte is eluted with a buffer whose pH is at least 2 units below the analyte pK_a , a buffer whose pH is 2 units above the column pK_a , or a buffer of high ionic strength (>0.1 M). The eluents can be totally aqueous or aqueous-organic mixtures; addition of an organic modifier such as methanol may improve analyte recovery.

Cation-exchange extraction. An aqueous, low ionic strength sample containing inorganic or organic cations is applied to an SCX, PRS, or CBA column preconditioned with methanol followed by a buffer whose pH is 2 units below the analyte pK_a and >6.8 for the CBA column. The sample pH is adjusted in the same manner. Interferences are eliminated by

elution with the sample buffer and with an organic solvent, if necessary. The analyte is eluted with a buffer at least 2 units above the analyte pK_a , a buffer of pH <2.8 for the CBA column, or a buffer of high ionic strength (>0.1 M). Addition of an organic modifier such as methanol may improve analyte recovery.

Examples of applications of SPE prior to TLC analysis include analysis for pesticides in fruits and vegetables according to the official German multimethod S19 using SPE on silica gel and amino cartridges prior to HPTLC with gradient elution AMD (60); oxygenated cholesterol derivatives in plasma using silica gel SPE (61); quinoline and quinuclidine alkaloids in pharmaceutical preparations using cation-exchange SPE (62); rutin in glycerinic plant extracts using Envi-18 (Supelco) cartridges (63); and aflatoxins in a variety of foods using phenyl, silica, C₁₈, and Florisil-C₁₈ cartridges (64). A strategy for choosing SPE cartridge elution solvents based on the PRISMA TLC mobile-phase optimization procedure was demonstrated for extraction of furocoumarin isomers and flavonoid glycosides from medicinal and aromatic plants (65).

The use of immunoaffinity columns for sample cleanup is among the newest sample preparation procedures. Immunoaffinity cleanup was used after methanol extraction for determination of aflatoxins B-1, B-2, G-1, and G-2 in various food matrices by TLC-densitometry (66).

Of the current sample preparation methods (46, 48), only SPE (above) and SEE have had substantial use in combination with TLC. Automated Soxhlet extraction, microwave-assisted extraction (MAE), and accelerated solvent extraction (ASE) have good potential for preparing solid samples for TLC analysis, but published methods have not yet appeared. Stahl first interfaced SEE with TLC in 1977, and there has been increasing interest in developing new methods in recent years. Examples of SFE-TLC analyses reported include cyanazine herbicide in soil (67); flavonoids in *Scutellariae radix* (68); aloin and aloe-emodin in consumable aloe products (69); semivolatile compounds in cassia and cinnamon (70); and residues of 20 pesticides of multiple classes in soil (71). Hydroperoxides in combustion products were separated from solid matrices using SEE with on-line transfer to TLC plates (72).

6. Additional Sample Preparation Procedures

Additional procedures performed prior to TLC analysis, depending on the sample type, include drying, grinding, freeze-drying (removal of water), drying of extracts (passage through a drying column or phase-separating filter paper or addition of a drying agent such as sodium sulfate), and the steps described below in this section.

Desalting is often required for samples such as urine, serum, and tissue culture media in order to eliminate streaking and the formation of unresolved zones in the TLC of amino acids, carbohydrates, and other hydrophilic compounds. Salts are removed from samples by performing ion exchange, using a desalting column, dialysis, and passage through a nonpolar sorbent. A simple desalting procedure suitable for 0.1–0.2 mL of urine, serum, or saline solution is the following. The sample is dried under air at 45°C and then extracted with 1 mL of 0.5% HCl in 95% ethanol for 24 h. The extract is evaporated to dryness and the residue dissolved in 100 μ L of ethanolic HCl prior to

spotting for TLC (73). The ion retardation resin AG 11 A8 (Bio-Rad Laboratories Inc.) and mixed bed cation/anion-exchange resins (e.g., Bio-Rad AG 501) have been used successfully for desalting samples prior to TLC.

7. Deproteinization

When proteins may interfere with TLC analysis, they must be removed by deproteinization procedures. A suitable procedure for an approximate 50 μL sample of serum involves addition of 100 μL of methanol to precipitate the protein followed by shaking and centrifugation of the mixture to obtain a clear supernatant. The technique has been used to deproteinize biological fluids prior to their analysis for drugs (74). Proteins in samples such as serum, urine, tissue, and milk can be precipitated by addition of trichloroacetic acid (75), perchloric acid, or sulfosalicylic acid followed by centrifugation and removal of the supernatant, which may or may not require further cleanup prior to TLC. Protein removal from various types of samples has also been carried out by pH modification, denaturation with chaotropic agents or organic solvents, addition of a compound that competes for binding sites, and the use of restricted-access media.

8. Derivatization

The preparation of derivatives in TLC was reviewed by Edwards (76), who documented the application of derivatization techniques to a wide range of compounds including amino acids, steroids, drugs, and environmental pollutants. Fluorescent derivatives for TLC were reviewed by Wintersteiger (77).

One of the major advantages of TLC is the use of derivatization postchromatography for the purpose of zone detection. This is normally achieved by spraying the layer with (or dipping it into) a solution of an appropriate reagent or reagents and then drying or heating to complete the reaction. Hundreds of such reagents have been described to cause zones to absorb visible or ultraviolet radiation or to become fluorescent for organic species in general or to react selectively with particular compound classes (see Sec. VIII.A). Examples include spraying with ninhydrin reagent to produce purple spots for amino acids, or with a solution of diazonium reagent (prepared from *p*-nitroaniline, HCl, and sodium nitrite) to detect phenols and aromatic amines as orange zones. Postchromatographic derivatization allows the reaction of all standards and samples simultaneously under the same conditions, and the separation properties of the solutes are not changed by the reaction.

Prechromatographic derivatization is advantageous when the parent compound is too volatile for TLC but the derivative is less volatile, the derivative is easier to separate from other sample constituents, the derivative has greater stability (e.g., resistance to oxidation or decomposition), the derivative is more successfully extracted and/or cleaned up, or the derivative is more sensitively and/or selectively detected. A disadvantage of prederivatization is that the introduction of usually high molecular weight functional groups into the derivative may equalize the chromatographic properties of similar substances and make separation more difficult. In addition, prederivatization of each sample prior to its application can be tedious and time-consuming, by-products of the reaction may interfere with the TLC separation, or the presence of excess reagent may

cause a background that interferes with quantification by scanning. It is possible in some cases to derivatize in situ prior to chromatography. This is usually done by applying a spot or band of excess reagent to the origin and overspotting the sample while the reagent zone is still moist, followed by application of heat to accelerate the reaction, if necessary. Zones of sample and reagent should be chromatographed on adjacent lanes for comparison. Many different kinds of in situ prechromatographic derivatization have been reviewed (78).

The following are examples of analyses that include the formation of derivatives prior to TLC: the formation of fluorescent dansyl derivatives for determination of biogenic amines in red wine (79) and other foods (80) and of colored thiocarbamoyl derivatives of biogenic amines (81); the use of *p*-benzoquinone for derivatization of 2-(methylamino)ethanol and other primary and secondary amines (82); the separation of *p*-dimethylaminobenzaldehyde from *p*-dimethylamino-cinnamaldehyde after derivatization with diphenylamine (83); determination of bisoprolol, labetalol, and propafenone as dansyl derivatives in pharmaceutical preparations (84); and determination of the toxin fumonisin B-1 in corn after immunoaffinity column cleanup and derivatization (85). In many cases, enantiomers have been resolved by TLC after the formation of derivatives, e.g., amino acids derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, and separated on RP plates (86). The latest methodology involves separation of enantiomers of compounds such as chiral drugs by TLC without their prior derivatization (87).

9. Evaporation of Solutions

Most sample preparation procedures require concentration or evaporation to dryness of sample extracts, combined partition solvent batches, or column effluents. It is important that evaporations be carried out without loss or degradation of the analyte, and studies may be required to determine which of the available methods is best to use in each particular situation.

A common method of concentration uses a rotary evaporator with an attached round-bottomed flask. A helpful variation is to place the solution in a Kuderna-Danish evaporative concentrator flask with attached lower calibrated tube (Kontes), so that the concentrate ends up in the tube and can be applied to the layer without transfer.

Nitrogen blowdown is the recommended method for concentration of small volumes of volatile organic solvents. Gas is supplied to the sample, held in a tube or vial, through Tygon tubing connected to a glass capillary. The sample is warmed in a 40–60°C water bath to speed evaporation. Various commercial devices that allow simultaneous blowdown of multiple samples are available.

10. Reconstitution of Evaporated Residues

It is common practice to evaporate solutions just to dryness and then dissolve the residue in an exact volume of the same or a different solvent, from which a known aliquot or the total sample is applied to the layer. The best initial zones on silica gel are obtained if the solvent is highly volatile and as nonpolar as possible, consistent with complete solubility and stability of the analyte(s). By use of a nonpolar solvent, purification can be achieved if some polar impurities in the residue are left undissolved (selective solvation). Solvents

with a high boiling point or polarity are difficult to remove from the sorbent during application. If a small amount of solvent is retained after application, it can adversely affect the separation by causing zone spreading or deformation or a different R_f value. Care must be taken, however, because hot air used to dry solvent at the origin can decompose labile substances on the surface of an active sorbent. A volatile sample solvent promotes the production of small, regular initial zones, but containers must be kept tightly sealed except when filling the sample application device.

IV. SORBENTS AND LAYERS

Sorbent materials and layers are described in Chapter 4 of this Handbook and Chapter 3 of Ref. 1 and in a review paper (88) and an encyclopedia article (89).

A great variety of commercial precoated layers are available for TLC on glass, plastic, or aluminum foil supports in 20×20 cm size. The most common layer thickness for analytical TLC is 250 μm , but cellulose and polyamide layers are often 100 μm . For mechanical stability, 0.1–20% of a gypsum (calcium sulfate), starch, or organic polymer binder [e.g., poly(acrylic acid)] is added to the sorbent slurry from which the layer is cast. Plates with gypsum binder, which are known as “soft layers” and are designated with a G, must be used with greater care than “hard” organic polymer-bound layers to avoid abrasive conditions. Gypsum binder allows the use of sulfuric acid charring techniques, and sample zones can be easily scraped from the glass support for subsequent elution of compounds from the sorbent. Binder-free silica gel plates containing a small amount of colloidal silica to aid layer adherence are also available. For detection of zones by fluorescence quenching, plates are impregnated with indicator compounds (e.g., manganese-activated zinc silicate) that cause the layer to fluoresce uniformly when exposed to 254 or 366 nm UV light. Glass is the most inert support material, and its planarity is advantageous when the layer will be scanned for quantitative analysis. Procedures and devices for preparing homemade plates are described in Chapter 3 of the third edition of Fried and Sherma (1). Homemade plates, the quality of which is almost never equivalent to that of commercial plates, are rarely made except when a needed layer is not available or cost is a major consideration.

To remove extraneous materials that may be present due to manufacture, shipping, or storage conditions, it is advisable to preclean plates before use. This has often been done by predevelopment to the top with dichloromethane-methanol (1:1) or the mobile phase to be used for the analysis. The following two-step HPTLC plate cleaning method has been proposed (90) for surface residue removal in critical applications when optimum sensitivity is required for detection and quantification: Develop the plate to the top with methanol, air dry for 5 min, totally immerse the plate in a tank filled with methanol, air dry for 5 min, oven dry for 15 min at 80°C, and cool in a desiccator before use. The routine activation of adsorbents at 70–80°C for 30 min, or at a higher temperature, is often proposed in the literature, but this treatment is not usually necessary for commercial plates unless they have been exposed to high humidity. RP plates do not require activation prior to use. Suggestions for initial treatment, prewashing, activation, and conditioning of different types of glass- and foil-backed layers have been published (91).

A. Adsorbents

Silica gel is by far the most frequently used layer material for adsorption TLC. Some characteristic properties, including porosity, flow resistance, particle size, optimum velocity, and plate height, have been tabulated for three popular brands of silica gel TLC and HPTLC plates (38). Separations take place primarily by hydrogen bonding or dipole interaction with surface silanol groups by using lipophilic mobile phases, and analytes are separated into groups according to their polarity. Typical properties of TLC silica gel are a silanol group level of approximately $8 \mu\text{mol}/\text{m}^2$; pore diameter of 40, 60, 80, or 100 Å; and specific pore volumes of 0.5–2.0 mL (89). Specific differences in the types and distributions of silanol groups for individual sorbents may result in selectivity differences, and separations will not be exactly reproducible on different brands of silica gel layers (25). Other TLC adsorbents include aluminum oxide (alumina), magnesium oxide [used mostly for carotenoid pigment separations (92)], magnesium silicate (Florisil) (93), polyamide, and kieselguhr (94).

Alumina (95) is a polar adsorbent that is similar to silica gel in its general chromatographic properties, but it has an especially high adsorption affinity for carbon-carbon double bonds and better selectivity toward aromatic hydrocarbons and their derivatives. The alumina surface is more complex than silica gel, containing hydroxyl groups, aluminum cations, and oxide anions, and pH and hydration level alter separation properties (25). It is available in basic (pH 9–10), neutral (7–8), and acid (4–4.5) forms. The specific surface area of aluminas range from 50 to 250 m^2/g (89). The high density of hydroxyl groups ($\sim 13 \mu\text{mol}/\text{m}^2$) leads to a significant degree of water adsorption, and alumina layers are usually activated by heating for 10 min at 120°C before use (89).

Poly amides 6 (Nylon 6; polymeric caprolactam) and 11 (polymeric undecanamide) have surface—CO—NH—groups and show high affinity and selectivity for polar compounds that can form hydrogen bonds with the exposed carbonyl groups. However, depending on the type of analyte and mobile phase, three separation mechanisms can operate with poly amide: adsorption, partition (normal- and reversed-phase), and ion exchange. This has led to separations of compounds from a wide array of chemical classes such as amino acids, phenols, phenolic compounds, carboxylic acids, cyclodextrins (96), coumarins, and flavonoids (97). Polyamide has been impregnated with various metal salts to improve the separation of sulfonamides (98). Separation numbers for a series of higher fatty acids and alcohols were determined to be 8–12 for polyamide and 4–9 for cellulose (99).

Homemade mixed sorbent layers have been used by various workers to increase the resolution of certain samples compared to that obtained on the separate phases. Binary layers that have been reported include silica gel-alumina (100), kieselguhr-alumina, alumina-calcium sulfate, magnesium-kieselguhr, cellulose-silica gel, poly amide-silica gel, polyamide-kieselguhr, poly amidecellulose, poly amide-glass powder (similar to silica gel), silica gel-kieselguhr (101), and alumina-cellulose (102). The properties of these mixed layers are usually somewhere between those of the two separate phases but are impossible to predict or explain with certainty. Information on and applications of mixed layers are mostly contained in older standard TLC texts and reviews.

B. Partition, Preadsorbent, and Impregnated Layers

Compounds that have the same polarity and functional group and migrate together on silica gel can often be resolved by partition TLC. Crystalline cellulose (AVICEL) or high-purity fibrous cellulose serves primarily as a support material for the NP liquid-liquid partition TLC of polar substances, such as amino acids (103), and water-soluble biopolymers, although adsorption effects cannot be excluded in many cases. The stationary phase is either water or an impregnated polar liquid such as dimethylformamide. Cellulose used to prepare thin layers differs from that in chromatography paper mainly by having shorter fiber length (2–20 μm), resulting in the same migration sequence for a series of compounds developed with a given mobile phase but less diffusion and higher efficiency than in paper chromatography.

Kieselguhr (diatomaceous earth) (104) and synthetically prepared silicon dioxide (Merck silica 50,000) (105) are small surface area, weak adsorbents that are used as the lower 2–4 cm inactive sample application and concentrating zone in the manufacture of silica gel and C_{18} preadsorbent plates. Samples applied to the preadsorbent region usually develop into sharp, narrow bands at the preadsorbent/sorbent interface, leading to efficient separations with minimum time and effort in manual application of samples and possible sample cleanup by retention of interferences in the preadsorbent.

Layers have been impregnated with buffers, chelating agents, metal ions, or other compounds to aid in the resolution or detection of certain compounds (see Ref. 106 for a review). If plates are prepared in the laboratory, the reagent is usually added to the stationary-phase slurry. Reagents are applied to precoated plates by spraying, brushing, horizontal or vertical dipping, development, or overdevelopment (107). Analtech precoated plates are available already impregnated with potassium oxalate to facilitate resolution of polyphosphoinositides, magnesium acetate for phospholipids, 0.1 M NaOH for organometallics and acidic compounds, silver nitrate for compounds with carbon-carbon double bonds such as fatty acids (107), and carbomer for mannitol and sorbitol analysis according to several Pharmacopoeia methods, as well as plates containing ammonium sulfate for detection of compounds as fluorescent or charred zones after heating (vapor-phase fluorescence detection). Other reagents that have been added to thin layers to improve separations include ion-pairing reagents (108), molybdic acid (for separation of carbohydrates), boric acid (carbohydrates and lipids), poly cyclic aromatic hydrocarbons (PAHs), (formation of charge transfer complexes with numerous organic compounds), surfactants (sulfa drugs and substituted pyrazoles) (109), EDTA (reduces tailing of drugs) (110), urea (wax esters and hydroxybenzenes), ferric ion (carboxy- and hydroxybenzenes), cupric ion (glucose and sorbitol), caffeine (PAHs), and ammonium sulfate (surfactants). The separation of amino acids and their derivatives and enantiomers by impregnated TLC was reviewed by Bhushan and Martens (110a).

C. High-Performance Layers

High-performance (HP) plates (10×10 or 10×20 cm) are produced from sorbents having narrow pore and particle size distributions and an apparent particle size of 5–7 μm instead of 8–10 μm for 20×20 cm TLC plates (23). Layer thickness is usually 100–200 μm for

HPTLC plates compared to 250 μm for TLC, but ultrathin (10 μm) layers of monolithic silica gel have recently been described (110b).

High-performance layers are more efficient, leading to tighter zones, better resolution, and more sensitive detection. Flow resistance is higher (migration time per centimeter is slower), but overall development time is shorter because smaller migration distances are used for HPTLC than for TLC (typically 3–8 cm versus 10–16 cm). The low flow rate through fine-particle HPTLC plates led to the development of forced-flow methods. Sample sizes are generally 0.2–1 μL for HPTLC and 1–3 μL for TLC, although the upper levels of these ranges can be exceeded when spotting with the Linomat instrument or using preadsorbent layers.

Silica gel is the most widely used type of HP plate, but other HP layers, including bonded phases, are also commercially available. Among the newest layers are Merck's TLC and HPTLC silica gel 60 plates (60 Å pore size) with imprinted identification codes for use in documentation when analyses are performed according to good manufacturing practice (GMP) and good laboratory practice (GLP) standards (52). Merck also sells two new HPTLC layers with spherical silica gel: HPTLC plates with LiChrospher Si60F_{254S} (0.2 mm layer thickness, 7–8 μm mean particle size), and HPTLC aluminum sheets with Si60F_{254S} Raman (0.1 mm layer thickness and 3–4 μm particle size). Layers with spherical particles offer better efficiency, spot capacity, and detection limits than those with nonspherical particles. The silica gel matrix on the sheets is designed to have the least possible spectral interference for direct coupling of TLC with Raman spectrometry (see Sec. VIII.B).

TLC and HPTLC are compared in Chapter 2 of Ref. 1.

D. Bonded Layers

Reversed-phase TLC, in which the stationary phase is less polar than the mobile phase, was originally carried out on silica gel or kieselguhr layers impregnated with a solution of paraffin, squalane, silicone oil, octanol, or oleyl alcohol. Analtech sells RP plates with hydrocarbon liquid phase physically adsorbed onto the surface of a silica gel layer. Impregnated plates of this kind require the use of aqueous and polar organic mobile phases saturated with the stationary liquid, and they cannot tolerate the use of nonpolar organic solvents, which will strip the coating from the support.

Bonded phases with functional groups chemically bonded to silica gel eliminate stripping of the stationary liquid from the support by incompatible mobile phases. Alkylsiloxane-bonded silica gel with CH_3 , C_2H_5 , C_8H_{17} , and $\text{C}_{18}\text{H}_{37}$ (111) functional groups are most widely used for RP-TLC of organic compounds (polar and nonpolar homologous compounds and aromatics), weak acids and bases after ion suppression with buffered mobile phases, and strong acids and bases using ion-pair reagents. Layers from different companies but with the same bonded group can have different percentages of carbon loading and give different results. The hydrophobic nature of the layer increases with both the chain length and the degree of loading of the groups. Alkylsiloxanebonded layers with a high level of surface modification are incompatible with highly aqueous mobile phases and are used mainly for normal-phase separations of low-polarity compounds (25). Problems of wettability and lack of migration of mobile phases with high proportions of water have been solved by adding 3% NaCl to the mobile phase

(Whatman layers) or preparing “water-wettable” layers with a slightly larger particle size, less exhaustive surface bonding, and a modified binder. The latter layers with a low degree of surface coverage and more residual silanol groups exhibit partially hydrophilic as well as hydrophobic character and can be used for RP-TLC and NP-TLC. Chemically bonded phenyl layers are also classified as reversed-phase, but their use has only seldom been reported in the literature.

Hydrophilic bonded silica gel containing cyano (112), amino (113), or diol (114) groups bonded to silica gel through a trimethylene chain $[-(\text{CH}_2)_3-]$ are compatible with aqueous mobile phases and exhibit multimodal mechanisms. Polarity varies as follows: unbonded silica > diol-silica > amino-silica > cyano-silica > reversed-phase materials (89). Cyano layers can act as a normal or reversed phase, depending on the characteristics of the mobile phase, with properties similar to a low-capacity silica gel and a short-chain alkylsiloxane bonded layer, respectively (25). Amino layers are used in NP and weak anion-exchange modes. In NP-TLC, compounds are retained on amino layers by hydrogen bonding as with silica gel, but the selectivity is different. Charged substances such as nucleotides or sulfonic acids can be separated by ion exchange using acidic mobile phases. Although there is limited retention in RP-TLC, the separation of oligonucleotides on amino layers based on differences in hydrophobic properties of the compounds has been reported. Diol plates can operate with NP- or RP-TLC mechanisms, depending on the mobile phase and solutes. Polar compounds show reasonable retention by hydrogen bond and dipole-type interactions in the former mode, and in the RP mode retention is low but higher than with amino layers. A study of mixed mechanisms on cyano, amino, and diol layers was reported (115).

E. Layers for Enantiomer Separations

Commercial layers are available for separation of enantiomers by the mechanism of ligand exchange under the names Chiralplate (Macherey-Nagel) and HPTLC CHIR (Merck). These consist of a glass plate coated with a reversed-phase silanized silica gel and impregnated with the Cu(II) complex of (2S, 4R, 2'RS)-N-(2'-hydroxydodecyl)-4-hydroxyproline. Separation is based on the formation of diastereomeric chelate complexes between the central cupric ion, the chiral selector, and the solute. Enantiometric resolution is achieved if the antipodes of the chiral solute form complexes of different stabilities. The history of chiral ligand exchange in TLC and column liquid chromatography has been reviewed (116).

In addition to ligand exchange, enantiomeric separations have been carried out using cyclodextrin-containing mobile phases with hydrophobic C_{18} (117) and cellulose triacetate (118) layers (inclusion TLC). Chiral selectors have been impregnated into silica gel layers for NP-TLC enantiomer separations, e.g., the macrocyclic antibiotic vancomycin for DL-amino acids (199) and L-lysine and L-arginine for β -adrenergic blocking agents (120). Unmodified cellulose has been used for separation of enantiomeric amino acids and peptides and other compounds (e.g., 121). Optical isomers can be derivatized and separated without using impregnated plates or a chiral mobile phase, e.g., amino acids derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide and separated by RP-TLC (86).

The newest approach is the preparation of molecularly imprinted polymers (MIPs) for use as chiral stationary phases in TLC. For example, the direct separation of enantiomers of adrenergic drugs on MIPs of (–)-pseudoephedrine and (–)-norephedrine was demonstrated as a rapid, sensitive, and reliable method for quality control of these compounds (121a). Beta-blocking drugs and nonsteroidal anti-inflammatory drugs have also been separated on molecularly imprinted chiral layers (121b).

Enantiomeric separations by TLC have been reviewed (122–124), and this topic is covered in Chapter 17 of this Handbook.

F. Miscellaneous Layers

Cellulose has been surface-modified to produce RP (acetylated cellulose), weakly basic anion-exchange [polyethyleneimine (PEI), aminoethyl (AE), diethylaminoethyl (DEAE), and ECTEOLA], or weakly acidic cation-exchange [cellulose phosphate (P) and carboxymethyl-cellulose (CM)] layers. These cellulose exchangers have open structures that can be penetrated by large hydrophilic molecules such as proteins, enzymes, and nucleic acids.

Polygram Ionex-25 precoated sheets (Macherey-Nagel) are polyester sheets coated with a mixture of silica, a polystyrene-based strong acid cation-exchange or strong base anion-exchange resin, and a binder. The cation exchanger has been used to separate and identify amino acids in biological samples (125), and both are suited to inorganic ion separations. A large variety of inorganic ion exchangers, such as titanium(IV) silicate (126), have been prepared and used mostly for metal ion separations.

Size-exclusion gel TLC has been carried out on dextran (Sephadex) gels with controlled pore sizes. These layers, which are used to estimate molecular weights and separate and determine biological macromolecules (e.g., enzymes and serum proteins), are used in totally swollen condition and developed continuously in the descending direction.

Combination layers with a C₁₈ strip adjacent to a silica gel layer (Whatman Multi-K CS5) or a silica gel strip adjacent to a C₁₈ layer (SC5) are available for 2-D TLC with diverse mechanisms (RP phase and adsorption).

G. Preparative Layers

Preparative silica gel plates are available precoated with a layer thickness of 500–2000 μm . Particle size is typically 5–40 μm with a 25 μm average, but Mallinckrodt-Baker manufactures a preparative plate with 5 μm spherical particles. Analtech offers a unique tapered layer for capillary flow preparative separations (see Sec. V.D) and precast HPTLC silica gel GF rotors (Fig. 2) with 1000–8000 μm nominal thickness for use with the Cyclograph and Chromatotron centrifugal forced-flow PLC instruments (see Sec. XI).

V. APPLICATION OF SAMPLES

Samples and standards prepared for TLC are dissolved in an appropriate solvent at a concentration that will allow eventual detection of the solutes of interest. Typically 1–5

μL containing 1 ng to 10 μg of solute is applied in the form of spots or narrow bands to TLC plates. Because the starting zones should be as small as possible for efficient separation, larger volumes are spotted by repeated applications of a small volume to the same origin with solvent evaporation between increments. Evaporation of the sample solvent must be complete so that the selectivity of the mobile phase and the spot position in the chromatogram are not altered. This drying is achieved and the sample application speeded up if a stream of cool or heated air or inert gas is gently blowing across the plate being spotted. After application, initial zones are usually thoroughly dried with a hair drier or in an oven. Ideally, the sample should be distributed homogeneously throughout the starting zone (38).

Sample volumes must be reduced to realize fully the greater efficiency of HPTLC layers, and 100–200 nL is typically applied. Detection limits are usually 5–10 times better for HPTLC than for TLC. Optimum initial spot size for HPTLC is about 1–1.5 mm, whereas initial spots for TLC are typically 3–6 mm. Initial zones that are overloaded with sample will form poorly separated tailed zones during development.

Sample application is one of the main sources of error in quantitative TLC, and great care should be taken to choose a reliable application device and optimize techniques if accurate and precise analyses are to be realized. Sample volumes must be accurately known, and exact posi-

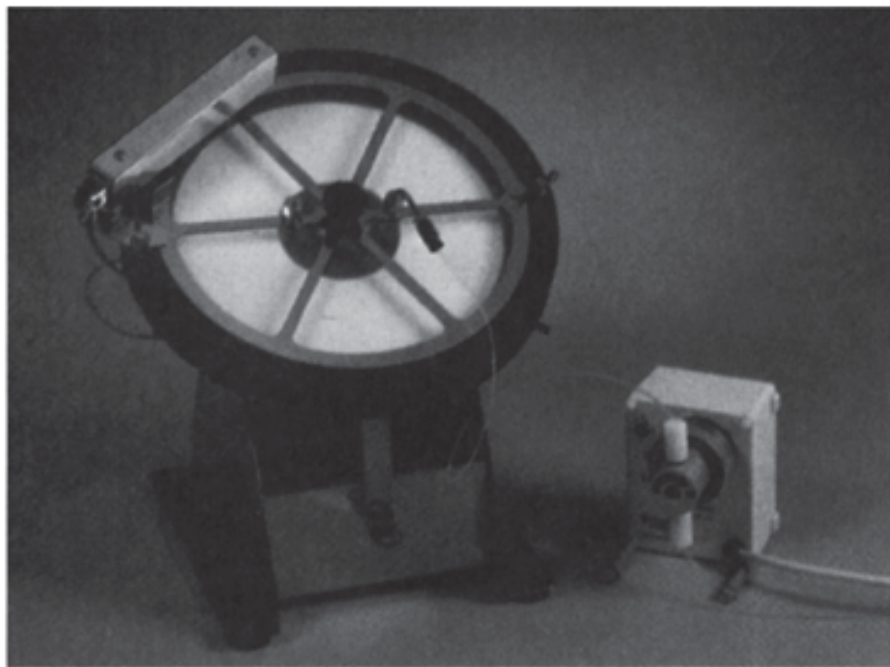


Figure 2 Precast silica gel GF rotor.
(Photograph supplied by Analtech.)

tioning of initial zones is critical when measurements are to be made by scanning. Automated sample application is preferred for best results in quantitative TLC.

A. Choice of the Sample Solvent

Samples and standards are best prepared in a solvent that dissolves the analytes completely, is volatile, has low viscosity, wets the sorbent layer, and is a weak chromatographic solvent for the analytes. In practice, it may be impossible to find a solvent with all of these properties. For silica gel TLC, it is important to use the weakest (least polar) solvent that allows quantitative dissolution and spotting of the sample, so that preliminary development and separation within the initial spot at the origin does not occur, resulting in significant loss in separation efficiency. The R_f of the compounds of interest should be <0.1 in this solvent. The choice of a weak solvent is more difficult for RP layers because solvents that wet the layer (e.g., acetonitrile, methanol) may be strong (nonpolar) enough to cause predevelopment of the spot. If all or part of the sample is solidified or adsorbed onto the layer surface, a slow dissolution effect can cause significant tailing of the spots.

B. Application of Spots

Instruments and techniques for a sample application are described in Chapter 5 of this Handbook and Chapters of Ref. 1.

Samples and standards are applied to the layer as small round spots by using one of a variety of application devices, for example, a wooden stick with flattened end, glass capillary pipet, or syringe with a 90° needle tip. Drummond microcap micropipets, available in virtually any size between 0.1 and 200 μL (Fig. 3), and 10–50 μL digital microdispensers (Fig. 4) are highly recommended for manual applications for both qualitative and quantitative TLC. For linear or circular HPTLC, initial zone diameter should not exceed 1.5 mm for maximum resolution. Spots for HPTLC can be applied to an exact layer position using a 100 or 200 nL Pt-Ir pipet held in a mechanical device that electromagnetically brings the pipet into reproducible contact with the layer without surface damage (Camag Nanomat). Camag and Desaga also supply completely automated devices with which selectable volumes of samples and standards are applied from vials

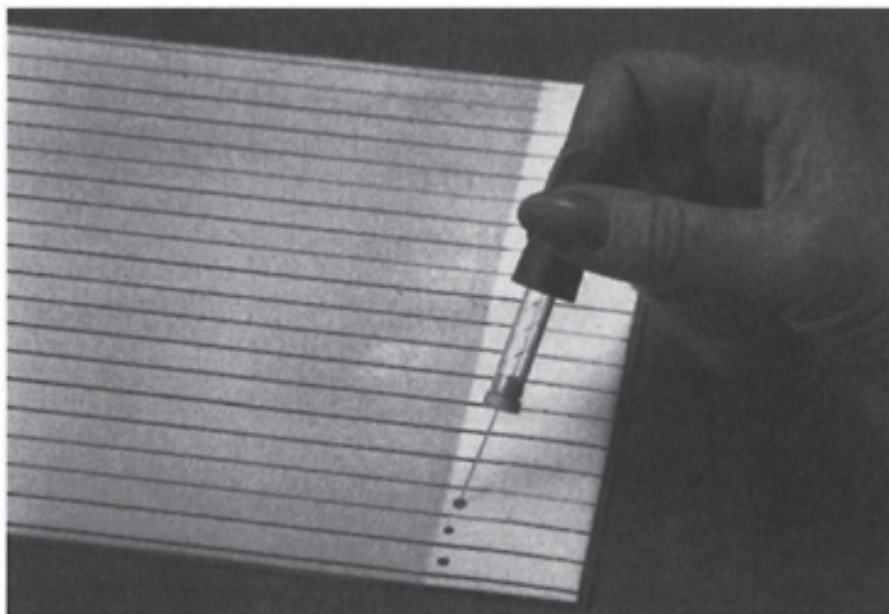


Figure 3 Application of initial zones to a glass-backed 20×20 cm channeled preadsorbent silica gel plate using a Microcap micropipet. (Photograph supplied by Analtech.)

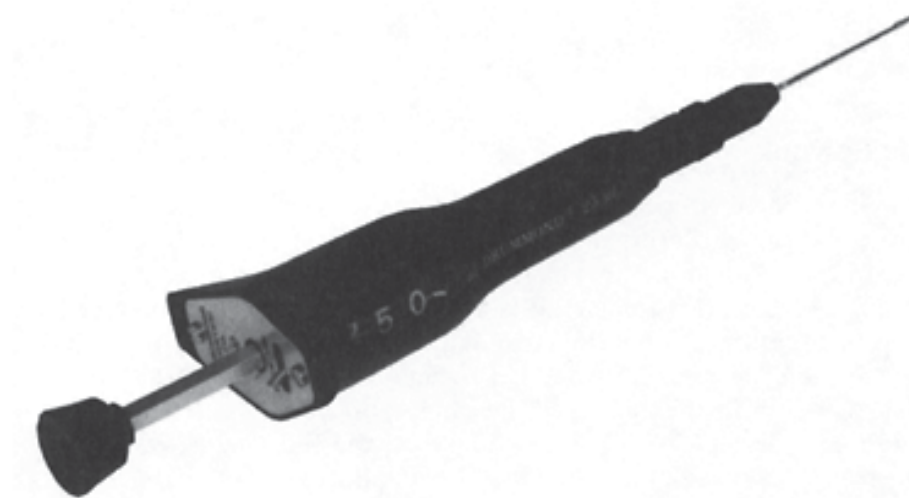


Figure 4 Twenty-five microliter digital microdispenser. (Photograph supplied by Drummond Scientific.)

as spots or bands in any order to specified layer positions through a capillary pipet that is rinsed with solvent between applications.

C. Formation of Bands

Bands or streaks of sample are applied manually, are applied automatically with the Camag Linomat, are formed automatically during development by use of plates with a preadsorbent or concentrating area (see Sec. IV.B), or are produced by predevelopment on conventional plates. Manual application essentially involves placing a contiguous series of spots from a syringe or micropipet side by side. Even with practice, it is difficult to do this uniformly and reproducibly on a conventional plate. The Linomat, which is based on movement of the plate underneath a fixed syringe from which a nitrogen atomizer sprays the sample onto the origin at a constant rate, is advantageous because larger sample volumes [40 μL or more (127)] can be concentrated during the application process compared to other HPTLC devices, and variable volumes of the same standard solution can be applied for calibration in densitometry.

In using preadsorbent plates, samples are spotted or streaked onto the preadsorbent area, and narrow, accurately aligned, homogeneous bands form automatically at the interface with the sorbent upon development. When laned or channeled plates are used, the length of the band is confined within the channel. Sample application is fast and simple for relatively large volumes (up to $\sim 50 \mu\text{L}$ for TLC and $25 \mu\text{L}$ for HPTLC). High efficiency can be obtained for HPTLC by spotting larger volumes of dilute solutions rather than nanoliter volumes of highly concentrated solutions. Crude samples can be directly spotted, and salts and other impurities may be retained in the preadsorbent and not interfere with sample resolution or detection. Figure 5 shows the sequence of zone separation in various stages of development on a preadsorbent TLC plate.

The final method for forming initial bands is to concentrate a large spot into a line by partial predevelopment of the layer with a strong solvent in which all components move with the solvent front. After the plate is dried, it is developed with the mobile phase needed to provide resolution.

It has been shown that bands give sharper separations and lower detection limits than spots and are advantageous for densitometry because the length of the scanner light beam can be made shorter than the length of the band (one-half to two-thirds of the original band length). This method of aliquot scanning minimizes the need to exactly position the zone within the beam.

D. Sample Application for Preparative Layer Chromatography

The amount of sample that can be applied to a preparative layer depends on the mixture to be separated, the layer, and the development method. Adsorption TLC permits the application of

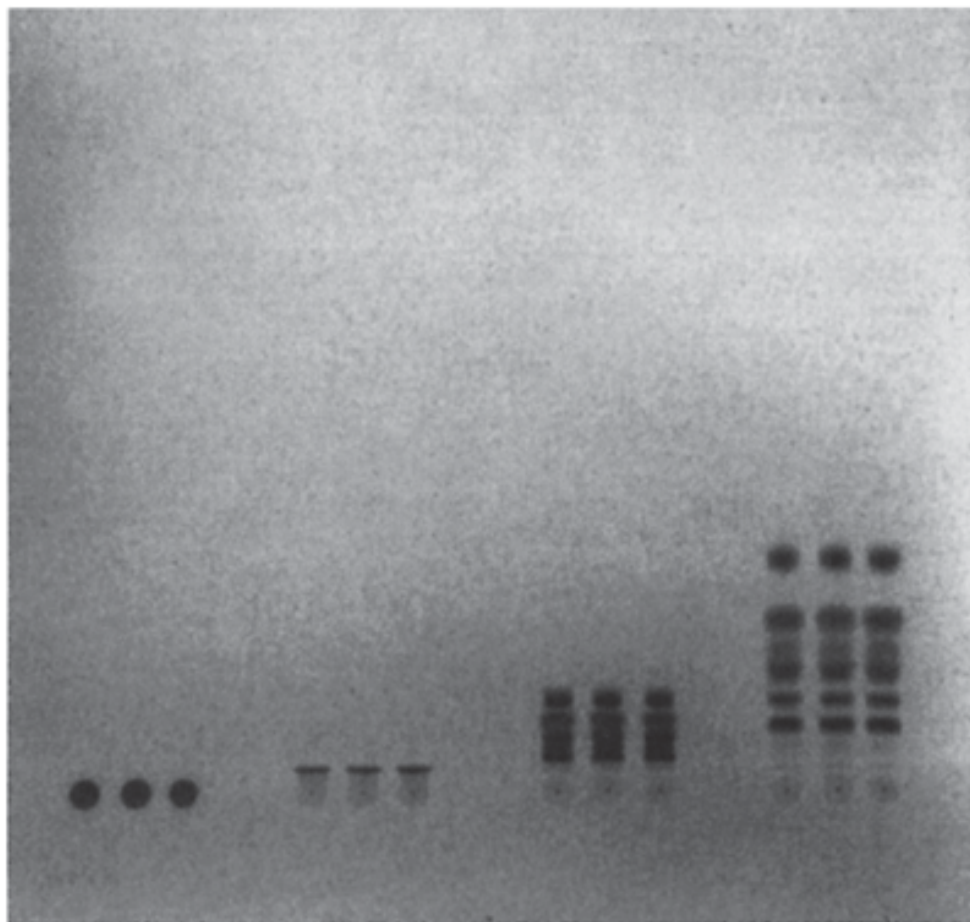


Figure 5 Sequence of development stages on a preadsorbent layer.
(Photograph supplied by Analtech.)

larger samples than partition TLC. Capacity increases roughly as a function of the thickness of the layer.

Samples for preparative layer chromatography are applied as bands across thicker layers. This can be done manually by allowing sample to flow from a pipet drawn along the edge of a ruler. The Camag Linomat, described above, is among the special instruments designed to apply samples more uniformly.

Analtech Preparative Uniplat-T wedge-shaped layers are tapered from a thickness of 1700 μm at the top to 300 μm at the bottom and have a 700 μm thick preadsorbent area at the bottom for manual or instrumental sample application. Sample concentration occurs in the preadsorbent zone, and low R_f bands tend to separate better in the thinner lower layer region.

VI. SELECTION AND OPTIMIZATION OF THE MOBILE PHASE

Selection of mobile phases is discussed in Chapter 6 of Ref. 1 and in Ref. 25. Criteria and strategies for optimization of mobile phases are covered in Chapter 3 of this Handbook, Chapter 3 of the previous edition of this handbook written by a different author, and Refs. 25, 128, 129, and 130. Solvent systems for different compound classes are given in the respective applications chapters in Part II of this Handbook and in the 29 *CRC Handbook of Chromatography* volumes edited by J.Sherma and G.Zweig between 1972 and 1993.

The flexibility of TLC relative to HPLC is enhanced by the greater choice of solvents available for preparing TLC mobile phases. The choice of solvents for HPLC is limited by the re-quirements for their chemical and physical properties imposed by the nature of the method. HPLC is a closed system operated under high pressure with on-line detection, most often using a UV monitor, and the column is continually reused. Solvent components with high vapor pressure (e.g., ethyl ether) or UV absorbance (benzene) or those that might degrade the column (NaOH) are difficult to use in HPLC but are readily applicable to TLC.

Single-development, capillary flow TLC typically produces <5000 theoretical plates and a zone capacity for baseline-resolved peaks of 10–14 (38). Therefore, selectivity, which is established by the choice of layer and mobile phase, is the most critical parameter in achieving the required separation. Mobile phases for TLC are selected in relation to the nature of the layer and mixture to be separated. The strength (polarity) of the mobile phase influences the R_f range of the solutes, while the chemical classification of the solvent components determines the interactions and selectivity of the system. Single solvents and solvent mixtures have been classified according to elution strength in relation to a particular sorbent. These “eluotropic series” are used along with knowledge of the solubility (polarity) characteristics of the mixture to select the chromatographic system to be used. As polarity increases, a solvent becomes stronger (increases R_f values) in normal-phase TLC, whereas solvents that are strong for RP-TLC are less polar. Retention in liquid chromatography is a complex process involving solute interactions in both the mobile and stationary phases. Assorted models of varying complexity have been proposed to attempt to explain and predict retention and separations, but the exact nature of the mechanisms is still incompletely understood (see Sec. 4.5 in Ref. 131 for an excellent discussion). Because of the similarity of results in comparable TLC and HPLC systems (Sec. XIII), analogous retention mechanisms are probably operative in the two methods.

Mobile phases are most often selected by consulting literature sources to find those that were previously used for separation of the compounds of interest or similar compounds. This is followed by a trial-and-error approach to modify the mobile phase for the particular layer and other local conditions being used, if necessary.

Systematic and computer-assisted approaches to mobile-phase selection and optimization have been developed based on solvent strength and selectivity parameters. Mixtures of solvents that differ in their interaction mechanisms and selectivity effects are used in these procedures, ranging from simple binary solvent combinations to mixtures of three solvents with a fourth weak, non-selective strength-adjusting solvent. Snyder has

arranged solvents in eight selectivity groups and within a selectivity triangle to simplify the systematic design of mobile-phase mixtures (see Ref. 25 for descriptions). Some of the strategies for solvent optimization are the PRISM A method (37, 132), the mixture design approach with the solvation parameter model (25, 133), a thermodynamic model (134), the Snyder—Soczewinski model (134), simplex (37), quality factor (37), window diagrams (25), overlapping resolution maps (25), and iterative procedures (25). The PRISMA model, which is a three-dimensional geometrical design that correlates the solvent strength and selectivity of the mobile phase, is the most successful and most widely used. It involves selection of three to five solvents for construction of the model plus a low, constant concentration of a modifier to improve the separation and reduce tailing, if necessary. A structured trial-and-error approach is used that is described in detail for use in TLC in Ref. 135. A fully automatic method for selection of mobile phases for silica gel TLC was described for tetrahydroisoquinolines and corresponding 1-ones using LSChrom software based on the Snyder theory (135a).

A. Solvents for NP-TLC

Poole and Dias (26) identify the following primary solvents for initial use in TLC mobile-phase optimization: hexane, toluene, methyl *tert*-butyl ether, dichloromethane, chloroform, ethyl acetate, acetone, pyridine, triethylamine, acetonitrile, methanol, 2-propanol, 2,2,2-trifluoroethanol, acetic acid, and water. All of Snyder's selectivity groups are represented by these solvents, which ensures exploration of the full range of solvent selectivity possibilities.

For silica gel TLC, the four solvents used most often are hexane as the diluent, with methylene chloride (selectivity group V, dipole interactions), chloroform (group VIII, proton donor), and methyl *tert*-butyl ether (MTBE) or diethyl ether (group I, proton acceptor or basic). The latter three solvents are located at the apexes of the selectivity triangle and are therefore most likely to enhance resolution. The systematic approach (136) is to prepare binary mixtures of each solvent with hexane in such proportions that R_f values are within the useful 0.15–0.7 range (0.3 optimal). If none of these gives the required separation, then equal-strength ternary mixtures of hexane with two of the other solvents are prepared, and, finally, a quaternary mixture of all four solvents, if necessary. In each case, solvent strength is controlled by the amount of hexane used, and selectivity is varied by changing the ratios of the other three solvents. Preparation of mixtures is facilitated by consulting an eluotropic series listing relative solvent strengths [P' values (137)] and calculating solvent strengths of mixtures as the sum of the volume fraction multiplied by the solvent strength for each component (138). Additional selectivity can be achieved by exploring other basic solvents in place of MTBE, e.g., triethylamine, pyridine, THF, or dimethylsulfoxide (DMSO). If the required separation is still not obtained, a change from acidic silica gel to amino-bonded silica (basic) or cyano-bonded silica (dipole-interacting) with four-solvent optimization should be tried next. If none of these normal-phase systems is successful, a reversed-phase system may be needed.

B. Solvents for Reversed-Phase TLC

Although the retention mechanisms on chemically bonded reversed phases are not clearly elaborated, R_f values for a series of solutes are usually reversed compared to the sequence on silica gel if water constitutes a large proportion of the RP mobile phase. It is also possible to obtain excellent separations on RP plates by using entirely organic mixtures as the mobile phase.

Two-solvent mixtures composed of water plus an alcohol, acetonitrile, acetone, dioxane, or an ether are widely used, with methanol-water (8:2) a convenient first-try solvent for C_8 or C_{18} layers. Solvent strength is varied by changing the water/organic modifier ratio, and selectivity by using a different modifier or modifier mixture. A more polar sample requires a weaker solvent (relatively more water). In general, small changes in mobile-phase strength have less effect on R_f in RP-TLC than in normal-phase TLC, making mobile-phase selection easier in the former case.

A four-solvent, seven-mixture optimization approach similar to that described in Section IV.A but based on Snyder's RP solvent strength (S) values was illustrated by Sherma and Charvat (140) for C_{18} RP-TLC using methanol, acetonitrile, and THF, with water as the weak, strength-adjusting carrier. Additional solvent modifiers recommended for RP-TLC include isopropanol, dimethylformamide, and DMSO.

Buffered weak acid and basic mobile phases or ion-pair reagents (strong acids and bases) are used to separate ionic and easily ionized compounds by RP-TLC (25). Hydrophilic quinolines were separated by adsorption-ion association on diol-silica layers by using solutions of the ion-pairing reagent di(2-ethylhexyl)orthophosphoric acid in polar solvents as mobile phases (141).

C. Solvents for Ion-Exchange and Gel TLC

Mobile phases for ion-exchange TLC are usually aqueous solutions with specified pH and ionic strength. pH determines the degree of exchange by controlling the ionization of solute and exchanger functional groups, whereas increasing ionic strength generally reduces the retention of solutes. Organic solvents have been added to increase selectivity in some ion-exchange systems.

Mobile phases for gel TLC must dissolve the sample and allow the gel network to swell. For gel permeation TLC on organic polymer layers (e.g., styrene-divinylbenzene), organic solvents such as THF are common, whereas aqueous buffers are employed for gel filtration chromatography on layers such as dextran.

VII. DEVELOPMENT TECHNIQUES

Layer development techniques are covered in Chapter 7 of Ref. 1.

A. Linear Development

Development of a TLC plate is most often carried out in the ascending mode by immersing the lower edge of the plate in the mobile phase in a rectangular glass chamber (N-chamber) (see A and B in Fig. 6). Chambers made from pressed glass, as shown in Fig. 6, or lightweight sheet glass are available commercially. "Saturated" or "unsaturated" chamber conditions can be used for development. In the former case, the mobile phase is poured into a chamber lined with a filter paper sheet or saturation pad, and the chamber is covered for a period of time (typically 15 min) to allow vapor equilibration. The chamber is quickly opened, the plate with applied initial zones is inserted, and the tank is covered again. For development under unsaturated conditions, mobile phase is poured into a chamber containing no paper liner, the plate is inserted, and the chamber is covered immediately. The chamber becomes progressively more saturated with increasing duration of the separation. Unsaturated chambers usually result in higher R_f values and lower efficiency (38).

Conditions inside TLC chambers during development with solvent mixtures are complicated because of the presence of three phases: the layer, liquid mobile phase, and vapor. Evaporation and condensation processes continually occur, and mobile-phase gradients are formed because the more polar components will be sorbed preferentially by the hydrophilic layer, causing the remaining solvent to be depleted in this solvent (solvent demixing). These gradients, which are not deliberately chosen or controlled as are mobile-phase gradients in HPLC (and occasionally in TLC; see Chap. 6), are detrimental to reproducibility of analyses but cause areas of different selectivity along the length of the layer that can be exploited for improving separations. Development times, separations, reproducibilities, and R_f values can be very different for the same systems in saturated and unsaturated N-chambers. Different types and sizes of developing chambers and small changes in the mobile-phase composition and/or temperature and relative humidity during development may cause dramatic changes in the retention characteristics of the compounds to be separated (40). Development conditions must be controlled and recorded if reproducible results are to be obtained from day to day in one laboratory or between laboratories. Procedures for standardizing TLC results have been described (142, 143). The most reproducible ascending capillary flow development conditions for TLC and HPTLC plates are achieved using the Camag Automatic Developing Chamber (ADC).

The bottom of the Camag twin-trough N-chamber is bisected by a glass ridge running along its center. It is used as a conventional saturated or unsaturated chamber by placing mobile phase only on the side where the plate will be inserted (4–20 mL is used depending on the plate size). In a second mode, one side is filled with mobile phase and the plate is placed into the other, empty, side. After equilibration of the chamber space and layer with vapors, development is started

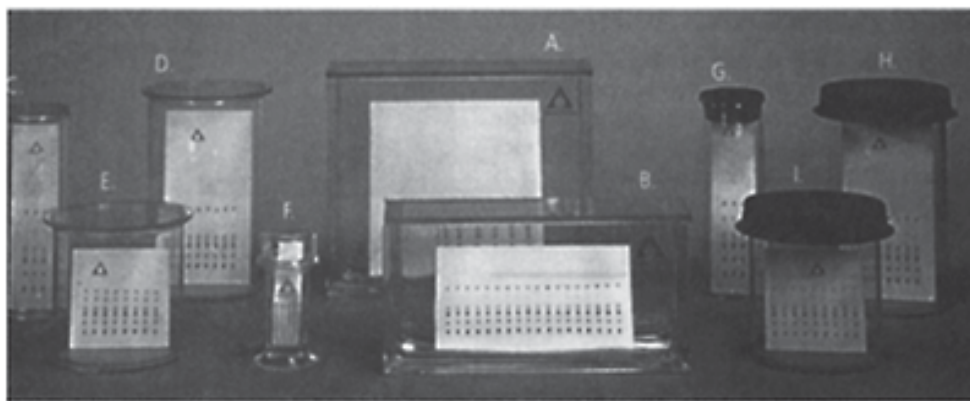


Figure 6 Assorted rectangular (A and B) and cylindrical (C-I) flat-bottomed glass chambers for development of TLC and HPTLC plates of different sizes. (Photograph supplied by Analtech.)

without removing the cover by carefully tipping the chamber to transfer mobile phase to the side with the plate. In a third mode, a different solvent or humidity-controlling sulfuric acid-water mixture is put on one side and the plate and mobile phase on the other to provide gas-phase conditioning during development. Desaga sells a thermostatted chamber for control of temperature during development (118) (see Sec. VILG).

Sandwich chambers (S-chambers), which have a second glass plate placed about 1 mm from the surface of the layer and therefore a very small gas space, can be used unsaturated, or saturated through the presence of a mobile-phase-soaked counter layer or filter paper sheet or pad. S-chambers today are mostly horizontal, and the Camag Linear Development Chamber and Desaga H-Separation Chamber are examples. The Camag chamber allows development of 70 samples simultaneously from both ends toward the center on a 20×10 cm HPTLC plate, or one-half that number of samples can be developed over the full length of the plate from one end only. Ascending and horizontal S-chambers have been described (144).

The Camag HPTLC Vario System is a horizontal chamber that has a wide variety of operational modes and applications. The plate is placed layer down over a tray with various compartments, which can hold different mobile phases, humidity controlling liquids, and volatile acids and bases whose vapors will impregnate and condition or preload the layer. Developing solvent is in a separate tray and is transferred to the layer by a wick. The Vario chamber can be used to test six mobile phases side by side on one plate for solvent optimization, to determine if layer pre-equilibration (preloading) is advantageous, to ascertain if S- or N-chamber configuration is best, and to test different humidity conditions.

Two new techniques involving horizontal development have been described. In “halfway development,” a new horizontal sandwich chamber is used to apply mobile phase to any part of a plate in order to redevelop a separated zone. This chamber can also be used for relay development of an overlength plate, programmed multiple development,

gradient development, band application, concentration, and micropreparative separation (144a). Flow TLC (FTLC) involves sample injection into mobile phase flowing over a horizontal layer with continuous optical detection at a fixed layer position. Mobile phase is evaporated from the end of the plate to maintain constant mobile-phase velocity depending on capillary effects (144b). Practical applications of these new techniques have not yet been demonstrated.

Ascending development of TLC sheets has been carried out in plastic bags for quality screening of pharmaceuticals under field conditions (145).

Displacement TLC uses three different mobile phases (carrier, displacer, and regenerant) and three main steps (loading the sample; development of the displacement train, collecting the fractions of the separated bands; and regeneration). The displacement system is generated in situ, when the mixture of carrier and displacer is separated as a result of the carrier running faster than the displacer-carrier mixture. The principles, techniques, and possibilities of displacement TLC have been described (146), but few practical applications have been reported.

B. Circular and Anticircular Development

Circular or radial development was first carried out in a Petri dish containing mobile phase and a wick that touches the layer, supported on top of the dish, at its central point. The Camag Li-Chamber and Anticircular U-Chamber have been used for circular and anticircular development, respectively, but these instruments are no longer listed in the Camag catalog. Except for the very occasional publication of research employing circular development (e.g., Refs. 147, 148), the method is almost never used currently for analytical TLC. However, circular chromatograms are produced by use of the modern preparative methods, e.g., multilayer OPLC (ML-OPLC) and micropreparative U-RPC (149).

C. Multiple Development

Thin-layer chromatography with multiple development often allows separation of complex mixtures or closely related substances not resolvable with a single development. The plate is repeatedly developed in the same direction, with the mobile phase dried between runs. Each subsequent development achieves zone reconcentration as the trailing edges of the zones move closer to the fronts, resulting in narrower bands and greater efficiency, resolution, and sensitivity. The classic multiple development method involves repeated development with the same mobile phase for the same distance. As an example of its use, double development was required for silica gel HPTLC assay of potassium salicylate in diuretic tablets and capsules (150).

Multiple development can also be performed with a change in the solvent composition and/ or migration distance for each step in order to optimize the separation of certain mixture components. Compounds that are difficult to separate require a large number of developments with a selective solvent that initially produces low R_f values; maximum zone center separation has been shown to occur when the zones have migrated 63.2% of the development distance (151). Quantitative measurement can be made at the end of any development stage when the different elements are separated optimally. The zone-

refocusing effect of multiple development has been illustrated by a densitogram of the HPTLC separation of 10 PTH-amino acid derivatives (13).

An apparatus comprising an N-type chamber with connections for adding and removing solvents and gas phases is available from Camag for AMD. AMD involves the use of a stepwise gradient of different mobile phases of decreasing strength in 10–30 successive developments in the same direction, increasing in length by 1–5 mm, to separate complex mixtures with a wide polarity range. The initial solvent, which is strongest, focuses the zones during the first short run, and the mobile phase is changed for each, or most, of the following cycles. Between runs the mobile phase is completely removed from the developing chamber and the layer is dried and activated by vacuum evaporation and then conditioned with a controlled atmosphere of vapors prior to the next development. The combination of the focusing effect and gradient elution results in high resolution and improved detection limits. Widths of separated zones are approximately constant at 2–3 mm, and separation capacity for baseline-resolved components is 30–40 (13). A typical universal gradient for a silica gel layer involves 25 steps with methanol, dichloromethane or *tert*-butyl ether, and hexane as solvents (152). A theoretical model has been presented for computer-aided optimization of AMD separations (153), and philosophies for method development in AMD have been discussed (38). Examples of recent analyses using AMD include sugars on diol layers with a 15-step acetonitrile–water gradient decreasing linearly from 35% to 15% water (154), beet and cane molasses in the sugar industry (155), and different lipid classes (156). AMD is described in Chapters 5 and 6 of this Handbook.

D. Continuous Development

Continuous development is another technique that increases separating power relative to conventional ascending unidimensional development. The top end of the plate is extended outside of the chamber so that mobile phase evaporates and its flow is continuous. Weak solvents are used to increase selectivity, and development distances are kept short so that development time does not become excessive. The method has been used mostly with HPTLC plates, for which Regis makes the Short Bed/Continuous Development (SB/CD) Chamber.

It has been shown that minimum analysis time is always shorter for continuous development than for conventional development when conditions are analyzed (157). Optimum conditions for the continuous TLC separation of steroids in terms of analysis time, plate length, and mole fraction of a binary mobile phase were determined using the overlapping resolution maps technique (158).

Although the SB/CD chamber is specified for use in the latest edition of the U.S. Pharmacopeia (USP 24/NF 19, (621)p. 1917), the method has little current use.

E. Two-Dimensional Development

In 2-D TLC, a sample is spotted in the corner of a layer and developed sequentially at right angles using two mobile phases that provide complementary retention mechanisms, with drying between the runs. With the correct choice of mobile phases, sample components will be distributed over the entire surface of the layer, increasing resolving

power by almost the square of that obtained in a one-dimensional system. The zone capacity will increase from 10–20 for capillary flow TLC to 100–250 for capillary flow 2-D TLC (30). Predicted zone capacity for 2-D TLC with forced flow or AMD is approximately 1500 (13), but this has not been tested. If the same mobile phase is used in both directions, or different mobile phases that result in differences in intensity rather than true orthogonality, the zones become distributed along the diagonal between the two development directions rather than over the whole surface, leading to a resolution factor of only $\sqrt{2}$ because of the increased migration distances for the sample. The use of bilayer plates and chemically bonded layers that separate according to different mechanisms depending upon the nature of the mobile phase (both described above), as well as other types of specialized layers (see below), are advantageous for 2-D TLC.

Disadvantages of the 2-D method include difficulty with interpretation of results, reduced reproducibility compared to one-dimensional TLC, poorer detection sensitivity because of greater diffusion during two developments, and the inability to carry out reliable in situ quantification because standards cannot be developed together in both directions and slit-scanning densitometers are designed to measure zones in a single layer track. Electronic scanning with a video densitometer is a possible solution, but routine, accurate, and precise quantitative analyses of 2-D chromatograms have not been demonstrated.

Computer-assisted methods were described for mobile-phase selection and optimization of mixtures of eight pesticides (159) and 10 antihistamines (160) in 2-D TLC.

Recent applications of 2-D TLC include the following separations: penicillium fungal extract on a cyano-derivatized silica gel layer (161); opiates on HPTLC silica gel (162); PAHs on C_8 and diol mixed phases (163); fatty acids on urea- and silver nitrate-impregnated silica gel for the first and second dimensions, respectively (164); parabens and carboxylic acid additives in pharmaceutical formulations on silica gel, with paraffin impregnation after the first run (165); and saponins on mechanically connected silica gel and C_{18} plates (166). Overpressured development has been occasionally used in 2-D TLC (167), and mass spectrometry to identify the separated zones (168).

Two-dimensional TLC along with multiple unidimensional, programmed multiple, and automated multiple development were covered in an encyclopedia article (169).

F. Forced-Flow Planar Chromatography

Forced-flow development is accomplished by use of centrifugal force (RPC) or pressure (OPLC). Analytical or preparative forced-flow planar chromatography (FFPC) can be carried out off-line (starting with a dry layer), on-line with a stationary phase previously equilibrated with mobile phase as in HPLC, or a combination of the two (e.g., off-line sample application and on-line separation and detection).

Rotation planar chromatography (RPC) is mainly a preparative method that has been described in Ref. 149. It uses U (ultramicro), M (micro), or N (normal) chambers, which differ mostly in the size of the vapor space. RPC in M or U chambers is applicable to a wide range of substance classes and polarities. Continuous development is possible with different types of the RPC techniques if an outer ring is scraped from the stationary phase and the development is stopped and the compound of interest scraped off when it reaches

this ring. From analytical TLC separations in saturated or unsaturated tanks, mobile phases can be transferred via analytical ultramicro U-RPC and M-RPC (separation distance 8 cm, average layer particle size 11 μm) to preparative U-RPC and M-RPC (10 cm, 14–15 μm), respectively, if the mobile-phase strength and selectivity are kept constant. The sample is applied as a circle in the center of the layer. Preparative RPC is covered in Chapter 11 of this Handbook.

Overpressured layer chromatography (OPLC) (see Chap. 7 in this Handbook) was invented to overcome the changing velocity of the mobile phase in the plate and to eliminate the vapor phase present in capillary development TLC. The process is as follows (5): Samples are applied to the dry layer, which is placed into the pressurized development chamber. The layer is tightly covered and is sealed on its sides by an elastic membrane (plastic sheet), which is pressurized by an inert gas or water cushion. The mobile phase is delivered directly to the layer by pumping at constant velocity through a slit in the membrane.

Modes of OPLC include linear, two-directional, long-distance, and circular. The delivery point of the mobile phase is varied for each of these. Use of commercial plates with sealed edges prevents the mobile phase from running off the plate in the linear mode. On-line operation for analysis of one sample involves mobile-phase flow from the OPLC apparatus directly to an HPLC detector; the plate is dried after development and is then scanned separately in off-line linear analysis of multiple samples. On-line OPLC is most similar to HPLC, but linear off-line OPLC of up to 18 samples with a run distance of 18 cm on a 20×20 cm plate is most commonly used. Two-directional OPLC is used with less complex samples for separation of up to 70 samples over an 8 cm run distance. Samples are applied on two parallel, vertical origin lines in the center of the layer, and mobile phase enters from a channel in between and simultaneously develops the samples toward the sides of the plate. Long-distance OPLC extends the migration distance by employing three to five stacked plates with slits to direct mobile phase from one plate to the next. Samples are spotted in a radial pattern around the center of the plate for circular OPLC. Mobile phase enters at the center, and low R_f compounds are especially well resolved in this mode. A statistical method of mobile-phase selection was developed specifically for OPLC (170), and the PRISMA method can also be used with unsaturated chambers for the initial trial-and-error experiments.

The latest instrument for analytical and semipreparative OPLC is the Personal Basic System (BS) 50 (OPLC-NIT Engineering Ltd., Budapest, Hungary) (171). It consists basically of a separation chamber and a liquid delivery system. The separation chamber contains a holding unit, hydraulic unit, layer cassette, and drain valve, and there is a pumping system to deliver the mobile phase and hydraulic liquid. The entire apparatus and development process are computer-controlled, and external pressure (up to 50 bar), mobile-phase flow rate and volume, and development time can be automatically programmed. With this OPLC apparatus, minimum values of reduced plate height are 2.1–3.5, depending on the operating conditions and layer properties. The corresponding range for a good HPLC column is 2.0–2.5, so efficiencies are comparable under optimum conditions (39).

The principles, techniques, and instrumentation for OPLC are reviewed in Refs. 39 and 172.

G. Gradient Development

The three types of gradients that have been used the most in TLC are mobile phase, stationary phase, and temperature. Planned mobile-phase gradients must be distinguished from the natural, uncontrolled gradients resulting from solvent demixing during development. AMD, mentioned in Section VII.C, involves development in a commercial instrument with a "universal gradient" starting with the most polar (strongest) mobile phase and becoming increasingly weaker in order to form focused, well-resolved zones.

The horizontal DS-Chamber (Chromdes, Lublin, Poland) is a Teflon sandwich chamber that is often used with stepwise gradient development (increasing mobile-phase strength) (173). The use of mobile-phase gradients was reported for separations of pigments by RP- and NP-TLC (174, 175); phenolic acids on silica, propylamine, and diol layers (176); and alkaloids on sodium bicarbonate-impregnated silica gel (177). Strategies for computer-aided optimization of gradient elution programs were published (178, 179).

Stationary-phase gradients involve a continuous or discontinuous change of sorbent composition, and they are used much less than mobile-phase gradients. Discontinuous gradients are produced by treating different layer areas with different reagents to alter the separation mechanism or by casting layers with different sorbent regions. The latter can be commercial bilayer plates (described above) or layers made by using a modified sorbent spreader. As an example, 2-D TLC with a sorbent gradient (C_{18} and silica gel) was applied to the analysis of saponins (166).

Most TLC is performed at room temperature in nonthermostatted chambers, but recent use of temperature-controlled TLC by one research group has been reported. These workers have described homemade and commercial devices that provide a temperature gradient for separations of chiral and nonchiral compounds (180), technical problems associated with temperature-controlled TLC (181), and studies of the interactions between native cyclodextrins and n-alcohols (182) and the retention and separation of cholesterol and bile acids (183) using thermostatted RP-TLC.

Gradient development in TLC is described in Chapter 6 of this Handbook.

VIII. DETECTION AND QUALITATIVE IDENTIFICATION OF ZONES

Detection and qualitative evaluation are covered in Chapters 8 and 9, respectively, of Ref. 1, in Ref. 184, and in Chapter 8 of this Handbook.

A. Detection (Visualization) of Zones

After development, the plate is dried with a hair drier, in an oven, or on a programmable plate heater (Desaga or Camag) to evaporate the mobile phase. Compounds are detected on layers by their natural color, natural fluorescence under UV light, or quenching of fluorescence on a phosphor-containing layer or as colored, UV-absorbing, or fluorescent zones after reaction with an appropriate reagent. Additional methods are based on radiochemical detection (e.g., autoradiography, scintillation counting, and direct in situ scanning) and on biological properties (e.g., enzyme inhibition, bioautography, and

immunostaining techniques). A significant advantage of TLC over HPLC is that detection is static rather than dynamic or on-line. This eliminates the time constraints of detection on the fly and permits flexibility through the utilization of a variety of compatible detection techniques and reactions in combination.

Chromogenic reagents typically have detection limits ranging from low nanograms to several micrograms; fluorogenic reagents, from high picograms to high nanograms; and enzyme inhibition, from low picograms to low nanograms. The selectivity of the latter two reagent types allows detection and confirmation at low parts-per-billion (ppb) concentrations in many samples, at which level most chromogenic reagents would not be effective. Certain compound classes lacking convenient chromophores are especially amenable to TLC analysis because of the ease of using postchromatographic chemical reactions for their detection; these include organic acids, lipids, carbohydrates, amino acids and peptides, and surfactants (38).

Descriptions of more than 450 detection reagents are available in Volume II of the *Handbook of Chromatography* (185). Reagents for specific compounds are found in the data tables of Volume I and later volumes of this series devoted to different chemical classes. The chapters in Part II of this Handbook contain descriptions of many detection reagents applicable to particular types of compounds. New detection reagents are updated biennially in the *Analytical Chemistry* review of TLC written by Sherma (11). Books have been published on physical and chemical detection methods in TLC (186, 187).

Compounds that are naturally colored are detected on layers directly, whereas compounds with native fluorescence are viewed under UV light (Fig. 7). In some cases, fluorescence is visible on a wetted plate only, so the layer is impregnated with a nonvolatile liquid. Certain compounds fluoresce only when the layer is adjusted to a specific pH value, e.g., quinine at low pH. Compounds that absorb UV light can be detected on layers containing an indicator (phosphor) that fluoresces upon excitation with 254 nm or 366 nm UV light. When irradiated, absorbing compounds diminish (quench) the uniform layer fluorescence and are visualized as dark zones on a bright (usually green) background. One of the most common fluorescent indicators is manganese-activated zinc sulfate, which is excited by 254 nm UV radiation.

Color, UV absorbance, or fluorescence can be induced by application of a detection reagent that chemically reacts with the compound(s) to be visualized. Less commonly, detection methods utilize the biological activity of the separated compounds. The most active current area of biological detection involves immunostaining (Western blotting). For example, glucuronides of glycyrrhetic acid were detected after silica gel TLC by transfer to a poly(vinylidene difluoride) (PVDF) membrane and treatment of the membrane with sodium periodate solution followed by bovine serum albumin (BSA), resulting in glucuronides of glycyrrhetic acid-BSA conjugate. Individual spots were stained with monoclonal antibody against glycyrrhizin (188). Steroidal alkaloid glycosides have also been detected by TLC immunostaining (189). TLC immunostaining has been combined with TLC blotting/secondary ion mass spectrometry (SIMS) in a number of analyses, e.g., the analysis of glycosphingolipids (190). In situ enzymatic reactions have been used for direct detection and quantification of toxicologically active compounds at low levels. As an example, 20–80 ng of pentachlorophenol could be quantified using a slit-scanning or video densitometer based on the degree of the compound's cholinesterase inhibition (191).



Figure 7 Viewing cabinet for inspecting TLC plates under 254 nm or 366 nm UV light. (Photograph supplied by Camag.)

The detection reagent solution is usually applied postchromatography by spraying or dipping the layer. Alternatively, the reagent may be preimpregnated into the layer prior to spotting and mobile-phase development, or it can be included in the mobile phase for impregnation during the development step. Volatile reagents may be applied by exposing the plate to their vapors. This is the case for iodine, a “universal” detection reagent for many organic compounds, and induced vapor-phase fluorescence (192). Charring with concentrated sulfuric acid is another general detection method. Many reagents are specific for compounds with certain functional groups. Reagent-free detection can be achieved with some compound-layer combinations by simply heating the plate (thermochemical reaction). Sugars have been detected as fluorescent zones by heating on amino layers and creatine as a quenched zone on silica gel F₂₅₄ layers (193).

For quantitative TLC, it is essential that the detection reagents be applied uniformly. Manual spraying is used most often, but a more homogeneous distribution of reagent in the layer may be achieved if the reagent can be applied by use of a manual dipping device (Desaga). Camag’s plate immersion device, which effects vertical plate movement at a uniform rate for precise dip application of reagents, and Desaga’s automatic, computer-controlled ChromaJet DS 20 spray apparatus have been used for performing quantitative analyses with improved accuracy and precision.

The high carbon content of RP layers hinders some common detection methods. Most charring reagents and those that do not wet the layer cannot be used. Reagents that have been successfully employed on C₁₈ layers include iodine, 10% phosphomolybdic acid in

ethanol with heating at 120°C (for lipids), 10% sulfuric acid in ethanol with heating for 2–3 min (general reagent), and fluorescamine (amino acids).

B. Qualitative Identification of Zones

A major use of TLC is to identify unknown sample components or to confirm the identity of compounds initially detected by GC or HPLC. Qualitative identification is based on characteristic colors produced by a selective detection reagent combined with R_f values. Identification can be aided by using more than one detection reagent, often applied in sequence to a single chromatogram. To have the highest confidence in the identification procedure, R_f values of sample zones should be compared to standards in more than one type of TLC system with different mechanisms, e.g., adsorption, normal- and reversed-phase bonded layers, and ion-exchange layers (194). Alternatively, multiple mobile phases can be used with one type of layer. For example, principal component analysis of standardized R_f values of 443 drugs and their metabolites chromatographed on silica gel in four mobile phases was employed for identification of unknown drugs in cases of overdose intoxication or poisoning (195).

In general, at least one spectroscopic method must be used in addition to TLC results in order to make a valid statement of identity (152). TLC has been combined off-line and on-line with UV-visible (UV-vis), fluorescence, NMR, IR, Raman, photoacoustic, line narrowing, and MS for compound identification. See Refs. 196 and 197 for reviews of these coupled methods.

Modern densitometers allow in situ measurement of visible and UV absorption and fluorescence excitation and emission spectra. Sample and standard spectra from the same layer should be directly compared for identification purposes. Lack of a match is definite proof that the compounds in the zones are different, but an apparent match may not be sufficient proof that the compounds are the same if the spectra are not characteristic of the total molecular structure (38). If the corresponding standards are not available for comparison with presumptive sample zones, UV-vis and fluorescence spectra are usually not sufficiently characteristic to identify unknowns by making structural assignments through spectral interpretation. Recording UV-vis spectra of separated zones both before and after pre- or postchromatographic derivatization increases the probability of correct zone identification (152). Novel library searching software was introduced for improved identification of compounds in autopsy urine samples by use of in situ UV spectra (198).

Virtually all combinations of TLC and NMR spectrometry for separated substance characterization have involved zone removal and extraction of the analyte with a solvent. A preliminary paper on high resolution magic-angle-spinning solid-state NMR for in situ compound identification was published (199), but no subsequent reports on the use of this approach have been found.

HPLC effluents have been deposited on a silica gel layer, which was not used for chromatography but acted as a substrate for analyte identification by fluorescence line-narrowing spectrometry (FLNS) (200). Off-line coupling of TLC and FLNS for high-resolution, low-temperature spectral characterization was reviewed (201). FLNS and PEI-cellulose TLC were coupled for low-picomole detection of DNA adducts using fluorescence background subtraction, time-resolved detection, and a new synchronous scanning procedure (202).

TLC is combined with Fourier transform infrared (FTIR) spectrometry off-line by scraping off the zone and eluting the compound onto a KBr pellet or on-line by subtracting a silica gel layer background spectrum from a spectrum of the zone, both measured in situ using diffuse reflectance FTIR (DRIFT) spectrometry (175). The silica gel TLC on-line method has been used to identify hydrocarbons in wastewater (203), the major marijuana metabolite in urine (on-line TLC-UV was also used) (204), impurities in chlordiazepoxide bulk drug powder and tablets (205), and impurities in flurazepam bulk drug powder and capsules (on-line TLC-UV and a specialized plate containing 50% magnesium tungstate were also used) (206). The development, techniques, and applications of HPTLC-FTIR on-line coupling have been reviewed (207).

Photoacoustic spectrometry was applied to the characterization of TLC plates with respect to surface and in-depth distribution of different compounds inside the sorbent (208), as well as for qualitative and quantitative analysis of compounds separated by TLC, including mapping techniques (see Ref. 209 for a review).

Surface-enhanced Raman scattering spectrometry (SERS) can be used to characterize nanogram to picogram amounts of solutes on silver-treated HPTLC plates using a visible (Ar ion, 514.5 nm) or near-IR (NIR) (Nd-YAG) laser for excitation. Methods for silica gel layer preactivation include dipping the plate into a solution of silver oxalate and subsequently pyrolyzing it to form silver particles on the layer (210), vacuum evaporation (211), and citrate reduction (212). It was found that the type of plate and the development procedure (traditional vs. OPLC) influenced significantly the quality of SERS spectra obtained (213). NIR-SERS was shown to be useful for both qualitative and quantitative analysis in a single step (214).

Thin-layer chromatography coupled with mass spectrometry (TLC/MS) is covered in Chapter 9 of this Handbook, and the following MS methods combined with TLC have been reviewed: fast atom or ion bombardment, matrix-assisted laser desorption ionization (MALDI), surface assisted laser desorption ionization (SALDI), and the electrospray (ES) interface (215).

Recent applications of off-line TLC/MS, which involves scraping of separated zones from the layer and extraction of the analyte from the sorbent, include analyses of organic reactions by TLC/MALDI time-of-flight (TOF) MS (216); the major ganglioside from crucian carp liver by TLC/ES-MS (217); deramciclane metabolites by OPLC/MS (218), fast atom bombardment (FAB) tandem mass spectrometry (MS-MS) (219), and digital autoradiography (DAR); numerous drugs and metabolites by TLC/electron impact (EI)-MS (220); caffeine in soft drinks by TLC/SPE-atmospheric pressure chemical ionization (APCI)-MS (221); and lipopolysaccharides from bacteria by MALDI (222).

On-line TLC/MS analysis without elution from the layer was reported for benzodiazepines by TLC/FAB-MS and MS-MS (223), for impurities in a newly synthesized drug by TLC/MALDI-TOF-MS (224), and for picogram levels of nucleotides (225) and pesticides (226) by TLC/ MALDI-MS. The application of SALDI was demonstrated for a wide range of organic compounds including peptides using silica gel with the surface covered by activated carbon particles and added glycerol (227). A hybrid plate for TLC/MALDI-MS that recovered about 100% of the analytes consisted of a silica gel layer and a MALDI layer configured adjacently on a common backing; after separation on silica gel, the plate was rotated 90° and the analytes eluted onto the MALDI layer (228). Plates are prepared for TLC/MALDI-TOF-MS by brushing them with a

supersaturated solution of matrix or electrospraying the matrix solution (229). TLC/MS was the subject of an encyclopedia article (230).

The following criteria for identification of an analyte by TLC or HPTLC were recommended (230a) after study by a board of European experts. The R_f value of the analyte should agree within $\pm 3\%$ compared to the standard material under the same conditions. The visual appearance of the analyte should be indistinguishable from that of the standard material. The center of the spot nearest that due to the analyte should be separated from it by at least half the sum of the spot diameters. For identification, additional cochromatography in the TLC step is mandatory. As a result, only the spot presumed to be due to the analyte should be intensified; a new spot should not appear. If full-spectrum detection is possible, the maximum absorption wavelength in the spectrum of the analyte should be the same as that of the standard material, within a margin determined by the resolution of the detection system. The spectrum of the analyte should not be visually different from that of the standard material.

IX. DOCUMENTATION OF TLC RESULTS

Methods for documentation of TLC results are described in Chapter 9 of Ref. 1 and Chapter 8 of this Handbook. Procedures were described for true color photodocumentation of 254 nm and 366 nm UV-irradiated chromatograms (231) and optimized black-and-white and color photography of colored, fluorescent, and fluorescence-quenched zones (232). A video image archival system made use of integrating cameras (233). A system comprising a computer, digital image scanner, and black-and-white and color printers was described for documenting visible TLC spots but not those detected under UV light (234). Commercial photographic and video documentation instrumentation for colored, fluorescent, and quenched zones is available from commercial sources, including Camag (235, 236). Various paper brands were tested for printing and color stability characteristics and the effects on archiving of chromatograms produced from the Camag video system using an ink jet printer (237).

X. QUANTIFICATION

Quantitative TLC is the subject of Chapter 10 of Ref. 1. The theory and techniques of densitometric TLC are elaborated in Chapter 10 of this Handbook, and instrumental aspects of quantification are presented in Chapter 5. Quantification of lipids and hydrocarbons and some other types of compounds has been carried out on rods of silica gel or other sorbent with direct interfacing to a flame ionization detector (FID) (the Chromarod or Iatroscan system). TLC-FID is covered in Chapter 13 and Chapter 19 on hydrocarbons in Part II of this Handbook but not in this chapter.

A. Introduction

Visual comparison of the spot intensity of a definite sample aliquot with the intensities of simultaneously developed reference spots containing known weights of analyte is a

simple, direct method for quantitative analysis. The method is only semiquantitative, with accuracy and precision in the 10–30% range, but this level is often adequate for the purpose intended. Visual comparison works best if amounts near the detection limit are applied and the sample is closely bracketed by standards. Visual estimation is specified in various pharmacopoeias for purity testing of both drug active raw materials and formulated products (238).

B. Zone Elution

The zone elution method involves the following steps: drying the layer, locating the separated analyte zone, scraping the portion of layer containing the analyte, collecting the sorbent, eluting the analyte from the sorbent, and measuring against standards by an independent microanalytical method such as solution absorption or fluorescence spectrometry, GC, HPLC, or voltammetry.

The chromatogram is dried to remove the mobile phase, components of which might interfere in the determinative step. The conditions of drying must not cause loss of the analyte by volatility or decomposition. Zones are located by direct observation for compounds that are naturally colored or fluorescent or those that quench fluorescence on phosphor-containing layers. Other compounds must be located by application of a visualizing reagent to samples that are chromatographed simultaneously on outside lanes of the layer to serve as a guide for the areas of the layer that are removed by scraping. The zones are scraped and transferred carefully to a suitable container. The analyte is eluted from the sorbent using a solvent that provides complete, or at least reproducible, recovery.

The zone elution quantification method is tedious and time-consuming and is likely to be inaccurate because of difficulties in locating the exact zone boundaries, loss of sorbent during scraping and collection, nonreproducible or incomplete elution from the sorbent, and background interferences due to eluted impurities from the sorbent. These errors are minimized if standards and samples are chromatographed, scraped, and eluted together as consistently as possible, and if an equal-size blank area of layer is scraped and eluted in the same way. Prewashing the layer by development with an appropriate solvent will help to minimize the blank value.

An apparatus was described to facilitate sample elution without transfer of the solid; a total solvent volume of only 60 μL was used, and recoveries were >90% (239). Camag sold the Eluchrom automatic elution instrument for a number of years, but it has been discontinued.

Despite its inconvenience, the basic TLC elution method, usually combined with UV-vis absorption or fluorescence spectrometry, is used advantageously to separate and quantify a great variety of analytes in laboratories not equipped with a densitometer. The spot elution method continues to be prescribed in some assay methods in the U.S. Pharmacopeia.

C. Slit-Scanning Densitometry

In situ measurement of zones with a densitometer is the preferred method for quantitative TLC with maximum accuracy, precision, selectivity, and sensitivity. Densitometry in TLC is reviewed in Ref. 240.

Substances separated by TLC or HPTLC are quantified by in situ measurement of absorbed visible or UV light or emitted fluorescence upon excitation with UV light. Absorption of UV light is measured either on regular layers or on layers with incorporated phosphor, the latter resulting in dark zones on a fluorescent background (fluorescence quenching). Only those substances that have absorption spectra overlapping the excitation spectrum of the phosphor will be detected and quantifiable by this method. Although it has been claimed in the literature that better quantitative results are obtained for direct measurement of UV absorption, more analyses have been reported based on fluorescence quenching (e.g., 52).

Scanning densitometers manufactured by different companies (e.g., Desaga) (Fig.8) have many common features. Halogen or tungsten lamps are used to provide light for the visible region, deuterium lamps for the UV region (absorption measurement), and mercury or xenon arc sources or a laser (241, 242) for fluorescence excitation. Filters or monochromators (prism or grating) are employed for wavelength selection, and a photomultiplier tube or photodiode detector for signal measurement. The plate, mounted on a movable stage controlled by stepping motors, is scanned with a fixed beam of monochromatic light in the form of an adjustable rectangular slit, the height of which is matched to the width of the largest spot or band. Most reported analyses have involved HPTLC plates and single-wavelength, single-track linear reflectance scanning, but transmission scanning is sometimes used (243). Signal diminution (absorbance) or increase (fluorescence) between the zone and a blank area of the layer is the measurement upon which quantitative analysis is based. The use of laned plates causes the initial and developed zones to be present in accurately known track locations, which is advantageous for setting up and operating both manual and automatic scanners.

Single-beam scanners may produce chromatograms with drifting baselines due to irregular or impure layers. Double-beam scanners are able to eliminate general plate background effects, but these are no longer manufactured or used to any extent. In dual wavelength scanning (175, 244), two monochromators alternately furnish the sample lane with a reference wavelength (minimal absorbance by the analyte zone) and a sample wavelength (maximum absorbance by the analyte). The reference wavelength cancels out background interferences contributed by the sample and corrects for layer irregularities. Zigzag (flying spot) scanning (244, 245), which uses a spot of light that moves over the zones with swings that correspond to the length of the slit, provides more reproducible readings for zones with irregular shapes or nonuniform concentration distributions. It has been claimed that simultaneous measurement of transmission and reflection will also diminish the effects of noise arising from an inhomogeneous plate background and improve sensitivity, but use of this procedure is seldom reported. A rapid, high-resolution fiber-optic diode array HPTLC scanner was described recently (246).

Computer-controlled densitometers can perform a number of functions: data acquisition by scanning over an entire plate following a preselected geometric pattern

with control of all scanning parameters; automated peak searching and optimization of scanning for each fraction located; multiple-wavelength scanning to find, if possible, a common wavelength for all substances to be

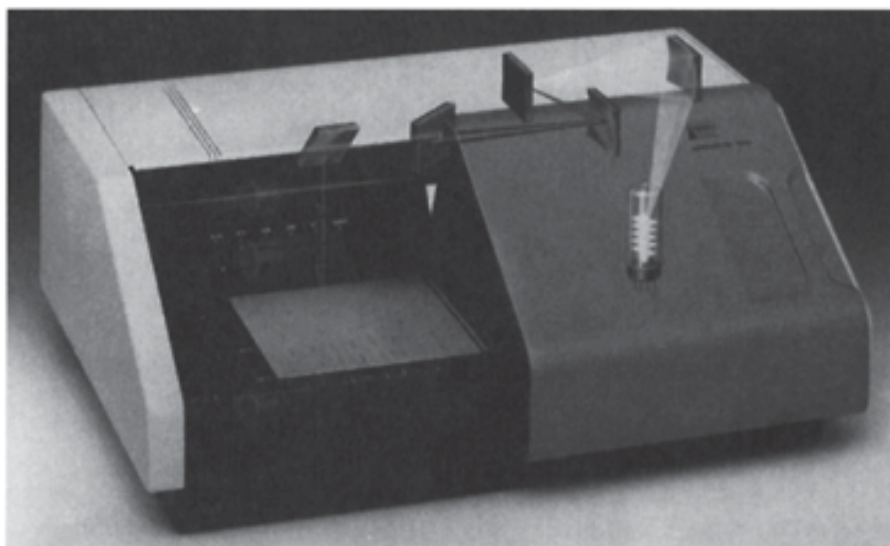


Figure 8 Photograph of the Desaga Densitometer CD 60 with a superimposed schematic diagram of the light path including (right to left) the source lamp, two mirrors, grating monochromator, mirror, beam splitter, plate with chromatogram to be scanned, reference and measuring detectors above the plate (reflection), and detector below the plate (transmission). (Photograph supplied by Desaga.)

quantified, to optically resolve fractions incompletely separated by TLC, and to identify fractions by comparison of spectra with those of standards cochromatographed on the same plate or stored in a spectrum library through pattern recognition techniques; baseline location and correction; computation of peak areas and/or heights of samples and codeveloped standards and processing of the analog raw data to quantitative digital results, including calculation of calibration curves by linear or polynomial regression, interpolation of sample concentrations, statistical analysis of reproducibility, and presentation of a complete analysis report; and storage of raw data for later reintegration, calibration, or evaluation with different parameters without rerunning the chromatogram. A high quality chromatogram with compact, regularly shaped and well-resolved zones

will lead typically to relative standard deviation (RSD) values in the range of 0.5–3% in quantitative HPTLC using a modern commercial computer-controlled densitometer.

The ability to spot unknowns and standards on the same plate and subject them to the same chromatographic conditions (in-system calibration) is an inherent advantage of quantitative TLC compared to sequential elution column chromatography. Systematic errors are minimized, an internal standard is less often required, and accuracy and precision values compare very favorably to those of GC and HPLC. Automatic instruments for sample application are necessary for the highest precision and accuracy in quantitative TLC. Because signal response is related to spot size for a fixed weight of analyte (247), it is usually recommended to apply a fixed volume of the sample and standard solutions of different concentrations to produce zones of constant size but varying intensity. However, the application of constant volumes appears not to be necessary for good results on laned preadsorbent plates if the developed bands spread across the width of each lane or when narrow bands are directly applied with the Linomat spray-on apparatus. In these cases, different volumes of a single standard solution can be applied.

Compared to absorption, fluorescence densitometry (248) has the advantages of higher sensitivity (often low picogram levels), calibration curves with a wider linear range (10^2 – 10^3), and improved selectivity because few compounds fluoresce and characteristic excitation and emission wavelengths can be chosen. Enhanced sensitivity has been obtained by impregnation of the dry plate with an antioxidant to reduce quenching from oxidation reactions or with a fluorescence-enhancing liquid such as paraffin, glycerol, or Triton X prior to scanning. Nonfluorescent compounds can often be converted to fluorescent compounds by pre- or postchromatographic derivatization with a fluorogenic reagent (e.g., dansyl chloride) or by treatment with ammonium hydrogen carbonate vapors at 100–150°C for 1–12 h to produce a reproducible fluorescent product. One of the most successful areas for application of fluorodensitometry is the quantification of toxins, e.g., deoxynivalenol in cereal products (249) (see Chapter 32 on toxins in Part II of this Handbook).

Absorption of light by zones on TLC plates is not described adequately by the LambertBeer law that is usually applied to solution spectrometry, because of the diffuse reflectance (scattering) by the sorbent particles. The Kubelka-Munk equation, which includes both light absorption and scattering coefficients, is usually applied as the basis of in situ TLC quantification, especially when reflected light is employed. This equation predicts a nonlinear relationship between the detector signal for reflectance measurements (peak area or height) and the amount of analyte (weight or concentration). Calibration curves obtained in practice using the reflectance or transmission scanning mode are unique for each analyte, have an intercept greater than (0, 0), and are essentially linear at low weights but tend to curve toward the weight axis at higher weights. If the calibration plot obtained by linear regression does not have a sufficiently high correlation coefficient (*r* value), then application of lower standard weights can be tried or the calibration curve can be fit to a polynomial function using the densitometer software. The external standardization method, with interpolation of the weight or concentration of unknowns from the calibration curve, has been used most often for quantitative densitometry. The internal standardization quantification method is used only occasionally, and the standard addition method not at all. The data pair sample

application scheme is preferred by some analysts, especially when maximum precision and accuracy are required in pharmaceutical assays. In the data pair method, all the standard and sample solutions are applied twice, with duplicates not next to each other but separated by half the width of the plate (19). The principles of quantitative analysis in TLC and HPTLC are reviewed in Ref. 250.

As an aid in method development, quantitative TLC has been treated in detail from the theoretical and practical viewpoints, including a description of protocols for sample calibration; for establishing resolution, sensitivity, detectability, and optimum scan rate; and for comparing the performance characteristics of different slit-scanning densitometers (247).

The development of formal validation [quality assurance (QA)] procedures for TLC has been addressed in the literature mostly in relation to pharmaceutical analyses, e.g., HPTLC assay of theophylline in an effervescent tablet (251). Guidelines formulated by the International Conference on Harmonization (ICH) for analytical procedures were adapted for TLC for use at different levels, i.e., qualitative identity testing, assay of active ingredients, semiquantitative limit tests, and quantitative determination of impurities, and described by Ferenczi-Fodor et al. (252). Basic acceptance criteria for evaluation of validation experiments, based on the authors' previous practical experience (253–255), were proposed for accuracy, precision (repeatability and intermediate precision), specificity, detection limit, quantification limit, linearity, and range, and selected parameters for robustness testing of given procedures and QA of quantitative TLC testing by control charts were described. Szepesi and Nyiredy (238) describe rules for validation of pharmaceutical analyses that include scanning every zone in triplicate to establish instrumental error, spotting the same volume of test solution in triplicate, and spotting three bracketing calibration standards that contain a known relationship to the expected test solution value, e.g., 80%, 100%, and 120%. As a recent example outside of pharmaceutical analysis, an OPLC method for determination of aflatoxins in wheat was validated fully, including robustness testing (256). Validation of data is necessary for analyses performed under the good laboratory practice (GLP) guidelines (38).

D. Image Analysis (Video Densitometry)

Video camera systems are available from several manufacturers for documentation and densitometric quantification of TLC plates. As an example, the Camag VideoScan instrument consists of a lighting module with short- and longwave UV and visible sources upon which the layer is placed, a charge-coupled device (CCD) camera with zoom and long-time integration capability, and a personal computer (PC) under MS Windows control with frame grabber, monitor, and printer. The available software for quantitative evaluation, such as Camag VideoScan software (257), allows display of the tracks of the chromatographic image acquired with the video camera as analog curves and calculation of their peak properties (R_f , height, area, height percent, and area percent). For quantification, the computer creates a standard curve from the areas or heights of the standards and interpolates unknown values from the curve. In addition to commercial instruments, laboratory-assembled computer-controlled CCD camera systems have been described for HPTLC quantification (257a).

Video scanners have certain advantages, including rapid data collection over the entire layer surface, simple design with virtually no moving parts, and unique software approaches for archiving and comparing chromatograms (258). However, current instruments are not capable of illuminating the plate uniformly with monochromatic light of selected wavelength, are less sensitive, and provide lower resolution for chromatogram recording than slit-scanning densitometers. Video densitometers can measure visible spots that are colored, fluorescent, or quench fluorescence on F-layers (259) in transmittance and reflectance modes (260), but they cannot perform spectral analysis. In addition to a CCD camera, video densitometry has been carried out using a flat-bed scanner and commercial software (261). With improvements in electronic scanning, it is very possible that video densitometers will replace mechanical scanning densitometers at some time in the future, with especially great potential for evaluation of 2-D chromatograms (13).

XI. PREPARATIVE LAYER CHROMATOGRAPHY

Preparative layer chromatography (PLC) is used to isolate 10–500 mg or more of material on layers thicker than those used for analytical TLC. As examples of commercial preparative layers, Whatman, Analtech, and EM Science (Merck) offer 0.5–2 mm layers composed of silica gel, alumina, cellulose, and C₁₈ bonded silica gel. Preparative layers of other sorbents and thicknesses may be available from other manufacturers and have been made in the laboratory. The presence of a fluorescent indicator facilitates nondestructive detection. The term “micropreparative layer chromatography (MPLC)” has been used for separations of up to 10 mg on a 0.25 mm analytical layer.

Samples are applied as streaks, either manually or with an instrument such as the Linomat, or by using a solid-phase sample application (SPSA) device (149). Ascending and horizontal development have been used most often, and the mobile phase is often chosen after preliminary tests on analytical plates. Incompletely separated bands are scraped and eluted and rechromatographed on a second plate (262). Multiple development and gradient elution have been applied for complex mixtures (263), and RPC (Fig. 2) and OPLC have also been used for PLC and MPLC. A recent application of centrifugal PLC is the fractionation of moderate molecular weight polysiloxanes for use as secondary standards for column gel permeation chromatography (GPC) (263a).

If the compounds to be recovered are not colored or fluorescent and do not absorb UV light, a detection reagent must be applied to the edges of the plate to locate the zones to be recovered. Plates with prescored edges facilitate this process. Pure compounds are recovered by scraping and elution with a suitable solvent (Fig. 9).

Layers for PLC are discussed in Section IV.G, and application of zones in Section V.D.

Procedures and apparatus for PLC are described in Chapter 12 of Ref. 1, in Chapter 11 of this Handbook, and in Ref. 149.

XII. RADIOCHEMICAL TECHNIQUES

Radio-TLC techniques are described in Chapter 12 of this Handbook and Chapter 13 of Ref. 1.

Thin-layer radiochromatography (TLRC), or radio-TLC, is used for separation, identification, and measurement of radioisotopes. The principal methods employed are autoradiography, zonal analysis, and the use of radiation detectors. An important application of TLRC is in the quality control and development of radiopharmaceuticals such as Tc-99m (264).

In autoradiography, an X-ray or photographic film is exposed to emissions from radioactive zones on the layer to produce an image on the film. The exposed film is developed by the usual photographic methods to reveal spots of varying darkness at the locations of the separated zones. The darkness, which is related to the amount of radioactivity, can be quantified by densitometry using a calibration curve prepared from a film exposed to zones of radioactive standards. A disadvantage is the long exposure period required for certain weakly radioactive isotopes. Two variations of direct-exposure autoradiography are direct exposure with an intensifying screen (plates coated with inorganic phosphors) and fluorography (impregnation of a scintillator into the layer followed by direct exposure).

For zonal analysis, zones are removed by scraping, the sorbent is transferred into vials, scintillation fluid is added, and the light emitted due to interaction of the radioactive nuclei with the fluid is measured with a scintillation counter. The study of bile acids in humans by liquid scintillation counting coupled with densitometry is an example of an application (265).

A variety of radiation detectors have been used for TLRC, including spark chambers, radioscaners, linear analyzers, a radioanalytic imaging system (Wallac Ambis), multiwire proportional counters, and phosphor imaging analyzers. All of these have been described in Chapter 12 of the first and second editions of this Handbook, but use of the latter two has been reported most often since the second edition was published and is discussed below.

The DAR is a two-dimensional position-sensitive multiwire proportional counter that measures all radioactive zones simultaneously on a 20×20 cm plate. The metabolism of the anxiolytic compound deramciclone was studied by using DAR after separation by conventional TLC (266) and in combination with TLC/FAB-MS-MS (219) and OPCL/MS (218, 267, 268). TLC and DAR were also coupled for the analysis of neutral C₁₄ lipids neosynthesized by the human sebaceous gland (269).

Phosphor or bioimaging analyzers operate by using a phosphor imaging plate made of fine crystals of BaF:Eu²⁺ (Fugi Photo Co., Ltd.) to store emitted beta energy from the layer; scanning

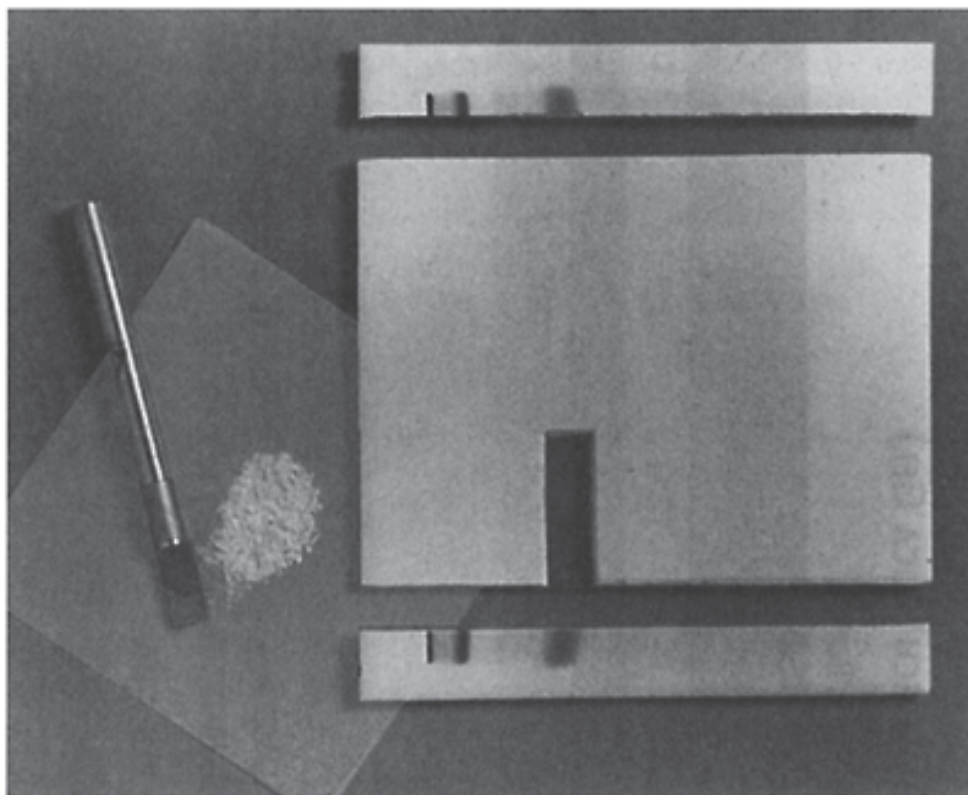


Figure 9 Prescored preparative plate with scraped zone. Edges are snapped off, detection reagent is applied, and the edges are realigned to locate the zones to be recovered by scraping. (Photograph supplied by Analtech.)

the plate with a laser in the reading unit; and measuring the resulting luminescence, which is proportional to the recorded radiation intensity, with a phototube. Radio-TLC with this detector has been used for detection as well as reliable quantification (270) of metabolites in pharmaco-kinetic studies (271) and of taxol and its metabolites in microsomal samples (272).

XIII. TRANSFER OF RESULTS FROM TLC TO HPLC

Two approaches for using TLC data as a pilot method for mobile-phase optimization in HPLC are correlation between retention parameters and thermodynamic description of adsorption systems with mixed mobile phases (273). The first method was used in a study involving silica gel, C₁₈, C₈, diol, NH₂, and CN layers, 62 pesticide standards, and a real sample by correlating k'_p retention values from TLC to k'_c values for HPLC by linear regression. Good results of transfer were obtained except with silica gel and diol, with

which some restrictions were needed because the HPLC and TLC sorbents were not of equal activity (274).

Multistep gradient elution RP-TLC on paraffin-impregnated silica gel of the colored pigments in red wine was shown to accurately predict the mobile-phase gradient for the same separation in C₁₈-HPLC (275). Successful transfer studies were also published for phenol and its methyl and chloro derivatives on bonded amino, cyano, and diol stationary phases (276) and for choosing binary gradient programs for HPLC of raw products in a synthesis research laboratory (277).

XIV. MULTIMODAL SEPARATION METHODS

The term “multimodal” has been used in two ways, to designate layers such as cyano-derivatized silica gels that can operate with two or more mechanisms (278) (see Sec. IV.D) and, in the context of this section, to specify multidimensional separations that are performed by on-line coupling of TLC, HPTLC, or OPLC with another technique in order to improve the separation capacity available from either of the individual methods. In the past, reports of the direct coupling of GC, SFE, and supercritical fluid chromatography to TLC have appeared, but recent publications are limited to combining HPLC and TLC.

Although the TLC-HPLC combination has been performed off-line by scraping and eluting TLC zones followed by injection into the HPLC instrument (279) or by TLC of collected column fractions (280), the most reported and most advantageous approach is when the two methods are coupled on-line using TLC as the second stage. This sequence allows utilization of the unique advantages of TLC, including further separation by a diverse mechanism, static detection with multiple methods, and storage on the layer of all zones in the column effluent fractions for evaluation without time constraints. A spray jet aerosol sample applicator (13, 281, 282) has been most commonly used to deposit column effluent onto the layer origin. As an example of an application, iprodione residues in vegetables have been determined by RP-HPLC followed by TLC-AMD (283).

XV. TLC DETERMINATION OF LIPOPHILICITY

The use of RP-TLC quantitative structure-activity studies aimed at determining lipophilicity has been reviewed (284, 285) but is not discussed in detail in this book. This parameter is important because it governs molecular properties such as penetration of bioactive compounds through hydrophobic cell membranes and uptake by target organs or organisms (i.e., biological activity).

Lipophilicity is evaluated on the basis of the linear relationship between R_m values and the concentration of organic solvent in the aqueous mobile phase in accordance with well-known TLC equations. The R_{m0} value, which is the theoretical R_m at 0% organic solvent or pure water, is determined by extrapolation (286, 287). Paraffin oil-impregnated silica gel (288) and C₁₈ bonded silica gel (289) layers are used in most cases, and the nature of the organic solvent has been found not to affect the measurement (290). Principal component analysis has also been used to evaluate lipophilicity (291, 292).

Many compound types have been studied, including 1,2,4-triazole (293) and furan (294) derivatives.

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2

Theory and Mechanism of Thin-Layer Chromatography

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I. INTRODUCTION

Chromatographic theory describes the physicochemical relationships that govern separations. Usually, semiempirical models of the chromatographic process are used that have a relatively simple thermodynamic background and give a bulk picture of the physical or chemical phenomena. Macroscopic models of the chromatographic process cannot mirror the respective separation mechanisms in any other way. Exceptions to this rule, if any exist, are rather negligible.

It is important to keep in mind two facts. First, one always has to be aware of the complexity of chromatographic processes and consequently of limitations of the existing semiempirical models. Second, one cannot forget that the study of chromatographic theory began only relatively recently and that there is much additional work to be done before it reaches its full potential.

In this chapter, basic knowledge about important physical phenomena in chromatography is introduced (Sec. II) as well as the main concepts regarding efficiency of separation (Sec. III). Further, the six overall semiempirical models of partition and adsorption chromatography are reviewed (Sec. IV), and their usefulness in everyday laboratory practice is discussed (Sec. V). Finally, the reader's attention is drawn to attempts that have been made to enhance performance of thin-layer chromatography (TLC) (Sec. VI).

II. BASIC PHYSICAL PHENOMENA

A. Capillary Flow

Transfer of a mobile phase through the thin layer is induced by capillary forces. Stationary phases (in adsorption, size-exclusion, and ion-exchange chromatography) and supports (in partition chromatography) are all microporous solids showing high specific surfaces (ranging from about 50 m²/g with celluloses to about 500 m²/g with silica), and for this reason they can be regarded as capillary agglomerations.

Solvents or solvent mixtures contained in the chromatographic chamber enter capillaries of a solid bed, attempting to lower both their free surface area and their free energy. The free-energy gain ΔE_m of a solvent entering a capillary is given by the relationship

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$$\Delta E_m = -\frac{2\gamma V_n}{r} \quad (1)$$

where γ is the free surface tension, V_n denotes the molar volume of the solvent, and r is the capillary radius.

From Eq. 1 it follows that the capillary radius r is very important for capillary flow and a smaller radius leads to more efficient flow. Preparation of commercial stationary phases and supports cannot provide pores that are all of ideally equal diameter, which results in certain side effects that contribute to broadening of the chromatographic spots. This problem is discussed in the next subsection.

B. Broadening of Chromatographic Spots

The most characteristic feature of chromatographic spots is that the longer the developing time and the greater the distance from the start, the greater their surface areas become. This phenomenon is not restricted to planar chromatographic methods but occurs in each chromatographic technique. Spot broadening is due to eddy and molecular diffusion, to the effects of mass transfer, and to the given mechanism of solute retention.

Eddy diffusion of solute molecules is induced by an uneven diameter of the stationary phase or support capillaries, which automatically results in an uneven flow rate of the mobile phase through the solid bed. In this way some solute molecules displace faster, whereas others are retarded, compared with the average displacement rate of the major portion of solute.

Molecular diffusion has nothing to do with the presence of a solid bed in the chromatographic system. It is the regular diffusion in the mobile phase, the driving force of each dissolution process, and for this reason it needs no further explanation.

The effects of mass transfer take place separately in the stationary and mobile phases. First let us describe the effect in the stationary phase. It can occur that for some energetic reason a fraction of solute molecules are "captured" by the stationary phase a little while

longer than the major portion of solute. Such retardation results in broadening of a chromatographic spot.

Two different effects of mass transfer are observed in the stagnant and flowing mobile phases. A certain amount of mobile phase can be trapped within the partially closed pores, and only gradually and slowly is it replaced by a fresh portion of mobile phase. This is what we call the stagnant mobile phase. If the solute molecules occasionally “dive” into such a blind pore, they will miss the main stream of the flowing mobile phase that carries the major portion of solute.

With the flowing mobile phase another phenomenon is observed. Those molecules that are in touch with the solid material move more slowly, while the others, passing through the center of the pores, displace more quickly. This friction-induced inequality of the flow rates additionally contributes to broadening of a chromatographic spot.

Mechanisms of solute retention, which are also responsible for spot broadening, differ from one chromatographic technique to another, and their role in this process is far less simple than that of diffusion and mass transfer.

All the aforementioned phenomena, which jointly contribute to spot broadening, used to be described as effective diffusion. This is a convenient term that, apart from being concise and informative, also underlines the fact that these phenomena occur simultaneously. Spot broadening results in mass distribution of solute in a given chromatographic spot. This distribution is presented by a respective concentration profile, which in practice can be established densitometrically. In Fig. 1, two examples of such concentration profiles are shown.

Numerous efforts have been undertaken to establish relevant theoretical models that could describe broadening of chromatographic spots and formation of the concentration profiles. The most interesting models are those that regard spot broadening as a two-dimensional process.

Two models of two-dimensional broadening of chromatographic spots were established by Belenky et al. (1, 2) and Mierzejewski (3). The common basic concept that enabled elaboration of these two models is Fick's second law, which describes the velocity of the concentration changes with a substance at a given point of a system:

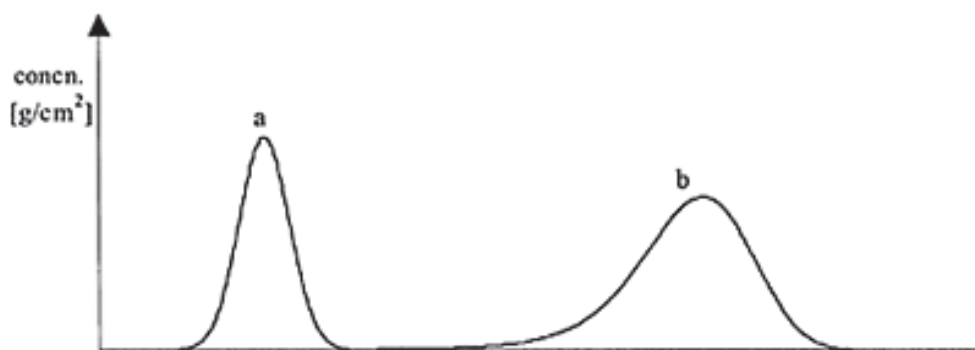


Figure 1 Two examples of concentration profiles: (a) symmetrical without tailing and (b) skewed with tailing.

$$\frac{\delta c}{\delta t} = -\operatorname{div} J \quad (2)$$

where c and t are concentration and time, respectively, and J denotes the mass flux of the investigated substance.

Upon the further assumptions of Belenky's dynamic model (1, 2), the following dependence was established, defining concentration of solute at time t and at a given point of the sorbent layer, described by the coordinates x and y (see Fig. 2):

$$c(x, y, t) = \frac{q}{4\pi R_f t \left[D_L \left(D_L + \frac{1 - R_f}{R_f} v^2 \tau \right) \right]^{1/2}} \cdot \exp \left[-\frac{1}{2} \left(\frac{(x - vt)^2}{2 \left(D_L + \frac{1 - R_f}{R_f} v^2 \tau \right) R_f t} + \frac{y^2}{2 D_L R_f t} \right) \right] \quad (3)$$

where q is the total amount of solute in a chromatographic spot; R_f is the basic TLC parameter introduced in Section III.C; D_L denotes the effective diffusion coefficient that characterizes broadening of a chromatographic spot; v is the migration velocity of the chromatographic spot center; and τ is the parameter representing a time lag in establishing equilibrium between the mobile and stationary phases (τ is also a function of the particle size of a solid bed).

From the main dependence of Belenky's model it follows that the concentration of solute in the chromatographic spot is described by a two-dimensional Gaussian distribution function, which can be rewritten in a simpler form:

$$c(x, y, t) = c_{\max} \exp \left[-\frac{1}{2} \left(\frac{(x - vt)^2}{\sigma_x^2} + \frac{y^2}{\sigma_y^2} \right) \right] \quad (4)$$

where

$$c_{\max} = \frac{q}{4\pi R_f t \left[D_L \left(D_L + \frac{1 - R_f}{R_f} v^2 \tau \right) \right]^{1/2}} \quad (5)$$

$$\sigma_x^2 = 2 \left(D_L + \frac{1 - R_f}{R_f} v^2 \tau \right) R_f t \quad (6)$$

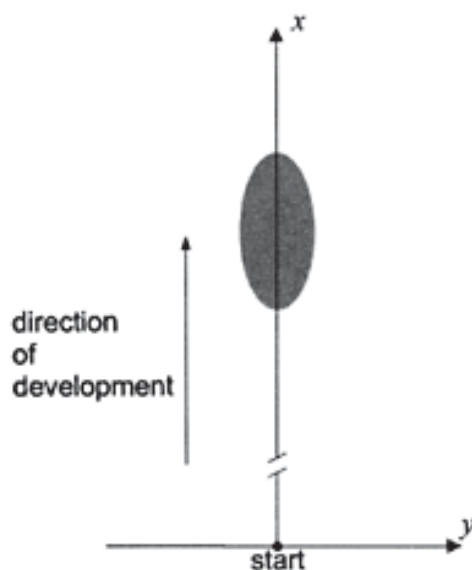


Figure 2 The diffused chromatographic spot. Illustration of Belenky's and Mierzejewski's models of spot broadening.

$$\sigma_y^2 = 2D_t R_f t \quad (7)$$

Mierzejewski's approach (3) to the problem was different. That author introduced four vectors denoting the speed of the two-dimensional effective diffusion of the solute: two of them parallel to the migration direction x but showing opposite turns ($\vec{H}_{x,1}$ and $\vec{H}_{x,2}$), and the analogous two vectors perpendicular to this direction ($\vec{H}_{y,1}$ and $\vec{H}_{y,2}$). His relationship for solute concentration at time t , and at the point described by the coordinates x and y , is

$$c(x, y, t) = \frac{q}{4\pi g w} \exp \left[- \left(\frac{(K+1)^2(x-l_s)^2}{16g w^2 K^{2(j-1)}} - \frac{y^2}{4g} \right) \right] \quad (8)$$

where

$$g = H_y^2 t (H_y = |\vec{H}_{y,1}| = |\vec{H}_{y,2}|); \quad w = \frac{H_{x,1} + H_{x,2}}{2H_y}; \quad K = \frac{H_{x,2}}{H_{x,1}}; \quad l_s = v t$$

Additionally, if $x \geq l_s$, $j=1$, and if $x < l_s$, $j=2$.

As can be deduced from Eq. 8, Mierzejewski's model also describes the concentration of solute in the chromatographic spot by a two-dimensional Gaussian distribution that can be presented in a simpler form:

$$c(x, y, t) = c_{\max} \exp \left[- \frac{1}{2} \left(\frac{(K+1)^2(x-l_s)^2}{\sigma_x^2} + \frac{y^2}{\sigma_y^2} \right) \right] \quad (9)$$

where

$$c_{\max} = \frac{q}{4\pi g w} \quad (10)$$

$$\sigma_x^2 = 8gw^2K^{2(j-1)} \quad (11)$$

$$\sigma_y^2 = 2g \quad (12)$$

As can be seen by observation of actual thin-layer chromatograms, many experimental concentration profiles can, in fact, be described by the Gaussian distribution curves.

C. Volatility of Solvents

Unlike the situation in column chromatography, the thin-layer microporous solid bed stays in unhindered contact with a usually voluminous space of the chromatographic chamber. The so-called sandwich chamber is an exception in this respect. Therefore, in thin-layer chromatography some special measures need to be undertaken to facilitate achievement of thermodynamic equilibria between the mobile-phase components in the gaseous and liquid forms. To make this point clear, let us imagine that to an empty chromatographic chamber we simultaneously introduce mobile phase and the chromatographic plate, automatically initiating the chromatographic process. What happens then in the “free” room over the mobile-phase surface? First it was occupied by air components and water vapors only, but, after addition of solvent or solvent mixture, it starts filling with the mobile-phase molecules. This process will last until saturation of the “free” room with the gaseous mobile-phase components is completed. Where do these gaseous mobile-phase components come from? Partially from the bulk liquid, and partially from the chromatographic plate surface. In this way we obtain an unwanted change of the mobile-phase composition directly within the pores of the solid bed. One can imagine how much this phenomenon affects separation and how damaging it proves to be for reproducibility of the retention data.

The mental experiment presented above was aimed at explaining the necessity of saturation of the chromatographic chamber with the gaseous mobile-phase components prior to initiation of the chromatographic process proper. In other words, it was meant to demonstrate the indispensability in this process of thermodynamic equilibrium between the gaseous and liquid mobile-phase components. Due to them, evaporation cannot affect the mobile-phase composition either in the bulk form or in the capillaries of the solid bed. Equation 13 gives the thermodynamic condition of these equilibria:

$$\mu_{i(g)} = \mu_{i(l)}, \quad i=1, 2, \dots, n \quad (13)$$

where $\mu_{i(g)}$ and $\mu_{i(l)}$ are the chemical potentials of the i th mobile-phase component in the gaseous and liquid form, respectively, and n denotes the number of components.

In Fig. 3, a scheme of the chromatographic system with the thermodynamic equilibria achieved between the gaseous and liquid mobile-phase components is presented.

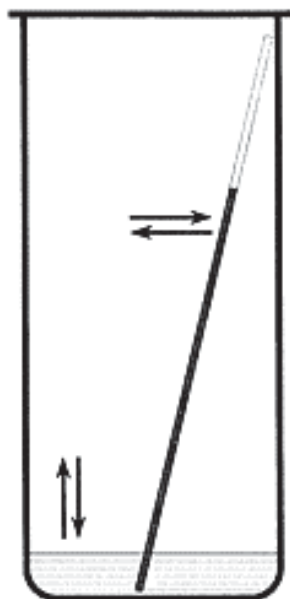


Figure 3 Scheme of thermodynamic equilibria between the gaseous and liquid mobile-phase components in a presaturated chromatographic chamber.

III. MEASURES OF CHROMATOGRAPHIC SYSTEM EFFICIENCY

A. Model of Theoretical Plates

The model of theoretical plates originates from the theory of distillation. It was adapted to chromatography in the pioneer work on the physicochemical foundations of this method accomplished by Martin and Synge (4, 5). The utility of this model in the highly sophisticated column techniques, e.g., gas or high-performance liquid chromatography, is long and indisputably recognized. The demand for the concept of theoretical plates in thin-layer chromatography seemed less in proportion to the comparatively lower separation efficiency of this method. In view of the recent and successful attempts to enhance efficiency in this field also (see Sec. VI), the idea of theoretical plates applied to thin-layer chromatography for the first time became actually and fully relevant.

Broadening of a chromatographic spot can be simply expressed in terms of the theoretical plate number N of the given chromatographic system:

$$N = \frac{16l_z}{w^2} \quad (14)$$

where l and z are the migration lengths of the mobile phase and solute, respectively, and w is the chromatographic spot width in the direction of the mobile-phase migration (see Fig. 4).

Although the numerical values of N attained for different solutes on the same chromatographic plate proved to coincide fairly well, they usually differ significantly from the analogous values characteristic of another plate type. For this reason, the quantity N can be regarded as an approximate measure of the separating efficiency of chromatographic plates. It is proportional to the migration length of the mobile phase l , so that, the z/w ratio being constant, an increase in l results in an increase of N and better separation. This proportionality of N and l is given by the relationship

$$N = \frac{l}{H} \quad (15)$$

where H is the so-called HETP value (i.e., height equivalent of a theoretical plate). The quantity

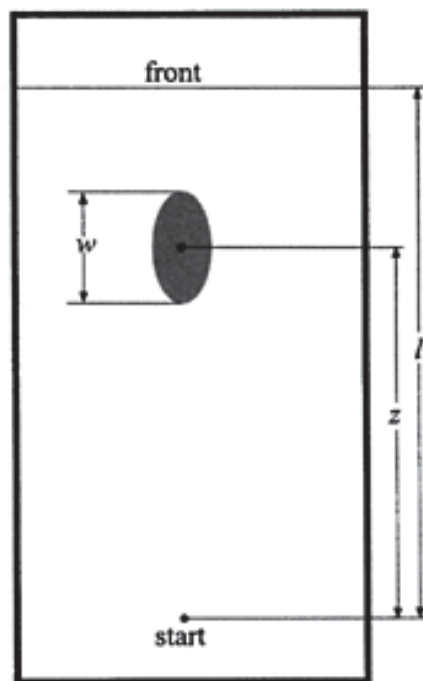


Figure 4 The thin-layer chromatographic parameters used in calculation of the theoretical plate number N .

H , or simply the plate height, measures the efficiency of a given chromatographic system per unit length of the migration distance, l , of the mobile phase. Small H values mean more efficient chromatographic systems and larger N values. The main goal of efforts to enhance performance of thin layers is the attainment of small H values and maximum N

values. As in other chromatographic techniques, the efficiency of a given TLC system is better (i.e., H is smaller) for

1. Smaller particles of stationary phases or supports
2. Lower mobile-phase flow rates
3. Less viscous mobile phases
4. Smaller solute molecules

B. Van Deemter Equation

In the preceding subsection the simplest measure of spot broadening was introduced in the form of the quantity H , the plate height. One of the most important chromatographic relationships, the Van Deemter equation, attempts to estimate the relative contributions of eddy and molecular diffusion, and of the effects of mass transfer, on H . It is an empirical equation, originally established for column chromatographic techniques but valid also for thin-layer chromatography.

The Van Deemter relationship can be written in the complete version,

$$H = Au^{0.33} + \frac{B}{u} + Cu + Du \quad (16a)$$

or simplified,

$$H = Au^{0.33} + \frac{B}{u} + Cu \quad \text{for } D \approx 0 \quad (16b)$$

where u is the flow rate of the mobile phase and A , B , C , and D are the equation constants, measuring contributions of the different spot-broadening processes to the quantity H . The effects of eddy diffusion and mass transfer on the flowing mobile phase are described jointly by A . The molecular diffusion is reflected in B , while C and D correspond to the effects of mass transfer in the stagnant mobile and stationary phases, respectively. The constants A , B , C , and D depend mostly on the parameters of the microporous solid, but they are also influenced by the nature of the solute and the mobile phase and by the working temperature of the chromatographic system.

Each constant of Eq. 16 can be defined as a function of certain properties of the chromatographic system. Let us briefly review the appropriate empirical relationships.

Giddings (6) proposed the following expression for A :

$$A = 2\lambda d_p \quad (17)$$

where d_p is the diameter of a solid particle and λ depends on the microscopic arrangement of solid bed.

B is given as

$$B = 2\gamma D_m \quad (18)$$

where D_m is the diffusion coefficient of the solute in the mobile phase and γ is a correction factor mirroring the nonlinearity of diffusion due to the labyrinthine arrangement of micropores.

C is described by the equation

$$C = \frac{\omega d_p^2}{D_m} \quad (19)$$

where ω is a proportionality factor. Similar to γ in Eq. 18, it also depends on the labyrinthine arrangement of micropores.

D is described by the relationship

$$D = \frac{\sigma d_f^2}{D_s} \quad (20)$$

where d_f is the thickness of the stationary-phase layer, D_s is the diffusion coefficient of the solute in the stationary phase, and σ is a proportionality factor.

C. Separation and Resolution

The R_f coefficient is the basic quantity used to express the position of solute on the developed chromatogram. It is calculated as the ratio

$$R_f = \frac{\text{distance of chromatographic spot center from start}}{\text{distance of solvent from start}} \quad (21)$$

Using symbols from Fig. 4, R_f can be given as

$$R_f = \frac{z}{l} \quad (21a)$$

R_f values are between 0 (solute remains on start) and 1.0 (solute migrates with front of mobile phase).

The traditional (and so far the only) method of determining the numerical values of analyte R_f coefficients quasi-automatically assumes the following preconditions:

1. Circular (or ellipsoidal) chromatographic band shape
2. Gaussian distribution of the mass of the analyte in this band

On the basis of these assumptions, the position of a band on the chromatogram is defined by measuring the distance between the origin and the geometrical center of the band. Despite the considerable imprecision of this definition for asymmetrical (i.e., tailing) and non-Gaussian bands, two features of the definition are very important:

1. The traditional definition regards the center of a chromatographic band as the point at which the local concentration of the analyte is the highest.
2. The traditional definition also regards the center of the chromatographic band as the center of gravity of the mass distribution of the analyte in the band.

For ideal, circular bands with Gaussian analyte concentration profiles, the band centers described by assumptions 1 and 2 are, in fact, identical.

For densitograms obtained from noncircular (i.e., tailing) bands with non-Gaussian concentration profiles, it can be stated that

The numerical value of the R_f coefficient for a given chromatographic band can be determined for the maximum value of the concentration profile of the band (which is the point at which the local concentration of the analyte is the highest). The R_f coefficient determined according to this definition can be denoted as $R_{f(\max)}$.

Alternatively, the numerical value of the R_f coefficient can be determined from the center of gravity of the distribution of analyte mass in the band. With nonsymmetrical chromatographic bands, this value cannot be identical with that obtained from the maximum of the analyte concentration profile. The R_f coefficient determined in this second manner can be denoted as $R_{f(\text{int})}$.

To determine the center of gravity of the analyte mass distribution in the chromatographic band, one has to establish the baseline, remove the noise from the densitogram, subtract the baseline signal, define the beginning ($i=0$) and end ($i=k$) of the chromatographic band, and, finally, calculate the position of its center of gravity by use of the relationship

$$d_{\text{sr}} = \frac{1}{\sum_{i=1}^k I \left(\frac{d_i + d_{i-1}}{2} \right) (d_i - d_{i-1})} \sum_{i=1}^k I \left(\frac{d_i + d_{i-1}}{2} \right) \left(\frac{d_i + d_{i-1}}{2} \right) (d_i - d_{i-1}) \quad (22)$$

$$= \frac{1}{S} \sum_{i=1}^k I \left(\frac{d_i + d_{i-1}}{2} \right) \left(\frac{d_i + d_{i-1}}{2} \right) (d_i - d_{i-1})$$

where S denotes the chromatographic band surface and $I(d_i)$ is the detector signal at a distance d_i .

With increasing use of scanners in thin-layer chromatography laboratories, it seems quite important to reconsider the definition of the R_f coefficient and practical ways in which it can be determined.

The main goal of chromatography is separation of a given solute mixture. However, it can happen that the chromatographic spots of two adjacent solutes overlap to a smaller or greater degree. Therefore, a demand arises for a measure of their separation. This demand is fulfilled by introduction of the quantity R_s , called resolution. The R_s of two adjacent chromatographic spots 1 and 2 is defined as being equal to the distance between the two spot centers divided by the mean spot widths (Fig. 5):

$$R_s = \frac{z_2 - z_1}{0.5(w_1 + w_2)} \quad (23)$$

The quantity R_s serves to define separation. When $R_s=1$, the two spots are reasonably well separated. R_s values larger than 1 mean better separation, and those smaller than 1, poorer separation. In Fig. 6, an example is given of separation as a function of resolution (R_s) and the relative spot concentration (understood as the ratio of the concentration profile maximum heights). From the example it becomes evident that spot overlap becomes

more disturbing when the concentration of solute in one spot is much greater than that in the other.

Utilizing the quantity R_f , Eq. 23 can be rewritten as

$$R_s = \frac{l(R_{f(2)} - R_{f(1)})}{0.5(w_1 + w_2)} \quad (23a)$$

where $R_{f(1)}$ and $R_{f(2)}$ are the R_f values of chromatographic spots 1 and 2, respectively.

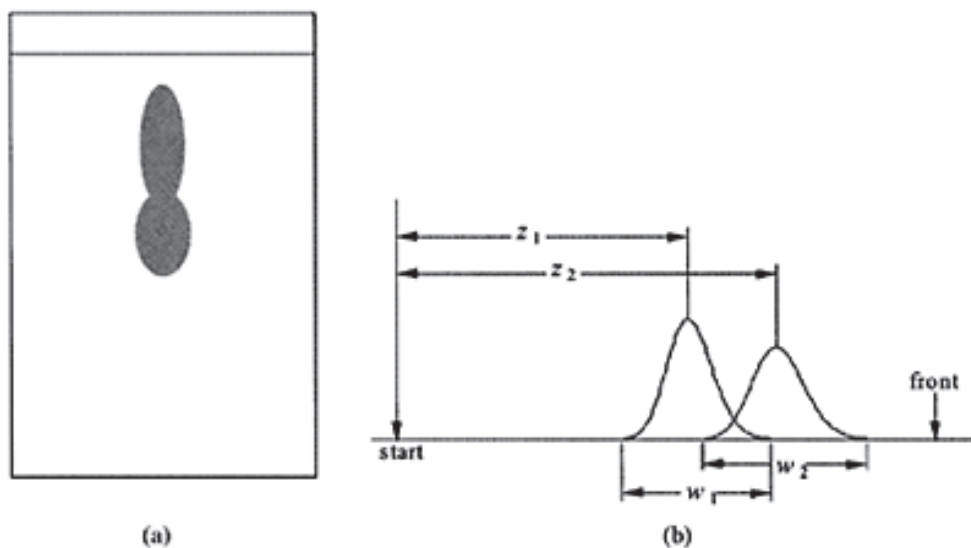


Figure 5 Illustration of resolution in thin-layer chromatography. (a) Chromatogram; (b) corresponding concentration profiles of chromatographic spots.

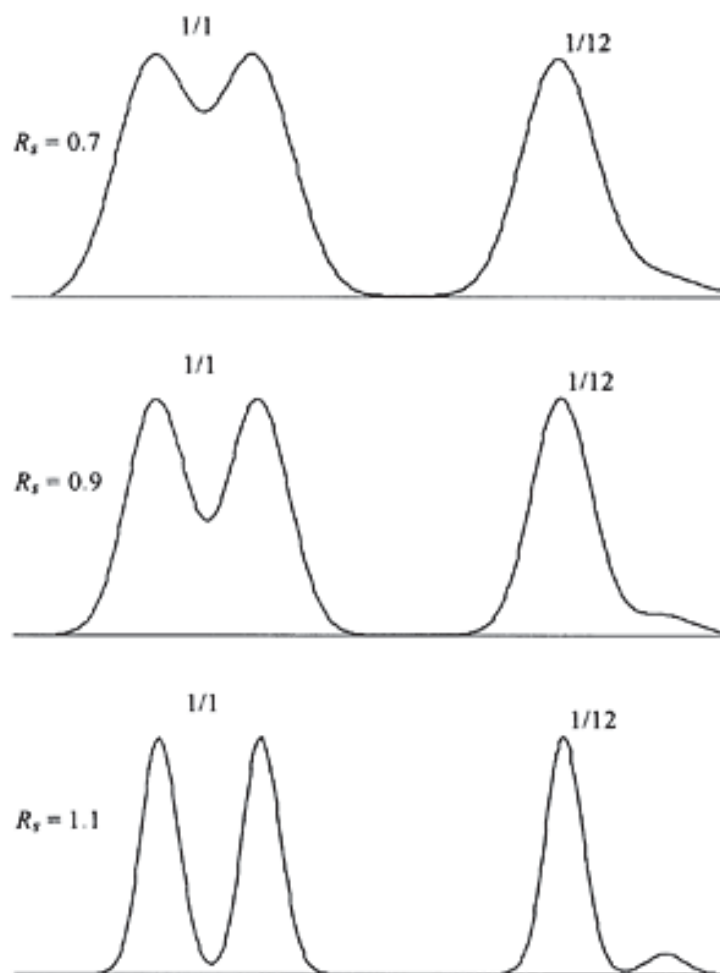


Figure 6 Separation as a function of R_s and the relative spot concentration (the ratio of the concentration profile maximum heights).

Assuming Gaussian concentration profiles of two closely spaced (i.e., overlapping) chromatographic spots and mean R_f value for both of them ($R_{f(1)} \approx R_{f(2)} \equiv \bar{R}_f$), Snyder (7) managed to transform Eq. 23 to the form

$$R_s = 0.25 \left(\overset{\text{(I)}}{\frac{K_2}{K_1}} - 1 \right) \sqrt{\overset{\text{(II)}}{\bar{R}_f} \overset{\text{(III)}}{N} (1 - \bar{R}_f)} \quad (24)$$

where K_1 and K_2 are distribution coefficients of solutes 1 and 2 between the stationary and mobile phases ("distribution" is used in a general sense and means partition, adsorption, or any other phenomenon, depending on the retention mechanism of a particular chromatographic technique).

Equation 24 is the thin-layer chromatographic version of a fundamental chromatographic relationship that allows discussion of spot resolution in terms of the influence of K_2/K_1 , N , and $\bar{R}_f$. Each of these three quantities is sensitive to changes in the

different factors, and Eq. 24 makes discussion of their relative importance for retention possible. Thus K_2/K_1 can monitor interdependence between the stationary and mobile phases, R_f can monitor elution strength of the mobile phase, and N depends on the length of the mobile-phase migration and on the plate height (i.e., l and H , respectively).

D. Selectivity of Separation

Selectivity of separation is seldom referred to in the case of thin-layer chromatography, although no serious reason can be given for avoiding this term. To the contrary, selectivity of separation is a useful chromatographic notion, no matter which particular technique, column or planar, is being considered. In the case of thin-layer chromatography, the separation factor α can be defined as

$$\alpha = \frac{K_2}{K_1} \quad (25)$$

which remains in full conformity with the definition used for the column techniques. In fact, the quantity α makes use of part of term I in Eq. 24, describing the resolution R_s of two overlapping chromatographic spots. It can be stated that with greater difference between distribution coefficients of solutes 1 and 2 (K_1 and K_2), greater selectivity of separation (α) and better resolution (R_s) are observed. With $K_1=K_2$, the two chromatographic spots entirely overlap ($\alpha=1$) and the respective spot resolution R_s is nil. According to Snyder and Kirkland (8), several options for increasing α are available, and these can be ranked in order of decreasing promise:

- Change of mobile-phase composition
 - Change of mobile-phase pH
 - Change of stationary phase
 - Change of temperature
 - Special chemical effects

IV. SEMIEMPIRICAL MODELS OF PARTITION AND ADSORPTION CHROMATOGRAPHY

Partition and adsorption mechanisms of solute retention are the two most universal mechanisms of chromatographic separation, both operating on physical principles. In fact, practically all solutes can adsorb on a microporous solid surface or be partitioned between two immiscible liquids. It is the main aim of the semiempirical chromatographic models to couple the empirical parameters of retention with the established thermodynamic quantities generally used in physical chemistry. The validity of these models for chromatographic practice can hardly be overestimated, because they often successfully help to overcome the old trial-and-error (or, elegantly said, empirical) approach to running the analyses.

A. Martin-Synge Model of Partition Chromatography

The basic principle of solute retention in partition chromatography is its distribution between the two immiscible liquids. Therefore, partition chromatography often used to be called liquid-liquid chromatography, even if the liquid stationary phase was replaced by a chemically bonded one.

Partition chromatography was the first among the chromatographic techniques to gain thermodynamic foundations, owing to the pioneering work of Martin and Synge (4, 5), the 1952 Nobel Prize winners in chemistry. It was their simple and simultaneously fruit-bearing idea to ascribe thermodynamic meaning to the so-called retardation parameter of the solute (i.e., R'_f or the thermodynamic R_f coefficient in thin-layer chromatography). The quantity R'_f is the idealized R_f value, undisturbed by the disadvantageous side effects accompanying the real chromatographic process. R'_f is related to R_f through the empirical dependence

$$R'_f = \xi R_f \quad (26)$$

where ξ is the disturbance factor [$1 \leq \xi \leq 1.6$ (9)].

According to Martin and Synge, R'_f can be viewed as

$$R'_f = \frac{t_m}{t_m + t_s} = \frac{n_m}{n_m + n_s} = \frac{m_m}{m_m + m_s} \quad (27)$$

(I) (II) (III)

where t_m and t_s denote time spent by a solute molecule in the mobile and stationary phases, respectively, n_m and n_s are numbers of solute molecules equilibrially contained in the mobile and stationary phases, and m_m and m_s are the respective mole numbers.

Term I of Eq. 27 can be understood as the relative time spent by solute molecules in the mobile phase, and terms II and III denote the molar fraction of solute in that phase. All the dependences are based on the assumption of partition equilibrium gained by the system. Equation 27 can further be transformed in the following way:

$$R'_f = \frac{m_m}{m_m + m_s} = \frac{c_m V_m}{c_m V_m + c_s V_s} = \frac{1}{1 + (c_s/c_m)(V_s/V_m)} \quad (27a)$$

where c_m and c_s are molar concentrations of solute in the mobile and stationary phases, respectively, and V_m and V_s are volumes of these phases.

The c_s/c_m ratio from Eq. 27a can be expressed as

$$K = \frac{c_s}{c_m} \quad (28)$$

where K is the equilibrium constant of partition, or simply the partition coefficient. Combining Eqs. 27a and 28, we obtain the final form of the Martin—Synge dependence:

$$R'_f = \frac{1}{1 + K(V_s/V_m)} \quad (27b)$$

This equation unites the retention parameter of solute, R'_f , with the established physiochemical quantity K , its thermodynamic meaning being

$$\ln K = \frac{\Delta\mu_p}{RT} \quad (29)$$

where $\Delta\mu_p$ is the chemical potential of partition.

The physical meaning of the partition coefficient K is fully analogous to that from the Nernst partition law, and consequently the numerical values of K obtained in the static experiment correspond well with those established chromatographically (10). This fact can be regarded as a favorable premise of the approaches aimed at prediction of the retention parameter R'_f on the basis of the known thermodynamic characteristics of partition.

B. Snyder-Soczewinski Model of Adsorption Chromatography

The basic principle of solute retention in adsorption chromatography is its distribution between the sorbent and the mobile phase. For this reason, adsorption chromatography is often called liquid—solid chromatography.

The semiempirical model of adsorption chromatography, analogous to that in Section IV.A, was established only in the late 1960s independently by Snyder (7, 11) and (12). The authors assumed that some part of the mobile phase rests adsorbed and stagnant on a sorbent surface. This adsorbed mobile phase formally resembles the liquid stationary phase in partition chromatography. Thus, instead of an inconvenient necessity of discussing solute concentration on a solid surface, one can introduce a quantity expressing its concentration in the adsorbed mobile phase. Otherwise, the Snyder-Soczewinski model benefits from the partition chromatographic concept of viewing the quantities R'_f and K_{th} (where K_{th} is the adsorption equilibrium constant, or simply the thermodynamic adsorption coefficient). The main relationship of the Snyder—Soczewinski model of adsorption chromatography is

$$R'_f = \frac{t_m}{t_m + t_a} = \frac{n_m}{n_m + n_a} = \frac{m_m}{m_m + m_a} = \frac{c_m(V_m - V_a W_a)}{c_m(V_m - V_a W_a) + c_a V_a W_a} \quad (30)$$

where V_a is the volume of the adsorbed mobile phase per mass unit of sorbent, and W_a is the considered mass of sorbent.

The final form of Eq. 30 is

$$R'_f = \frac{1}{1 + K_{th}[V_a W_a / (V_m - V_a W_a)]} \quad (30a)$$

where $K_{th} = c_a / c_m$.

In chromatographic practice, usually $V_a W_a \ll V_m$ and $K_{th}[V_a W_a / (V_m - V_a W_a)] \gg 1$, and therefore Eq. 30a can be rewritten in the simplified version

$$R'_f \approx \frac{1}{K_{th}(V_a W_a / V_m)} \quad (30b)$$

In most cases Eq. 30b describes the experimental results well enough, and there is no urgent demand for its complete form (i.e., for Eq. 30a). The approach to adsorption chromatography proposed by Snyder and Soczewiński proved effective in many respects and enabled quantification of the important chromatographic parameters such as sorbent activity and the elution strength of solvents. These problems are discussed more extensively in Section V.

C. Snyder Concept of Solvent Polarity and Selectivity

The original Snyder-Soczewinski model assumes competition between the solute and the solvent molecules for the active sites on the solid surface of stationary phase, its outcome quantitatively related to the net energy of adsorption (i.e., to the difference between the adsorption energies of the solvent and the solute; for more details see Sections V.A and V.B). However, the net energy concept encompasses a more detailed nature of these forces that are responsible for the process of adsorption. This deficiency is a particular shortcoming with the solvents, which to a large extent govern solute retention owing to their overwhelming excess over the solute molecules in the chromatographic systems.

In order to develop a quantitative measure of the solvent's relative ability to intermolecularly interact with the solutes as proton acceptors, proton donors, and strong dipoles, Snyder established a new semiempirical model (13, 14) coupling the solvent's polarity index (P') with the so-called corrected gas-liquid partition coefficients or solubility constants (K_g'') of the selected test solutes: ethanol (a model proton donor), dioxane (a model proton acceptor), and nitromethane (a model strong dipole). The main relationship of this approach is

$$P' = \log(K_g''_{\text{ethanol}}) + \log(K_g''_{\text{dioxane}}) + \log(K_g''_{\text{nitromethane}}) \quad (31)$$

where K_g'' is a measure of the excess retention of the given solute (i.e., ethanol, dioxane, and nitromethane) relative to an n -alkane of equivalent molar volume.

The individual terms of the trinomial given by Eq. 31 divided by the polarity index (P') are the selectivity parameters, x_e , x_d , and x_n :

$$x_e = \frac{\log(K_g''_{\text{ethanol}})}{P'} \quad (32a)$$

$$x_d = \frac{\log(K_g''_{\text{dioxane}})}{P'} \quad (32b)$$

$$x_n = \frac{\log(K_g''_{\text{nitromethane}})}{P'} \quad (32c)$$

The magnitudes of x_e , x_d , and x_n represent the fractions of P' contributed by interactions associated with ethanol, dioxane, and nitromethane, respectively.

Although the introduced concept of solvent polarity and selectivity cannot be regarded as a semiempirical model of adsorption or partition chromatography in its own right, it certainly remains in the mainstream of Snyder's viewing the role of the solvents in the process of retention as a valuable supplement to the approach presented in the preceding subsection.

D. Scott-Kucera Model of Adsorption Chromatography

The approach of Scott and Kucera (15, 16) aimed to define the equilibrium constant of solute distribution, K , for example, K_{th} from Eq. 30a, between the stationary and mobile phases in terms of the balance of forces between the molecules of the solute and the molecules of each phase. They defined the distribution coefficient K of a solute between the two phases in the following way:

$$K = \frac{\text{total forces acting on the solute in the stationary phase}}{\text{total forces acting on the solute in the mobile phase}} \quad (33)$$

$$= \frac{\text{forces between solute and stationary phase} \times \text{probability of interactions}}{\text{forces between solute and mobile phase} \times \text{probability of interactions}}$$

Considering the situation with respect to adsorption chromatography, Eq. 33 can be rewritten as

$$K_{th} = \frac{F'_p(P'_p) + F'_d(P'_d)}{F_p(P_p) + F_d(P_d)} \quad (33a)$$

where F'_p and F'_d are the polar and dispersive forces, respectively, between the solute molecules and the stationary phase; F_p and F_d are the polar and dispersive forces, respectively, between the solute molecules and the mobile phase; and P'_p , P'_d , and P_p , P_d are the probabilities of the solute molecule interacting with the polar and dispersive moieties of the stationary and mobile phases, respectively.

The probability of interaction of a solute with one of the phases is some function of the absolute temperature, proportional to the concentration of the interacting moieties in each of the respective phases:

$$K_{th} = \frac{F'_p f_1(T) c'_p + F'_d f_2(T) c'_d}{F_p f_3(T) c_p + F_d f_4(T) c_d} \quad (33b)$$

where c'_p , c'_d and c_p , c_d are the concentrations of polar moieties and dispersive moieties in the stationary and mobile phases, respectively, and T is the absolute temperature.

If the hypothesis is made that the dispersive forces result from mass interaction, then c_d is proportional to the density of the dispersing medium, which can be expressed as a concentration in terms of the mass per unit volume. Thus,

$$c_d = Ad \quad (34)$$

where A is a constant and d is the density of the low-polarity solvent. Inserting Eq. 34 in 33b, we obtain

$$K_{th} = \frac{F'_p f_1(T) c'_p + F'_d f_2(T) c'_d}{F_p f_3(T) c_p + F_d f_4(T) Ad} \quad (33c)$$

The authors further assumed that the dispersive forces on highly active sorbents, if present at all, do not have a significant effect on solute retention, which in the case of, e.g., silica, allows simplification of Eq. 33c:

$$K_{th} = \frac{F_p' f_1(T) c_p'}{F_p f_3(T) c_p + F_d f_4(T) A d} \quad (33d)$$

(33d)

The quantity K_{th} , as defined by Scott and Kucera, can be correlated with the basic retention parameter of solute, i.e., the R_f coefficient, with the help of Eq. 30a or 30b.

E. Kowalska Model of Adsorption and Partition Chromatography

In Kowalska's approach (17, 18) to adsorption and partition chromatography, the basic consequences were drawn from the effect of spot broadening. The author pointed to the fact that broadening of a chromatographic spot was due to the effective diffusion, and in this respect it resembled dissolution. Therefore, the change in the chemical potential accompanying the transfer of solute from the start to the chromatographic system, $\Delta\mu_i$, could be given by the relationship

$$\ln x_i f_i = \frac{\Delta\mu_i}{RT} \quad (35)$$

where x_i and f_i are the molar fraction and activity coefficient of solute, respectively, in the chromatographic "binary solution."

The "binary solution" concept assumes two components of a system, i.e., "solute" and "solvent." "Solute" is understood in a traditional way to be the chromatographed substance, and the stationary phase is considered the "solvent." The effects of the mobile phase (and, in partition chromatography, of the support) are expressed in an indirect way through the activity coefficient.

The molar fraction of solute, x_i , is defined as

$$x_i = \frac{c_i}{c_i + c_{ch}} \quad (36)$$

where c_i and c_{ch} are the molar concentrations of the chromatographed substance and the stationary phase (i.e., of the "solute" and "solvent"), respectively, in the chromatographic spot; c_i and c_{ch} can further be defined as

$$c_i = \frac{n_i}{v_i} \quad \text{and} \quad c_{ch} = \frac{n_{ch}}{v_i} \quad (37)$$

where n_i and n_{ch} are the molar aliquots of solute and solvent, respectively, contained in the chromatographic spot, and v_i is the spot volume (see Fig. 7).

Assuming thermodynamic equilibria within the thin-layer chromatographic system and the nonsymmetrical way of expressing the chemical potential of the "solute," its activity coefficient f_i was derived as equal to

$$f = 1 + \frac{c_i}{c_{ch}} \quad (38)$$

The approach proposed by Kowalska can be regarded as the only semiempirical model of the chromatographic process based on the effect of spot broadening. Its practical usefulness is discussed in Section V.

F. Kowalska Model of Retention with Use of Multicomponent Mobile Phases

The Kowalska model of adsorption and partition chromatography presented in the preceding subsection was not a proper retention model simply because it did not couple any recognized retention parameter of the solute with the thermodynamic magnitude of the chemical potential.

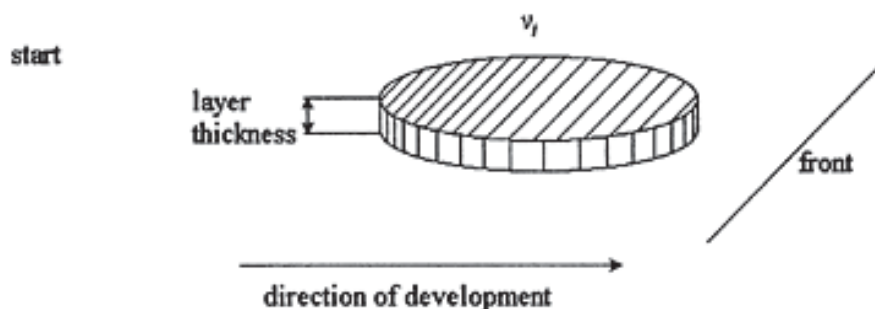


Figure 7 The chromatographic spot as a three-dimensional structure (of volume v_i) in chromatographic “binary soluton” model.

However, it positively emphasized the very specific role played by the mobile phase in the transfer of solute molecules through the chromatographic system. The molecular level conclusions drawn with the aid of that earlier approach (see Sec. V.E) plus the systematically growing importance of the chemically bonded stationary phases (applied in what is formally considered as partition, or synonymously liquid-liquid, chromatography, but what in fact is the liquid—solid or adsorption mode) gave rise to the unified (adsorption/partition) retention model focused on chromatographic systems that employ multicomponent mobile phases.

The new model was first introduced in Ref. 19 and aimed at a new physicochemical interpretation of the R_f coefficient. Accepting the indisputable value of the R_f coefficient for the theory and practice of chromatography, it must be emphasized that the physicochemical contents of this factor have not as yet been sufficiently studied and utilized. In Ref. 20, a new general definition of the R_f coefficient was given in the form

$$R_f = \sum_i \chi_i \beta_i \Delta \mu_{i/st\ ph} q_i \quad (39)$$

where i denotes the mixed mobile phase moieties, χ is the volume fraction of a given moiety, β denotes the degree of dissociation of the respective H-bonded moiety, $\Delta \mu_{i/st\ ph}$ is the respective standard chemical potential of the solute partitioning between the i th liquid

moiety and stationary phase, and q is the respective proportionality coefficient. When mentioning the mobile phase moieties, it needs to be explained that in the discussed model the recognized thermodynamic concept was introduced by mentally dividing the multicomponent mobile phases into the individual liquid moieties. For example, in the methanol-water mixture, three moieties can be distinguished:

Pure methanol (1)

Pure water (2)

The mixed H-bonded methanol-water moiety (3)

Then the general definition of the R_f coefficient was elaborated into a number of particular relationships referring to the common binary (and ternary) mobile phases employed in adsorption and partition chromatography. The most important relationships are listed below.

Mobile phases: methanol-water and methanol-buffer (21, 22):

$$R_f = \sqrt{x_1}A + \sqrt{x_2}B + C \quad (40)$$

where x_1 and x_2 are the volume fractions of methanol and water (or buffer), respectively, and A , B , and C are the equation constants with profound thermodynamic meaning.

Mobile phases: acetonitrile-water and acetonitrile-buffer (23, 24):

$$R_f = x_1A + \sqrt{x_2}B + \sqrt{2.92x_2n''} + x_2C + D \quad (41)$$

where x_1 and x_2 are the volume fractions of acetonitrile and water (or buffer), respectively; A , B , C , and D are the thermodynamically relevant equation constants; and n'' refers to the average self-associated water cluster.

Mobile phases: tetrahydrofuran-water and tetrahydrofuran-buffer (25, 26):

$$R_f = x_1A + \sqrt{x_2}B + \sqrt{4.51x_2n''} + x_2C + D \quad (42)$$

where x_1 and x_2 are the volume fractions of tetrahydrofuran and water (or buffer), respectively; A , B , C , and D are the thermodynamically relevant equation constants; and n'' refers to the average self-associated water cluster.

Mobile phases: aliphatic alcohol- n -paraffin hydrocarbon (27):

$$R_f = \sqrt{x_1}A + x_2B + C \quad (43)$$

where x_1 and x_2 are the volume fractions of alcohol and hydrocarbon, respectively, and A , B , and C are the thermodynamically relevant equation constants.

V. PRACTICAL CONSEQUENCES OF ESTABLISHED MODELS

Consequences of the established models are manifold, and their importance is both theoretical and practical. In the following subsections, we focus attention on the main practical aspects of the approaches that have been introduced.

A. Quantification of Sorbent Activity

Sorbent activity depends on three parameters:

1. Specific surface area
2. Density of the free (i.e., unoccupied) active centers per unit of sorbent surface area
3. Energy of intermolecular interactions between a solute molecule and a given type of sorbent active center

Specific surface area depends on the chemical structure of the sorbent (silica, alumina, cellulose, etc.) and on the technology of its manufacture. It can be measured and expressed numerically.

The density of the free active centers per unit of sorbent surface area also depends on the chemical structure of the sorbent and, in addition, on the number of molecules other than those of the solute or mobile phase occupying sorbent active centers. These are mostly water molecules, which block (deactivate) active centers on a sorbent surface, and the degree of deactivation usually depends on the storage conditions of the precoated chromatographic plates. The density of the free active centers can also be measured and expressed numerically.

The energy of intermolecular interactions between a solute molecule and a given type of sorbent active center depends as much on the chemical nature of the sorbent as on the nature of the solute itself. Therefore, with a given sorbent the energy of intermolecular interactions differs from one solute to another.

As can be easily deduced, sorbent activity cannot be quantified in the absolute, but in relative values only. The most complete approach to this problem was derived from the Snyder—Soczewiński model of adsorption chromatography, and it is discussed briefly here.

The thermodynamic adsorption coefficient K_{th} (see Eq. 30a) can be defined as

$$\log K_{th} = \frac{\Delta\mu_a}{2.303RT} \quad (44)$$

where $\Delta\mu_a$ is the chemical potential of adsorption.

Simplifying Eq. 44, we can write

$$\log K_{th} = \Delta E \quad (44a)$$

where ΔE (that is, $\Delta\mu_a/2.303RT$) is the dimensionless energy of adsorption. It equals the difference between the energies of adsorption of the solute (E_{Xa}) and the solvent (E_{Sa}) (a one-component mobile phase is assumed). The quantity E_{Xa} is a function of the sorbent surface energy A_i and the physicochemical properties of a solute X . Similarly, the quantity E_{Sa} depends on the magnitude A_i and on the physicochemical properties of a solvent S . Summing up, we can write

$$E_{Xa} = f(A_i)f(X) \quad (45)$$

$$E_{Sa} = f(A_i)f(S) \quad (45a)$$

$$\Delta E = E_{xa} - E_{Sa} = f(A_i)[f(X) - f(S)] \quad (46)$$

Equation 30a can be rewritten as

$$\log \frac{1 - R'_f}{R'_f} = \log \frac{V_a W_a}{V_m - V_a W_a} + \log K_{th} \quad (30c)$$

Combining Eqs. 30c, 44a, and 46, we obtain the relationship

$$\log \frac{1 - R'_f}{R'_f} = \log \frac{V_a W_a}{V_m - V_a W_a} + f(A_i)[f(X) - f(S)] \quad (30d)$$

This equation can be rewritten in the concise form

$$R'_m = \log \frac{V_a W_a}{V_m - V_a W_a} + \alpha' f(X, S) \quad (30e)$$

where $\alpha' = f(A_i)$ and $f(X, S) = f(X) - f(S)$. Thus, α' is a function of the sorbent surface energy independent from the properties of the solute. It is known as the activity coefficient of the sorbent, and determination of its numerical values can be regarded as quantification of sorbent activity.

The right-hand side of Eq. 30e consists of three terms that define separate contributions from the phase ratio, sorbent activity, and the so-called solute-solvent relationship $f(X, S)$ to the overall retention of solute. The numerical values of R'_m , V_m , V_a , and W_a can be established experimentally. Two unknowns in Eq. 30e, namely α' and $f(X, S)$, cannot be determined simultaneously from the same relationship. It was Snyder's (7) idea to overcome this difficult problem in the following way.

Through intensive drying, the sorbent can eventually achieve its full activity, which means that each active center of a sorbent sample is free of deactivating water molecules. The activity coefficient α' of this sorbent is assumed to be equal to 1. Then the fully active sorbent can further be used for determination of the solute-solvent relationship $f(X, S)$ with a number of test solutes. The respective results are collected for the sake of illustration in Table 1 (28). With the numerical values of $f(X, S)$ both known and independent of the degree of sorbent deactivation, one can again use Eq. 30a for the determination of α' with any given sorbent sample. Obviously, the numerical values of $f(X, S)$ have to be measured separately for each individual type of sorbent (silica, alumina, cellulose, etc.) obtained in a given manufacturing procedure.

B. Quantification of Solvent Elution Strength

Solvent elution strength is among the most important factors governing solute retention. Solvents with too little elution strength are incapable of moving solutes from the origin, whereas those that

Table 1 Numerical Values of $f(X, S)$ for Test Solutes Chromatographed in *n*-Hexane on Alumina and Silica

Test solute	$f(X, S)$	
	Al ₂ O ₃	SiO ₂
Styrene	2.34	1.71
Durene	2.30	1.80
Naphthalene	3.10	2.02
Azulene	3.56	2.35
Acenaphthylene	3.65	2.29
Phenanthrene	4.34	2.55
Anthracene	4.60	2.60
Pyrene	4.77	2.57
Fluoranthene	4.94	2.79
Chrysene	5.64	3.09
<i>m</i> -Terphenyl	4.78	3.13
Triphenylene	5.64	3.15
Benzantracene	5.65	3.09
1,2- or 3,4-Dibenzopyrene	6.40	3.28

Source: Data from Snyder (28).

are too strong push solutes with the mobile phase front. In other words, weak mobile phases cannot significantly affect intermolecular interactions between solute molecules and the stationary phase, whereas the strong ones practically annihilate such interactions. Therefore, the proper choice of a single eluent, or eluent mixture, with respect to the analyzed substance and the stationary phase is crucial for the successful outcome of the chromatographic process.

Quantification of solvent elution strength is based on the Snyder-Soczewiński model of adsorption chromatography. A possibility of appropriate quantification is offered by Eq. 46. For the sorbent activity coefficient $\alpha'=1$, Eq. 46 can be rewritten in the form

$$\Delta E = f(X) - f(S) = f(X, S) \quad (46a)$$

Equation 46a describes the difference between the adsorption energies of solute and equivalent amount of solvent (one solute molecule can replace one or more solvent molecules on the sorbent surface, depending on the stoichiometry of a given process). Thus, ΔE can be regarded as the net adsorption energy of the solute. With a simplifying assumption as to the monocomponent mobile phase, we can further write (7)

$$\Delta E = f(X, S) = S^0 - A_s \varepsilon^0 \quad (47)$$

where S^0 [$\equiv E_{Xa} = f(X)$] is the adsorption energy of the solute, A_s denotes the cross-sectional area of its molecule, and ε^0 is the adsorption energy of the solvent per unit of sorbent surface area [$A_s \varepsilon^0 = E_{Sa} = f(S)$]. ε^0 is usually referred to as solvent elution strength, or simply solvent strength.

Equation 47 is a function of three parameters, S^0 , A_s , and ε^0 , and therefore the question arises as to how to conveniently express solvent elution strength in terms of ε^0 . Choosing an aliphatic hydrocarbon as a test compound, one automatically attains the situation in which $S^0 \approx 0$. The quantity A_s can be evaluated from the molecular parameters of the test compound. Thus, ε^0 remains the only unknown of the simplified relationship

$$\Delta E \approx -A_s \varepsilon^0 \quad (47a)$$

and it can be established experimentally.

Elution strength of the simplest liquid aliphatic hydrocarbon, *n*-pentane, is equal to 0, and this particular solvent begins what is usually called the eluotropic series. Numerical values of solvent elution strength ε^0 determined for the most common chromatographic solvents on alumina are collected in Table 2. To obtain the analogous set of data for silica, Snyder advises multiplying the data from Table 2 by a factor of 0.77.

The concept of solvent elution strength ε^0 is one way of quantifying solvent polarity. This polarity is a very important factor in establishing the chromatographically advantageous equilibria according to the scheme



Thus the solvent elution strength ε^0 became a cornerstone of the new semiempirical strategy of predicting multicomponent mobile-phase composition, and this problem is discussed in Section V.D.1.

C. Quantification of Solvent Polarity and Selectivity

The main idea of this approach was to compare the variety of solvents most often used as components of mixed mobile phases with respect to their polarity and simultaneously as proton acceptors, proton donors, and intermolecularly interacting dipoles. Three test solutes were selected, upon which the polarity and selectivity scale was built: ethanol as a model proton donor, dioxane as a model proton acceptor, and nitromethane as a model strong dipole. Based on an extensive experimental study by Rohrschneider (29) that determined the solubility constants K_g (the so-called gas-liquid partition coefficients of the test solute distributed between the gas phase and

Table 2 The Eluotropic Series of Solvents and Solvent Elution Strength ε^0 Determined in Alumina

Solvent	$\varepsilon_{\text{Al}_2\text{O}_3}^0$	Solvent	$\varepsilon_{\text{Al}_2\text{O}_3}^0$
<i>n</i> -Pentane	0.00	1,2-Dichloroethane	0.49
<i>n</i> -Hexane	0.01	Ethyl methyl ketone	0.51
<i>n</i> -Heptane	0.01	Acetone	0.56
Cyclohexane	0.04	Dioxane	0.56
Carbon disulfide	0.15	Ethyl acetate	0.58
Carbon tetrachloride	0.18	Methyl acetate	0.60
Isopropyl ether	0.28	1-Pentanol	0.61
2-Chloropropane	0.29	Dimethylsulfoxide	0.62
Toluene	0.29	Aniline	0.62
1-Chloropropane	0.30	Nitromethane	0.64
Chlorobenzene	0.30	Acetonitrile	0.65
Benzene	0.32	Pyridine	0.71
Bromoethane	0.37	2-Propanol	0.82
Diethyl ether	0.38	Ethanol	0.88
Chloroform	0.40	Methanol	0.95
Dichloromethane	0.42	Ethylene glycol	1.11
Tetrahydrofuran	0.45	Acetic acid	∞

Source: Data from Snyder (7).

the solvent in a sealed flask and determined by gas chromatographic analysis of the gas phase) for the aforementioned three test solutes and over 80 solvents, Snyder managed to devise a chromatographically useful scale of the polarity indices P' and the selectivity parameters x_i (13, 14). The backbone of his approach was the relationships

$$P' = \log(K_g'')_{\text{ethanol}} + \log(K_g'')_{\text{dioxane}} + \log(K_g'')_{\text{nitromethane}} \quad (31)$$

$$x_e = \frac{\log(K_g'')_{\text{ethanol}}}{P'} \quad (32a)$$

$$x_d = \frac{\log(K_g'')_{\text{dioxane}}}{P'} \quad (32b)$$

$$x_n = \frac{\log(K_g'')_{\text{nitromethane}}}{P'} \quad (32c)$$

Snyder's principal objective was to remove the dependence of the magnitude of K_g on the molecular weights of solvent and solute (14). The effect of the solvent molecular weight was removed by multiplying K_g by the molar volume V_s (mL/mol) of the solvent, leading to the partially corrected magnitude K'_g :

$$K'_g = K_g V_s \quad (49)$$

The molecular weight effect of the solute on its K'_g value can likewise be removed by dividing K'_g by the estimated K'_g value (K_v) of an n -alkane whose molar volume is the same as that of the solute:

$$K''_g = \frac{K'_g}{K_v} \quad (50)$$

or

$$\log K''_g = \log K'_g - \log K_v \quad (50a)$$

In this way, Snyder "purified" Rohrschneider's results of the effect of mass interaction, thus better exposing the energetics of the differentiated intermolecular interactions between solute and solvent.

Although solvent elution strength (ε^0) and polarity index (P') can be considered as two quasiequivalent ways of quantifying solvent polarity, the physicochemical relevance of P' is greater, simply because it offers deeper insight into the nature of the forces that ultimately play the most crucial role in the displacement mechanism of solute retention or in the otherwise rather neglected solute-solvent interactions. In other words, the two different solvents can be equally polar (thus yielding similar R_f values for the test solute) and yet considerably different in terms of their molecular level roles in the process of retention. This difference usually results in the differentiated selectivity of separation attained with the aid of these two solvents.

D. Optimization of Mobile Phases

Optimization of resolution and selectivity is a practical goal in thin-layer chromatography. The proper strategy is dictated by the equation

$$R_s = 0.25 \left(\frac{K_2}{K_1} - 1 \right) \sqrt{R_f N (1 - R_f)} \quad (24)$$

From this relationship it follows that thin-layer efficiency (plate number N) and composition of mobile phases (monitored through K_2/K_1 and R_f) can be optimized separately. Enhancement of thin-layer performance in terms of increasing N is the subject of Section VI, whereas the approaches aiming to optimize the composition of mobile phases are discussed below.

1. Snyder's Approach (Solvent Elution Strength)

The most universal approach to optimization of mobile phase composition is a simple consequence of the idea of solvent elution strength introduced by Snyder (7). Combining Eqs. 44a, 46, and 47, we can view the thermodynamic adsorption coefficient K_{th} as a function of solvent elution strength, ϵ^0 :

$$\log K_{th} = \alpha'(S^0 - A_s \epsilon^0) \quad (51)$$

If one solute is developed in two different monocomponent mobile phases 1 and 2 using the same sorbent, the following equations can be written:

$$\log K_{th(1)} = \alpha'(S^0 - A_s \epsilon_1^0) \quad (51a)$$

$$\log K_{th(2)} = \alpha'(S^0 - A_s \epsilon_2^0) \quad (51b)$$

and, finally, subtracting Eq. 51b from Eq. 51a, we obtain

$$\log \frac{K_{th(1)}}{K_{th(2)}} = \alpha' A_s (\epsilon_2^0 - \epsilon_1^0) \quad (51c)$$

where ϵ_1^0 and ϵ_2^0 are solvent strength values for solvents 1 and 2, respectively. Equation 51c allows comparison of the influence of solvents 1 and 2 on solute retention, which is indirectly expressed in the form of the quantities $K_{th(1)}$ and $K_{th(2)}$ (see Eqs. 30a and 30b). Proper adjustment of the numerical K_{th} values is really important for separation, and the optimum working conditions are attained within the range

$$1 < K_{th} \frac{V_a W_a}{V_m} \leq 10 \quad (52)$$

The practical nature of Eq. 52 is better perceived if it is rewritten as (see Eq. 30b):

$$0.1 \leq R_f < 1.0 \quad (52a)$$

Considering the large number of solutes and complex mixtures that are separated by TLC, it is necessary to take advantage of multicomponent mobile phases to improve the fine-tuning of the necessary retention. The most commonly used are binary and ternary mobile phases, although in some special cases four-component, or even more complex, mixtures cannot be avoided. To make the choice of a multicomponent mobile phase less empirical, it would be useful to know in advance its elution strength ϵ^0 . Unfortunately, the experimental determination of ϵ^0 for these phases is almost impossible, owing to the endless combinations of components and their volume ratios.

In the early 1980s, Snyder (30–32) succeeded in deriving appropriate semiempirical relationships to describe and allow calculation of the elution strength ϵ^0 of multicomponent mixtures.

The solvent strength ε_{AB} of a binary solvent mobile phase can be related to the mole fraction of the stronger solvent B (N_b) in the mobile phase, the ε^0 values of two pure solvents that constitute the mobile phase (ε_A and ε_B), and the area n_b required by a molecule of solvent B on the sorbent surface:

$$\varepsilon_{AB} = \varepsilon_A + \frac{\log(N_b \times 10^{\alpha' n_b (\varepsilon_B - \varepsilon_A)} + 1 - N_b)}{\alpha' n_b} \quad (53)$$

The solvent strength ε_{AB} of a binary solvent mobile phase can also be related to the thermodynamic adsorption coefficients K_{th} of some solute in that mobile phase (K_{AB}) and in pure solvent (K_A):

$$\varepsilon_{AB} = \varepsilon_A + \frac{\log[K_A/K_{AB}]}{\alpha' A_s} \quad (54)$$

The quantity A_s , which is the molecular area of the solute, can have any value, so let it equal n_b :

$$\varepsilon_{AB} = \varepsilon_A + \frac{\log[K_A/K_{AB}]}{\alpha' n_b} \quad (54a)$$

Relationships analogous to Eq. 54a are valid in the case of ternary, quaternary, and even more complex mobile phases:

$$\varepsilon_m = \varepsilon_A + \frac{\log[K_A/K_m]}{\alpha' n_b} \quad (54b)$$

where ε_m is the solvent strength of a multicomponent mobile phase and K_m is the thermodynamic adsorption coefficient of some solute in that phase.

In the case of simple solutes (e.g., aliphatic hydrocarbons) showing adsorption energies $S^0 \approx 0$, the quantities ε_{AB} and ε_m can help to predict the R_f parameter. Combining Eqs. 30b and 51 gives

$$R_f \approx \frac{1}{10^{-\alpha' A_s \varepsilon^0} (V_a W_a / V_m)} \quad (55)$$

where ε^0 is the solvent strength of a multicomponent mobile phase (ε_{AB} for the binary phases and ε_m for more complex ones).

Prediction of solvent elution strength ε^0 and the retention parameter R_f made with the help of Eqs. 53–55 cannot be regarded as error-free. The observed differences between the experimental and calculated ε^0 and R_f values are in the first instance due to the simplicity of the assumed intermolecular interaction model in systems composed of solute, solvent, and mobile phase (see Eqs. 45, 45a, and 46). In fact, the model discussed fully ignores self-association of solute and solvent as well as mixed intermolecular interactions that simultaneously engage the solute and the mobile phase. For the aforementioned reason, the most successful optimization of the mobile phase can be attained for those solutes and solvents that are practically unable to interact intermolecularly (such as hydrocarbons). Still, the importance of Snyder's approach is undeniable as an easy-to-apply strategy for multicomponent mobile-phase optimization.

2. Snyder's Approach (Solvent Polarity and Selectivity)

In analogy with the eluotropic series presented in Table 2, Snyder managed to compare solvents according to their polarity indices (P') (13, 14). In Table 3, an example is given of this alternative comparison, showing the polarity indices (P') and the selectivity parameters (x_i) of selected common liquids.

Physicochemical relevance of the selectivity parameters concept gained convincing experimental confirmation in the sense that among solvents of similar functionality a strikingly great similarity of the selectivity parameters was observed (14) (see Table 4). This observation can be translated into the language of molecular level phenomena as the best proof of the predominant importance of functionality in intermolecular interactions of the solute—solvent and stationary phase—solvent types.

Upon careful consideration of the selectivity parameters (x_i), Snyder managed to divide solvents into eight classes of similar compounds (see Table 5 and Fig. 8).

In the case of liquid binary mixtures (composed of the solvents A and B), the respective polarity index (P'_{AB}) shows a straight-line dependence on its composition (13):

$$P'_{AB} = \phi_A P'_A + \phi_B P'_B \quad (56)$$

Table 3 The Polarity Indices (P') and the Selectivity Parameters (x_e , x_d , and x_n) of Selected Solvents

Solvent	P'	x_e	x_d	
<i>n</i> -Hexane	0.1			
Cyclohexane	0.2			
Carbon sulfide	0.3			
Carbon tetrachloride	1.6			
Isopropyl ether	2.4	0.48	0.14	0.38
Toluene	2.4	0.25	0.28	0.47
Chlorobenzene	2.7	0.23	0.33	0.44
Benzene	2.7	0.23	0.32	0.45
Diethyl ether	2.8	0.53	0.13	0.34
Chloroform	4.1	0.25	0.41	0.33
Dichloromethane	3.1	0.29	0.18	0.53
Tetrahydrofuran	4.0	0.38	0.20	0.42
1,2-Dichloroethane	3.5	0.30	0.21	0.49
Ethyl methyl ketone	4.7	0.35	0.22	0.43
Acetone	5.1	0.35	0.23	0.42
Dioxane	4.8	0.36	0.24	0.40

Ethyl acetate	4.4	0.34	0.23	0.43
Dimethylsulfoxide	7.2	0.39	0.23	0.39
Aniline	6.3	0.32	0.32	0.36
Nitromethane	6.0	0.28	0.31	0.40
Acetonitrile	5.8	0.31	0.27	0.42
Pyridine	5.3	0.41	0.22	0.36
2-Propanol	3.9	0.55	0.19	0.27
Ethanol	4.3	0.52	0.19	0.29
Methanol	5.1	0.48	0.22	0.31
Ethylene glycol	6.9	0.43	0.29	0.28
Acetic acid	6.0	0.39	0.31	0.30
Water	10.2	0.37	0.37	0.25

Source: Data from Snyder (14).

Table 4 Similarity of Selectivity Parameters of Solvents of Similar Functionality

Solvent functionality	Average parameter values		
	x_e	x_d	x_n
Alcohols	0.54±0.03	0.19±0.01	0.27±0.02
Alkyl ethers	0.48±0.05	0.15±0.03	0.37±0.02
Ketones	0.35±0.01	0.22±0.01	0.42±0.01
Esters	0.34±0.00	0.25±0.01	0.41±0.01
Phenyl alkyl ethers	0.27±0.01	0.30±0.02	0.43±0.01
Aromatic hydrocarbons	0.25±0.02	0.29±0.02	0.46±0.01

Source: Data from Snyder (14).

where ϕ_A and ϕ_B are, respectively, the volume fractions of solvents A and B, and P'_A and P'_B are, their respective polarity indices.

Optimization of the chromatographic process with the aid of the Snyder concept of solvent polarity and selectivity in fact means optimization of the separation selectivity. This goal can be attained with the help of so-called isoelutotropic mixtures, i.e., mixed mobile phases that, in spite of having compositions different from that of the original mobile phase, preserve equal elution strength.

Let us consider the adsorption and the normal-phase partition chromatography systems employing binary mobile phases composed of solvents A and B (with the nonpolar solvent A, for which $P \approx 0$). If we want to change the separation selectivity of this system,

the simplest way is to employ the isoeluotropic mixture in which solvent B is replaced by solvent C. The volume fraction of solvent C can be estimated from the relationship

$$\phi_C = \phi_B \frac{P'_B}{P'_C} \quad \text{for} \quad \phi_A P'_A \approx 0 \quad (57)$$

If we want to change the selectivity of a mixed mobile phase in a reversed-phase partition chromatography mode, we also need to employ an isoeluotropic mobile phase. The common strategy is to replace the less polar component of the starting binary mixture with another solvent. For example, if the starting mobile phase was a methanol–water mixture, methanol has to be replaced with acetonitrile or tetrahydrofuran. Then the volume fraction of the new mobile phase can be estimated from the relationship

Table 5 Classification of Solvent Selectivity

Group	Solvents
I	Aliphatic ethers, tetramethylguanidine, hexamethyl phosphoric acid amide, trialkylamines
II	Aliphatic alcohols
III	Pyridine derivatives, tetrahydrofuran, amides (except formamide), glycol ethers, sulfoxides
IV	Glycols, benzyl alcohol, acetic acid, formamide
V	Methylene chloride, ethylene chloride
VI	(a) Tricresyl phosphate, aliphatic ketones and esters, polyethers, dioxane (b) Sulfones, nitriles, propylene carbonate
VII	Aromatic hydrocarbons, halo-substituted aromatic hydrocarbons, nitro compounds, aromatic ethers
VIII	Fluoroalkanols, <i>m</i> -cresol, water, chloroform

Source: Data from Snyder (14).

When 1:1 complexes (AZ, AS, and SZ) are formed and the polar solvent S is diluted with an inert solvent *N* (e.g., an aliphatic hydrocarbon), then a simple relationship is obtained for the quantity R_m of solute Z:

$$R_m = \log \frac{1 - R_f}{R_f} = \log \frac{x_{AZ}}{x_Z + x_{SZ}} = \log \frac{K_{AZ}x_{AS}}{K_{AS}x_S(1 - K_{SZ}x_S)} \quad (60)$$

where x denotes molar fraction. For example, x_{AZ} is the concentration of the molecules of solute Z temporarily immobilized by hydrogen bonding with sorbent surface. It is assumed that the probability of adsorption of solvated molecules (SZ) is much lower than that of molecules that are nonsolvated (Z).

If it is additionally assumed that the solute is only weakly solvated by the solvent ($x_{SZ}=0$, $K_{SZ}=0$), then Eq. 60 simplifies to

$$R_m = \log \frac{K_{AZ}x_{AS}}{K_{AS}x_S} \quad (60a)$$

Equation 61a can be rewritten in the form

$$R_m + \log \frac{x_S}{x_{AS}} = \log \left[\frac{1 - R_f}{R_f} \left(\frac{x_S}{x_{AS}} \right) \right] = \log K_{AZ} - \log K_{AS} \quad (60b)$$

which is identical with Snyder's Eq. 46a, because

$$\log K_{th} = \Delta E = \log \left[\frac{1 - R_f}{R_f} \left(\frac{x_S}{x_{AS}} \right) \right]$$

$$S^0 = \log K_{AZ}, \quad A_S^0 = \log K_{AS}$$

4. The Adsorption-Partition Model

Another approach was introduced to enable modeling of solute retention in TLC with chemically bonded stationary phases (37). The authors of this model intended to reflect the physicochemical nature of the retention process more closely than in any other approach currently used. This retention model is capable of quantitative description of the two parallel processes occurring in the course of solute migration through the stationary-phase bed. One of these complementary processes can be described as intermolecular interaction of a solute with the chemically bonded organic ligands, according to the Snyder—Soczewiński model.

On the basis of this long-accepted assumption, the amount of adsorbed solute can be expressed as

$$q'_1 = Kc_1 \quad (61)$$

where $K = \exp(p_1 + p_2\phi)$, q'_1 denotes the concentration of the solute molecules physically (e.g., as a result of dispersive forces) connected to the chemically bonded ligands, c_1 denotes the concentration of this solute in the mobile phase, ϕ is the volume fraction of the active (i.e., strong) liquid component of the mobile phase, and p_1 and p_2 are the equation constants.

The competitive process consists of intermolecular (mostly polar) interactions of a solute with the free (i.e., nonbonded) silanols on the surface of the silica matrix. This complementary mechanism was modeled with the aid of a simple stoichiometric isotherm, taking into account the adsorption both of the solute molecules and of the components of a mixed mobile phase:

$$q_1'' = \frac{q_s K_1 c_1}{K_1 c_1 + K_2 c_2 + K_3 c_3} \quad (62)$$

where c_1 , c_2 , and c_3 are concentrations of the solute and of the components of the binary mobile phase, respectively; q_s is the saturation capacity of solid phase; and K_1 , K_2 , and K_3 are the equilibrium constants for the solute and the mobile-phase components, respectively. Because of the typically very low concentrations of the solute, the first term in the denominator can be ignored.

The overall mechanism of solute retention is given as the sum of the two contributions:

$$q_1 = q_1' + q_1'' = \left(\exp(p_1 + p_2 \varphi) + \frac{q_s K_1}{K_2 c_2 + K_3 c_3} \right) c_1 \quad (63)$$

It is well established that the retention coefficient k is proportional to the derivative of the solute concentration in the solid phase with respect to the solute concentration in the mobile phase:

$$k = \Phi \frac{\partial q_1}{\partial c_1} \quad (64)$$

The proportionality factor Φ (usually referred to as the phase ratio) is the volume ratio of stationary phase to mobile phase.

Keeping in mind that the retardation factor R_f is defined as $R_f = 1/(1+k)$ and assuming that the mobile-phase components form an ideal mixture, the following relationship for R_f can finally be derived from Eqs. 63 and 64 (37):

$$R_f = \frac{1}{1 + \exp(p_1 + p_2 \varphi) + 1/[p_3 \varphi + p_4(1 - \varphi)]} \quad (65)$$

where the phase ratio Φ is incorporated in the unknown terms p_i . The performance of this model was extensively tested on many experimental results (37) taken from the literature and relating to (a) the chemically bonded 3-cyanopropyl stationary phase with 2-propanol-*n*-hexane as the mobile phase (NP-TLC), (b) the chemically bonded octadecyl stationary phase with methanol-water as the mobile phase (RP-TLC), (c) silanized silica with methanol-water as the mobile phase (RP-TLC), and (d) silanized silica impregnated with paraffin oil as the stationary phase and methanol-water as the mobile phase (RP-TLC).

The outcome of this test led to the general conclusion that the fit of the experimental data to Eq. 65 was outstanding. A typical comparison of experimental and theoretically predicted data is shown in Fig. 9.

5. Other Approaches

The general approach to solute distribution between the stationary and mobile phases proposed by Scott and Kucera (15, 16) can also find application in the prediction of elution strength with binary solvent mobile phases. To demonstrate such a possibility, Eq. 33c is rewritten as

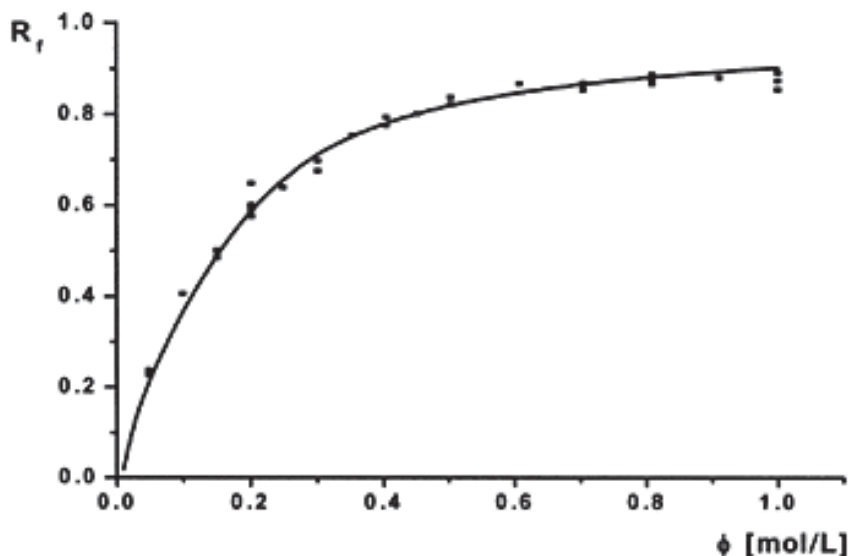


Figure 9 Relationship between R_f and ϕ for 1-naphthol chromatographed in system (a).

$$\frac{1}{K_{th}} = \frac{F_p f_3(T) c_p}{F_p' f_1(T) c_p' + F_d' f_2(T) c_d'} + \frac{F_d f_4(T) A_d}{F_p' f_1(T) c_p' + F_d' f_2(T) c_d'} \quad (33e)$$

Then it is assumed that in the case of binary mobile phases composed of one low-polarity and one semipolar or high-polarity solvent, the polar forces acting in that mobile phase on solute molecules are due basically to the polar component, whereas the dispersive forces are due mostly to the low-polarity component.

To examine the influence of different concentrations of polar or semipolar solvents in the same dispersing medium (e.g., an aliphatic hydrocarbon) on solute retention, Eq. 33e can be given in the simplified form

$$\frac{1}{K_{th}} = a c_p + b \quad (33f)$$

where a and b are equation constants.

A minimum of two known concentrations of polar solvent (c_p) allow establishment of numerical values of a and b for a given solute, stationary phase, and dispersing (i.e., nonpolar) medium. With the numerical values of a and b already established, the R_f value

for any other concentration of the same polar solvent can be predicted according to the relationship (see Eq. 30b)

$$R_f \approx \frac{V_m(ac_p + b)}{V_a W_a} \quad (66)$$

If, on the other hand, it is intended to examine the influence of changing dispersive forces on solute retention, then Eq. 33e can be rewritten as

$$\frac{1}{K_{th}} = a'd + b' \quad (33g)$$

where a' and b' are equation constants.

Polar interactions must be kept constant, which means that for a given phase system, c_p of the polar component must be kept constant. Dispersive forces are changed through changing the low-polarity component of the binary mixture (e.g., the hydrocarbon). For two different low-polarity components, numerical values of a' and b' (characteristic of a given solute, stationary phase, and polar solvent) can be established. With these data, the solute R_f value for a binary mobile phase with still another low-polarity solvent can be predicted (c_p of the polar component has to be maintained constant). The basis of such a prediction is furnished by the dependence

$$R_f \approx \frac{V_m(a'd + b')}{V_a W_a} \quad (66a)$$

Good correlation was observed between experimental R_f values and those predicted according to the assumed theoretical model (15, 16).

The Kowalska model of solute retention with use of multicomponent mobile phases (19–27) points out the fact that the generally accepted interpretation of the R_f coefficient does not fully exhaust the potential physicochemical contents of this factor. It anticipates eventual future models also immersed in the fundamentals of physical chemistry but refraining from the assumptions made by Martin and Synge and their successors.

Moreover, the relationships that form part of the Kowalska model (e.g., Eqs. 40–43) are more flexible and hence more accurate than the relationships offered by the other approaches discussed in this chapter. This is due to the fact that they (a) strongly depend on the chemical nature of the mixed mobile phases and (b) they couple together the R_f coefficient with the mobile-phase composition in a manner that is nonlinear in principle (an important feature that does not always occur with the remaining models of solute retention, no matter how much closer this nonlinearity is to the empirical practice of chromatography than the straight-line simplifications). Thus, it seems reasonable to expect that Eqs. 40–43 can be employed in the interpretational methods of selectivity optimization at least as successfully as any other already established retention model, and occasionally even more successfully.

E. Considerations on the Molecular Level

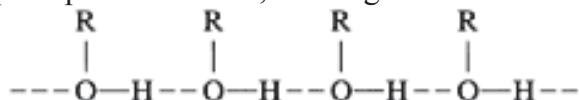
1. Role of Intermolecular Interaction Based of Chemical Potential Concept

Assuming the additive nature of the chemical potential, the basic relationship of Kowalska's model (see Sec. IV.E) can be rewritten as

$$\ln(x_i f_i) = \frac{\sum_k \Delta\mu_{(k)i}}{RT} \quad (35a)$$

where $\Delta\mu_{(k)i}$ is the partial change in chemical potential accompanying the transfer of the k th molecular fragment of the i th solute from the origin to the chromatographic system (calculated per mole of the k th fragment). Thus, $\Delta\mu_{(k)i}$ is an energetic measure of the efficiency of intermolecular interactions between this fragment and the rest of the chromatographic system considered as a whole. Table 6 gives an example of the numerical values of $\Delta\mu_{\text{CH}_2}$ and $\Delta\mu_{\text{OH}}$ determined for a homologous series of fatty alcohols on stationary phases of increasing activity using mobile phases of increasing polarity.

As can be seen from Table 6, the energetic values, which are not dimensionless and relative but on the contrary are absolute, are more persuasive and can be better integrated with general knowledge of physical chemistry. Two border cases of $\Delta\mu_{\text{OH}}$, obtained on low- and high-activity sorbents with the use of low- and high-polarity mobile phases, can be considered. Values range from about +15 to -15 kJ/mol, which coincides well with the absolute value of the hydrogen bond enthalpy for alcohols. This fact can be interpreted in the following way. An alcohol sample on the origin of a chromatogram can be regarded as a quasi-pure substance, forming chainlike self-associates:



In this way practically all OH groups are simultaneously involved in two hydrogen bondings. Transfer of alcohol to the low-polarity/low-activity chromatographic system involves dissociation of the chain multimers (disruption of two hydrogen bonds) followed by intermolecular interaction with the sorbent active center (formation of one hydrogen bond). The balance of this process consists of the disruption of one hydrogen bond, which in energetic terms equals +15 kJ/mol.

Transfer of alcohol to the high-polarity/high-activity chromatographic system proceeds through an identical initial stage, i.e., through dissociation of the chain multimers, which results in disruption of two hydrogen bonds. Then the alcohol OH groups form one hydrogen bond to anchor on the sorbent surface and two more with molecules of polar solvent (there are a maximum of three hydrogen bonds in which one OH group can be involved). Thus, in this case a balance is reached with the formation of one hydrogen bond, which corresponds to -15 kJ/mol. The scheme in Fig. 10 furnishes an illustration of the aforementioned differentiated behavior of the

Table 6 Numerical Values of $\Delta\mu_{\text{CH}_2}$ and $\Delta\mu_{\text{OH}}$ for the Homologous Series of Fatty Alcohols on Stationary Phases of Increasing Activity Using Mobile Phases of Increasing Polarity

Stationary phase	Mobile phase	$\Delta\mu_{\text{CH}_2}$ (kJ/mol)	$\Delta\mu_{\text{OH}}$ (kJ/mol)
Cellulose paper	Decalin	-0.57	+15.12
Cellulose powder	Decalin	-0.22	+11.16
Magnesium silicate	$\text{C}_6\text{H}_6 + (\text{CH}_3)_2\text{CO}$, 9:1 (v/v)	-0.28	-7.79
Alumina	$\text{C}_6\text{H}_6 + (\text{CH}_3)_2\text{CO}$, 9:1 (v/v)	-0.12	-12.46
Silica	$\text{C}_6\text{H}_6 + \text{CH}_3\text{OH}$, 9:1 (v/v)	0	-15.31

Source: Data from Kowalska (38, 39).

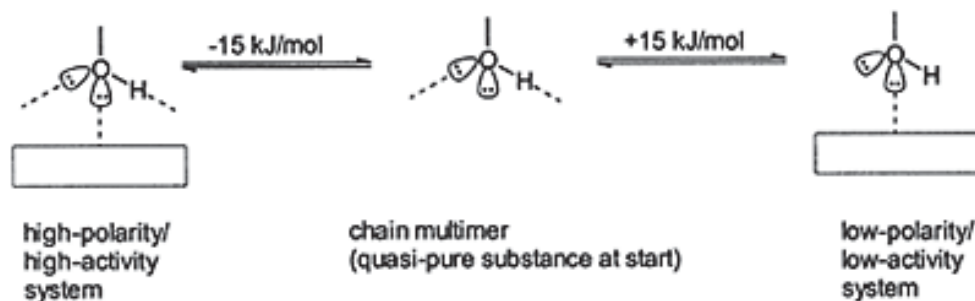


Figure 10 Behavior of an OH group in chromatographic systems differing with respect to sorbent activity and mobile-phase polarity.

OH group in chromatographic systems that differ profoundly in the activity of sorbents and the polarity of mobile phases.

The example discussed in this section is a rare case, in which we can deduce on a molecular level the nature of the chromatographic process investigated even if the applied model is macroscopic.

2. Role of Intermolecular Interactions—Multilayer Adsorption

When higher fatty acids are chromatographed on a cellulose layer with a nonpolar mobile phase, the densitograms obtained are similar to those presented in Fig. 11 (43, 44). Higher fatty acids form associative multimers by hydrogen bonding because of the presence of the negatively polarized oxygen atom from the carbonyl group and the positively polarized hydrogen atom from the carboxyl group. Direct contact of the higher fatty acids with an adsorbent results in forcible opening of the rings of most the cyclic

dimers (e.g., because of inevitable intermolecular interactions as a result of hydrogen bonding with the hydroxyl groups of the cellulose), thus considerably shifting the equilibrium of self-association toward the linear associative multimers.

The capacity of carboxylic acid analytes to form associative multimers (which can also be viewed as multilayer adsorption) is a cornerstone of the approach introduced (43, 44). This phenomenon was depicted with the aid of three similar isotherm models. The most convincing of these is founded on the following premises:

1. Analyte molecules are adsorbed on the active sites of an adsorbent. The kinetic rates of adsorption and desorption are infinitely fast.

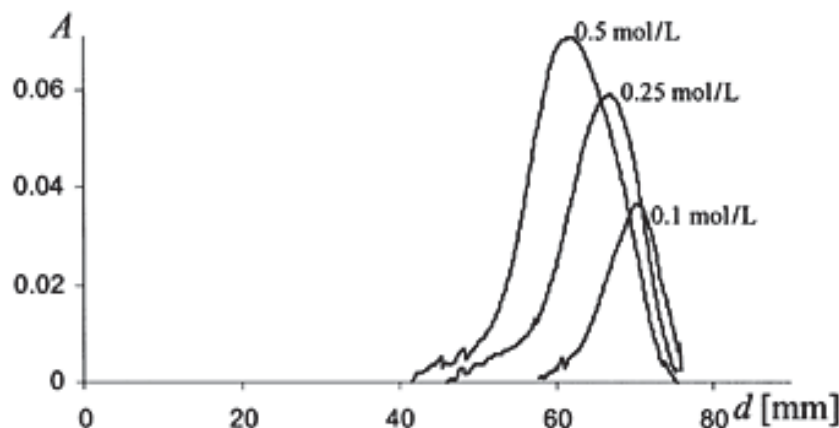


Figure 11 Densitograms obtained for succinic acid. Sample concentrations were 0.1, 0.25, and 0.5 mol/L. Stationary phase: cellulose; mobile phase: 1,4-dioxane.

2. Analyte molecules are adsorbed on a previously adsorbed monolayer. The kinetic rates of dimerization and dimer dissociation (i.e., of the reverse process) also are infinitely fast.
3. This chain process of stepwise building of consecutive adsorption layers can be continued ad infinitum.

These assumptions lead to the derivation of the isotherm equation (44)

$$q = q_s \frac{KC(1 + 2K_pC + 3(K_pC)^2 \cdots)}{1 + KC + KCK_pC + KC(K_pC)^2 \cdots} \quad (67)$$

where K is the equilibrium constant for the adsorption-desorption process on the active sites of the adsorbent; K_p denotes the equilibrium constant for dimerization, trimerization, etc.; q is the concentration of analyte on the adsorbent surface; q_s is the saturation capacity; and C is the concentration of analyte in solution.

The possibility of qualitative modeling of the experimentally observed peak profiles, presented in Fig. 11, was evaluated on the basis of the model (43, 44)

$$\frac{\partial C}{\partial t} + w \frac{\partial C}{\partial x} + \Phi \frac{\partial q}{\partial t} = D_x \frac{\partial^2 C}{\partial x^2} + D_y \frac{\partial^2 C}{\partial y^2} \quad (68)$$

with the assumed boundary conditions

$$\left. \frac{\partial C}{\partial x} \right|_{x=0, x=x_1} = \left. \frac{\partial C}{\partial y} \right|_{y=0, y=y_1} = 0 \quad (69)$$

where w is the average flow rate of mobile phase; C and q are, respectively, the concentrations (mol/dm^3) of analyte in the mobile phase and on the adsorbent surface; D_x and D_y are, respectively, the effective diffusion coefficients lengthwise (x) and in the direction perpendicular to the plate axis (y); Φ is the so-called phase ratio; and x_1 and y_1 are the plate length and width, respectively. It was assumed that at time $t=0$ analyte is concentrated in a rectangular spot at the start of the chromatogram.

The simulation depicted in Fig. 12 was obtained by solution of the Eq. 68 model in conjunction with the Eq. 67 isotherm and assuming three-layer adsorption as a maximum. Constants in the equation of the adsorption isotherm and the effective diffusion coefficients were chosen to reproduce the shapes of the lengthwise cross sections of the chromatographic bands obtained in the experimental densitograms (Fig. 11).

From Fig 12 it is apparent that the adsorption fronts are considerably less steep than the desorption fronts and that the adsorption fronts simulated for different initial concentrations of

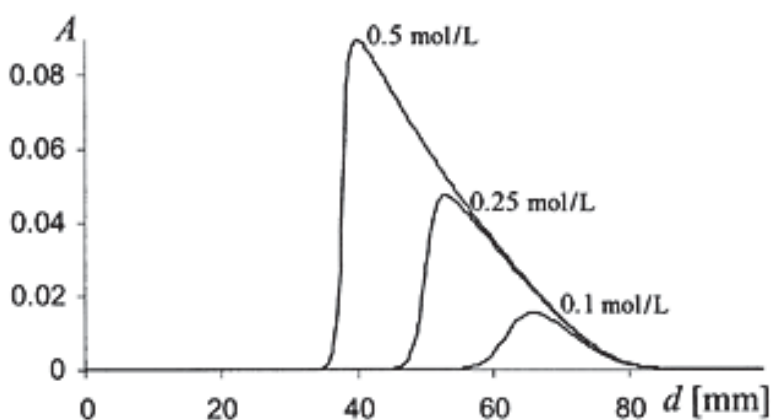


Figure 12 Lengthwise cross section of the simulated chromatogram, according to the model given by Eq. 68 in conjunction with the isotherm given by Eq. 67. Concentrations of the applied solutions were 0.5, 0.25, and 0.1 mol/L.

the spots overlap. Similar behavior is apparent in the typical experimental densitograms given in Fig. 11. In all of these densitograms, the adsorption fronts for the different

concentrations of acid also overlap. The experimental R_f values determined in the two alternative ways, i.e., from the concentration profile maxima and from the gravity centers of chromatographic bands, also decrease with increasing analyte concentration (43, 44). Such behavior of the R_f coefficients, qualitatively consistent with the theoretical data presented in Fig. 12, cannot be explained by assuming classical Langmuir, Freundlich, or similar isotherms.

Satisfactory qualitative agreement between the experimental and theoretical concentration profiles of polar analytes suggests that their retention is substantially affected by lateral interactions, which are probably even more complex than is assumed in this isotherm model. Overlapping of the adsorption fronts and the behavior of the R_f coefficients can be explained only on the basis of lateral interactions among the adsorbed molecules.

VI. ATTEMPTS TO ENHANCE THIN-LAYER PERFORMANCE

Enhancement of thin-layer performance is basically understood as an increase in the theoretical plate number N for a given type of chromatographic plate. The quantity N was defined in Section III.A as

$$N = \frac{l}{H} \quad (15)$$

From Eq. 15, it can be seen that an increase in N can be attained in two ways, i.e., through increasing l or decreasing H .

Elongation of the migration path l is usually achieved through a continuous flow of the mobile phase along the length of the chromatographic plate. This continuous development can be done using the traditional stationary phases or supports.

A decrease in the quantity H cannot, however, be achieved without a change in the basic physical parameters of the chromatographic system. The theoretical plate height H can be suppressed by decreasing the diameter of the solid bed particles d_p (see Eqs. 17 and 19) and decreasing the thickness of the stationary phase layer d_f (see Eq. 20). Practical transformation of these conclusions into independent chromatographic techniques is briefly sketched in the following sections.

A. High-Performance Thin-Layer Chromatography

It is not the aim of this section to present any details of the history or state-of-the-art procedures for HPTLC. HPTLC is a relatively young thin-layer technique (established about 1974), which is still undergoing improvements and gaining in popularity. According to Kaiser (40), HPTLC involves the combined action of several variables, including

1. An optimized coating material with a separation power superior to that of the best HPLC separation material
2. A new method of feeding the mobile phase
3. A novel procedure for layer conditioning

4. A considerably improved dosage method
5. A competent data acquisition and processing system

The secret underlying an optimized coating is a perfectly uniform surface of the thin layer. This can be attained using fine-particulate sorbent materials in the adsorption mode or very fine and spherical nonporous SiO_2 carriers with a bonded chemical phase in the partition mode. These microparticulate solids additionally demonstrate a narrow distribution of particle dimensions (i.e., all particles are of practically equal size), which allows a much greater density of packing of the HPTLC layers compared with normal ones. Thus, one can easily understand that the enhanced performance of HPTLC is mainly due to decreases in the quantities d_p and d_f (Eqs. 17, 19, and 20) compared with regular thin-layer chromatography. In other words, HPTLC takes advantage of a decrease in H . fs

B. Overpressured Thin-Layer Chromatography

The overpressured thin-layer mode of planar chromatography was introduced by Hungarian scientists (41, 42) in the 1970s. In overpressured thin-layer chromatography (OPTLC) the vapor phase has been eliminated, the sorbent layer being completely covered with an elastic membrane under external pressure. Thus, the mobile phase migrates through the thin layer due to the "cushion system" at overpressure. In this way, OPTLC combines advantages of the continuous development technique, mentioned before (increase in l), with elimination of the free space in the chromatographic chamber, which is also typical of the column techniques. This is done in an effort to enhance the theoretical plate number N of a regular thin layer, although high-performance plates are used as well.

C. Centrifugal Layer Chromatography

Centrifugal layer chromatography (CLC) (45) is a preparative circular chromatographic technique in which the mobile phase flow is induced by centrifugal force. The sample is applied near the center of a rotating disk covered with adsorbent material. Concentric zones of substances migrate toward the outside of the plate during elution. The circles elute sequentially from the disk and can be recovered separately. Thus CLC can also be regarded as a continuous development mode (increase in l).

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3

Optimization

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I. INTRODUCTION

The problem of optimization can be traced back to the advent of chromatography as an analytical method. Separation optimization is related to the proper choice of the parameters influencing the separation. Optimization is treated separately by every chromatographic method, taking into account the specific problems encountered in the fields of gas and liquid chromatography. Even in liquid chromatography, the subject of optimization is different in planar chromatography (1, 2) from that involved in column liquid chromatography (3, 4), and only a few authors have approached this subject as a general case (5). Simultaneously with the widespread use of computers in analytical laboratories, the topic has attracted more and more attention, and a great number of software packages have been developed to help the analyst in the optimization separation parameters (6–8). Some forms of optimization are generally necessary in planar chromatography if the separation of all compounds is required, especially when the number of components is larger than a small fraction of the spot capacity of the system.

In thin-layer chromatography, only a few factors need be taken into consideration for optimization, because most of them are fixed for theoretical or practical reasons even though the system is complex. The most important factors are the solvent system and its composition, the optimum time, the temperature, and, in some cases, the relative humidity. Temperature is not used as an optimizing factor because in most cases the variation of temperature in the normal temperature work range has no influence on the minimum time of analysis necessary to obtain a defined value of resolution. Relative humidity is an experimental variable difficult to change within narrow ranges, and therefore its use is not recommended in optimization. In conclusion, the most important factor that must be taken into account in the optimization of a thin-layer chromatography system is the composition of the mobile phase, which is often the only component seriously considered.

This chapter describes the methods used for mobile-phase optimization, including not only those developed for thin-layer chromatography but also those developed for liquid chromatography and applied to thin-layer chromatography. These methods are applied in

both one- and two-dimensional TLC. Furthermore, the chromatographic response functions used to reflect the quality of separation are reported. Automated multiple development is described as a method for separation optimization.

II. CHROMATOGRAPHIC RESPONSE FUNCTION

Simple optimization methods are used for the separation of simple mixtures. In the case of complex mixtures, some sophisticated strategies have been developed to optimize the mobile-phase composition. These methods are intended to find the maximum or minimum of an “objective function” called the chromatographic response function (CRF) or criteria function, which expresses the quality of separation by a single number. The chromatographic response function also expresses the goals of chromatographic separation in mathematical terms. Because there is no one CRF to satisfy all needs, a great number of CRFs have been designed and tested. The selection of a proper CRF is a crucial step in optimization strategy; its choice depends on the overall goal of the separation, and it has been demonstrated that the result of an optimization procedure depends on the criteria function selected.

The most widely used CRF is the resolution between adjacent peaks, but this function contains no information about the number of peaks eluted. The resolution should be calculated with the equation

$$R_s = 2 \frac{R_{f,i+1} - R_{f,i}}{W_{i+1} + W_i} \quad (1)$$

where w_i is the width of the i th peak.

Because of its simplicity and popularity, this function is widely used in research as a criteria function for different methods of optimization (9–12). Resolution-based criteria have the disadvantage of inexact determination of the width of a spot.

When separation of all solutes is desirable, in which case the resolution function is useful as an overall CRF, the resolution of each peak may be combined into a resolution product function (Eq. 2) (13, 14), resolution sum function (Eq. 3) (15), or resolution relative product (Eq. 4) (16). These global resolution functions give a resolution value for the entire chromatographic separation.

$$R_p = \prod_i R_{s,i} \quad (2)$$

$$R_s = \sum_i R_{s,i} \quad (3)$$

$$\text{RRP} = \frac{\prod_i R_{s,i}}{\sum_i R_{s,i}/(n-1)} \quad (4)$$

Another CRF that uses resolution is the modified chromatographic resolution statistic (Eq. 5) used by Lukulay and McGuffin (17) for the optimization of the mobile phase.

$$\text{CRS} = \left[\sum_i \left(\frac{R_{s,i} - R_{s,\text{opt}}}{R_{s,i} - R_{s,\text{min}}} \right)^2 \left(\frac{1}{R_{s,i}} \right) + \sum_i \frac{R_{s,i}^2}{n_p \bar{R}_s^2} \right] \left(\frac{t}{N} \right) \quad (5)$$

In Eq. 5, $R_{S,opt}$ is the optimum resolution, $R_{S,min}$ the minimum acceptable resolution, $\bar{R}$, the average of peak pair resolution, n_p the number of peak pairs on a given chromatogram, t the time of analysis, and N the number of actual peaks. This function reflects the extent of separation between adjacent peak pairs, the uniformity of the spacing between peaks, and the total analysis time. The CRS takes a minimum value when all peaks are well resolved and uniformly spaced on the chromatogram.

The retention factor (R_f) is used as the basic criterion in many CRFs, such as $\Delta R_{f,min}$ (Eq. 6) (18), ΔR_f product (Eq. 7) (18), multispot response function (Eq. 8) (19), separation response (Eq. 9) (20), chromatographic response function (Eq. 10) (21), performance index (Eq. 11) (22), and informational entropy (Eq. 12) (23), and these functions are often used in optimization procedures.

$$\Delta R_{f,min} = |R_{f,i+1} - R_{f,i}| \quad (6)$$

$$R^* = \prod \Delta R_f \quad (7)$$

$$MRF = \frac{[(hR_{f,max} - hR_{f,n})(hR_{f,1} - hR_{f,min})] \prod_{i=1}^{n-1} (hR_{f,i+1} - hR_{f,i})}{[(hR_{f,max} - hR_{f,min})/(n+1)]^{n+1}} (\%) \quad (8)$$

$$D = \left[\sum_{i=1}^n \left(R_{f,i} - \frac{i-1}{n-1} \right)^2 \right]^{1/2} \quad (9)$$

$$CRF = \prod_i \frac{\Delta R_{f(i+1,i)}}{\bar{R}_{f(i+1,i)}} \quad (10)$$

$$I_p = \left(\frac{\sum (\Delta hR_{f,i} - \Delta hR_{f,i})^2}{n(n+1)} \right)^{1/2} \quad (11)$$

$$s_m = \left(\frac{\sum (\Delta hR_{f,i} - \Delta hR_{f,i})^2}{n+1} \right)^{1/2} \quad (12)$$

The definitions of symbols from Eqs. 6–12 are the following: ΔR_f is the difference between a spot and its neighbor, $hR_f = 100R_f$, $hR_{f,max}$ and $hR_{f,min}$ can be selected to eliminate the region near the solvent front and the origin, $hR_{f,1}$ is the lowest hR_f value, $hR_{f,n}$ is the highest hR_f value, n is the number of equally spaced components, k is the number of all possible combinations of peak pairs in a solute mixture, and $\Delta hR_{f,i}$ and $\Delta hR_{f,i}$ are the measured interval between two adjacent peaks and the theoretical interval between any two adjacent peaks in the case of an ideal separation, respectively.

The function $\Delta R_{f,min}$ has the disadvantage that it takes into consideration only the most poorly separated pair of spots, and the overall chromatogram looks as bad even when all the other pairs of spots are well separated. The maximum value of $\prod \Delta R_f$ is obtained when the spots in the chromatogram are as uniformly spaced as possible, but the main inconvenience of this criterion is that it does not take into consideration the shape and width of an individual spot. This criterion partially overcomes the drawback of $\Delta R_{f,min}$. The multispot response function takes the maximum value of 100% when all components are equally spaced from the chosen boundaries and from each other, and the criterion is

equal to zero if the spots do not occur within the preset interval. The separation response tends to minimum in the optimum case when the components are equally spaced in the unit interval and they are arranged in ascending order. The performance index and informational entropy reflect the uniformity of the separation and are very useful in estimating the chromatographic separation.

Complex CRFs are used in optimization when an unequivocal determination of a single physical value is difficult. Some of these functions that are frequently used in the optimization of mobile-phase compositions are presented in the following equations.

$$I = - \sum \left(\frac{n_k}{n} \right) \log_2 \left(\frac{n_k}{n} \right) \quad (13)$$

$$I\bar{R}_i = I \sum \frac{R_{i,j}}{n} \quad (14)$$

$$IE = \sum p_i^2 \quad (15)$$

$$CRF = \sum \alpha_i \left(\frac{P_i}{P_d} \right) \quad (16)$$

In the above equations, n is the total number of components, n_k is the number of separated compounds, p_k is the probability of finding a peak in a group, P_i and P_d are the peak separation between an adjacent pair of peaks calculated from a densitogram and the desired peak separation, respectively, and α_i ($0 \leq \alpha_i \leq 1$) is the weight, which is visually determined by examination of the shape of the spot ($\alpha_i = 1$ if the shape is perfectly round).

The amount of information (Eq. 13) (24) and the informational energy (IE) (Eq. 15) (25) use the discontinuities of probabilities related to some arbitrary “groups” of retention parameter values illustrating the multicomponent separation, and these functions are not affected by peak widths. That is why the functions are not very sensitive, especially in the case of mixtures containing a small number of components. The product of the mean resolution of all consecutive peaks and the amount of information (Eq. 14) (26) reflects the overall separation of all pairs of adjacent peaks. The CRF (Eq. 16) (27) takes into consideration the shape of the spots, and it reflects the manner in which this shape differs from perfect roundness and whether the spots are or are not overlapped.

Moreover, some functions are proposed as criteria for separation quality, especially for two-dimensional TLC. For example, such functions are the distance function (Eq. 17) and the inverse distance function (Eq. 18) (28), the planar response function (Eq. 19) (29), and those introduced by Gonnord et al. (Eqs. 20 and 21) (30).

$$DF = \sum_{i=1}^{n-1} \sum_{j=i+1}^n S_{ij} \quad (17)$$

$$IDF = \sum_{i=1}^{n-1} \sum_{j=i+1}^n \frac{1}{S_{ij}} \quad (18)$$

$$PRF = \sum_{i=1}^{n-1} \sum_{j=i+1}^n \ln \frac{S_{ij}}{S_{SPEC}} \quad (19)$$

$$D_A = \sum_{i=1}^{n-1} \sum_{j=i+1}^n [(x_i - x_j)^2 + (y_i - y_j)^2] \quad (20)$$

$$D_B = \sum_{i=1}^{n-1} \sum_{j=i+1}^n \left(\frac{1}{(x_i - x_j)^2 + (y_i - y_j)^2} \right) \quad (21)$$

The separation was optimized either by the maximization of DF and D_A or by the minimization of IDF and D_B . To prevent IDF and D_B from assuming too large a value, it is necessary to assign an arbitrary separation distance to unresolved peaks. In the case of PRF, all peak pairs with a distance S_{ij} greater than the desired minimum separation (S_{SPEC}) were assigned the value of S_{SPEC} , and such pairs have no influence on the value of the function.

The functions presented here are only a few of the criteria functions used in optimization of TLC separations with no attempt to exhaust their list. Some CRFs attempt to catch in their value a single essential quality of the whole multicomponent separation, whereas others combine several desired qualities of chromatographic separation.

Cimpoiu and Hodisan (31) concluded that a given chromatogram is "optimum" if it fulfills the following conditions: The number of separated compounds must be maximum, the peak width must be as small as possible, the separation coordinates of all individual peaks must be distributed throughout the chromatogram as uniformly as possible, the separation of all adjacent pairs of peaks must be the best, the solvent system and the stationary phase used must have a maximum separation potential, and the separation time must be the shortest possible. To satisfy all these conditions, different simple criteria functions could be coupled to form an overall CRF that is a combined function representing a well-balanced sum of simple functions, such as (32, 33)

$$F = 0.4 \frac{s_m}{10} + 0.2 \frac{10}{I} + 0.2 \frac{10}{IR_s} + 0.2 \frac{1}{100 RRP} \quad (22)$$

$$F_{obj} = an + bIR_s + c \frac{10}{IE} + \frac{d}{I_p + \varepsilon} \quad (23)$$

where n is the number of components observed as peaks (zones); a , b , c , and d are arbitrary weighting factors; and ε is a very small, arbitrary value (10^{-5}) used to eliminate the indetermination of the fourth term (I_p becomes zero in the case of optimum separation).

The function F_{obj} (Eq. 23) was used in mobile-phase optimization for the separation of some 1,4-benzodiazepines (33), and a plot of the fractions between the values of functions and their final (optimum) value during the optimization (Fig. 1) shows the evolution of individual functions to their final limit. It can also be concluded that the most sensitive functions proved to be IR_s and I_p . These functions had reverse fluctuations, but the trend was to the same "optimum." The functions IE seemed to be insensitive and therefore had a smoothing action, but their ultimate confirmatory action proved helpful in making the final decision. This function becomes insensitive

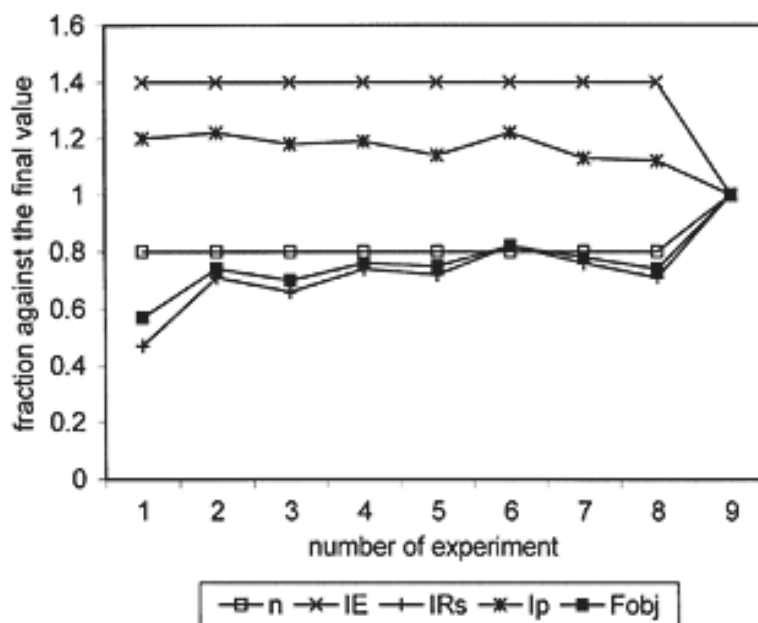


Figure 1 Plot of the fraction between the values of functions and their final (optimum) value versus the number of solvents tested. (From Ref. 33.)

only when the number of components in the mixture is small. Consequently, the authors concluded that the essential factor is not the sensitivity (the slope) of the function but the ability to give strictly different values for different solvents during the optimization steps.

In conclusion, the first step in any optimization procedure is the choice of solvent system with maximum separation potential, but once the components of the mobile phase have been determined, the CRF becomes necessary. The selection of criteria functions is a very important step in the optimization procedure, and the use of complex CRFs with a great number of factors seemed to make the final result safer.

III. OPTIMIZATION METHODS

The intuitive approaches of solvent selection and optimization of developing conditions are used only in separations of simple mixtures with a small number of components. In such cases, some chromatographic chambers have been developed with which a great number of solvents can be tested, and all approaches give results within a few hours when employed in a systematic manner. For example, in the Vario KS chamber, six to 10 solvent strengths can be evaluated in parallel, and complete optimization can be accomplished with three or four plates (34, 35). Methods based on several simultaneous experiments using multicomponent isocratic mobile phases are suitable for the optimization of ternary and quaternary solvent systems (36).

These methods are time-consuming when applied to complex mixtures that have a great number of components. More complex optimization strategies and approaches were

developed for such mixtures, and some of them are presented below with no attempt to be exhaustive.

A. Window Diagrams

Laub and Purnell (37) developed the window diagrams method for optimization of separation by gas-liquid chromatography, and this method has since been widely used in both gas chromatography and high-performance liquid chromatography (HPLC). Until recently, window diagrams were rarely used in TLC or HPTLC (38). The difference between the retention parameters is used as the chromatographic response function, and for two components it is given by the equation

$$\Delta R_f = \frac{k'_2 - k'_1}{(1 + k'_2)(1 + k'_1)} \quad (24)$$

The capacity factors k' could be calculated as functions of the mole fraction (X_s) of polar solvent in the mobile phase system:

$$\log k' = a \log X_s + b \quad (25)$$

ΔR_f was plotted against solvent composition (X_s or e_{AB}^0), and if all peak pairs are considered, the plot represents the window diagram by means of which the optimum solvent composition is identified. The maximum values of ΔR_f ($\Delta R_{f,\max}$) tend to assemble around a peculiar mole fraction of the solvent system, and this represents the optimum composition of the mobile phase. The advantage of this method lies in the fact that the global optimum can be easily located by eye or by computer (39).

The window diagrams method is seldom used in the case of ternary or quaternary solvent systems because these mobile-phase systems allow a large variety of intermolecular interactions. In such cases, the relationship between the retention parameter and mobile-phase composition is given by Eq. 26 (40), but a local optimum can be attained instead of the global optimum.

$$R_f = a_0 + a_1 X_s + a_{11} X_s^2 \quad (26)$$

The coefficients from Eq. 25 (a and b) and Eq. 26 (a_0 , a_1 , and a_{11}) have been determined by preliminary experiments. Other approaches such as the sequential simplex algorithm, PRISMA method, overlapping resolution mapping scheme, taxonomy, and principal components analysis have been used for the optimization of such mobile-phase systems, and these methods are discussed below.

B. Sequential Simplex Method

The sequential simplex method was introduced by Spendley et al. in 1962 (41) and was then used in analytical chemistry by Long (see Ref. 42). It is simple and fast and can be used in automatic optimization (43, 44).

The sequential simplex method is based on a geometric figure in the variable space of a criteria function, a figure whose number of vertices is greater by one than the number of

variables. The CRF is evaluated in each vertex of the figure, the most unfavorable vertex corresponding to the worst response is rejected, and then a new favorable vertex is established by searching the direction that is experienced by this unfavorable vertex and the centroid of the other vertices. The new simplex is thus determined, and the algorithm is repeated until the optimum response is obtained.

The method described by Spendley et al., the fixed-size sequential simplex method, is an algorithm consisting simply of reflection rules, and for this reason the method is slow and a false optimum could be attained. Moreover, the simplex with more than four dimensions does not cover the entire field of criteria functions in all cases, and the moment when the optimum has been attained is not very clear.

The method presented by Nelder and Mead (45) is a variable-size simplex algorithm consisting of reflection, expansion, and contraction rules, and the simplex can be accelerated in favorable directions and slowed down in unfavorable directions (Fig. 2). The first simplex was the triangle XYZ , the most favorable vertex is X , and Z is the most unfavorable vertex. The reflection, expansion, and contraction of simplex can be calculated by the following equations, which generate new simplexes.

$$R = C + (C - Z) \quad (27)$$

$$E = R + (C - Z) \quad (28)$$

$$C_R = C + \frac{C - Z}{2} \quad (29)$$

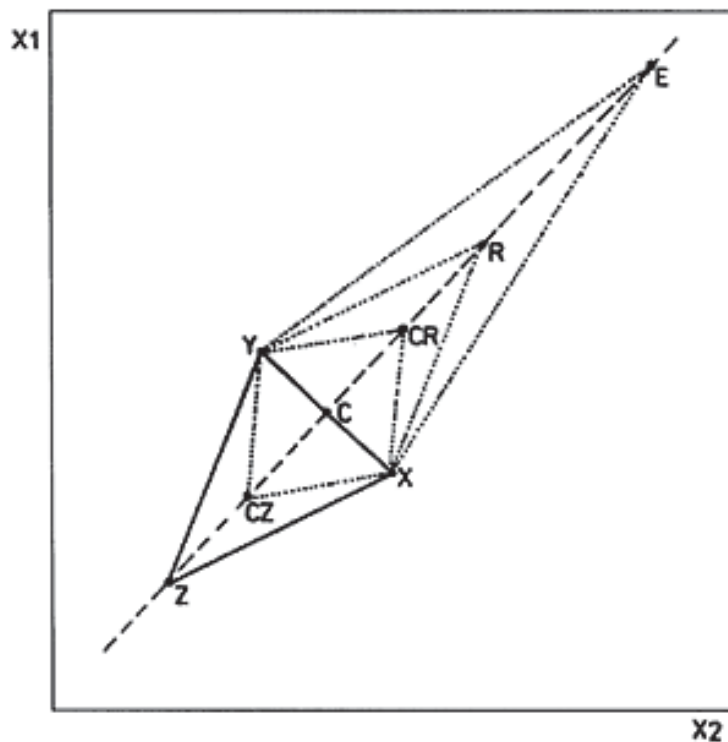


Figure 2 Simplex generation.

$$C_z = C - \frac{C - Z}{2} \quad (30)$$

The vertex R is obtained after the first reflection, and the vertex E , representing an expansion, is obtained if vertex R is more favorable than vertex X . If vertex R is more unfavorable than vertex F , the simplex must be contracted, which yields either the vertex C_R if $R > Z$ or the vertex C_z if $R < Z$.

False responses can be detected if the vertex found to be the most favorable in $k+1$ simplexes is re-evaluated. The simplex is stopped when the step size becomes less than some predetermined value, when the differences in responses approach the value of experimental uncertainty, or when an adequate response has been achieved. In the case when the vertex lies outside the boundaries of the factors, a most undesirable response is assigned to that vertex, the simplex is forced back inside the boundaries, and the new vertex is determined by a contraction. Because of its simplicity and efficiency, the sequential simplex method is one of the most applied methods in both isocratic development chromatography and gradient development chromatography.

One widely reported disadvantage of the simplex method is that a local optimum is obtained instead of the global optimum. For example, Morita et al. (27) used the simplex and PRISMA methods to optimize the mobile phase for separation of six red pigments. In this case, Eq. 16 reflects the quality of separation. The separation performed with the mobile-phase composition determined by the PRISMA method is better than the separation with the mobile-phase composition obtained by the simplex method. The authors stated that these results are due to the fact that either the response surface was too flat around the optimum area and consequently the response differences could not be distinguished from experimental error, or the optimum obtained with the simplex method was not the global optimum but a local one. It is possible to obtain a global optimum if the overall criteria functions are used to reflect the quality of chromatographic separation (32, 46). In order to be sure that the global optimum is found, the simplex should be initialized at several different starting points (15).

C. Prisma Method

The PRISMA method was developed by Nyiredy and coworkers (47–49) to optimize the mobile-phase system in TLC. Ten preliminary experiments are carried out with 10 solvents, chosen from the selectivity groups of Snyder (50), to select suitable solvents. For normal-phase TLC, the solvent strength has to be either reduced by dilution with hexane or increased by addition of water or acetic acid so that the R_f values of the compounds are in the range of 0.2–0.8. Two to five solvents can be selected for construction of the PRISMA model, which is a three-dimensional geometric design that correlates the solvent strength with the selectivity of mobile phase (Fig. 3). The lengths of the edges of the prism correspond to the strength of the solvent, and because different solvents usually have different solvent strengths, the model consists of three parts: the base or platform, the regular part of the prism, and the irregular part of the prism (frustum). The base represents the modifiers that can be added in low and constant concentrations to improve the separation and reduce tailing. In normal-phase chromatography, the regular part is used for mobile-phase optimization of nonpolar and

moderately polar compounds, and the frustum is used to optimize the separation of polar compounds, whereas for reversed-phase chromatography, the regular part of the prism is used to optimize both polar and nonpolar compound separations.

For polar compounds, optimization is started by selecting combinations corresponding to the center point and three other points close to the apexes of the top irregular triangle of the model. The initial solvent composition for the separation of nonpolar and moderately polar compounds corresponds to the center of the triangular top face of the regular part of the prism. This composition is diluted so that the R_f values will be in the range 0.2–0.8. Three other compositions of mobile phase with this solvent strength, corresponding to the selectivity points close to the apexes of the triangle, are tested. All the selectivity points can be described by three numbers so that the selected points are $P_s=333$ for the center of the triangle and $P_s=811$, 181, and 118 for those close to the apexes of the triangle. If the obtained separation is insufficient, other selectivity points are tested around the solvent combination that gave the best separation, and this process is repeated until the best solvent composition is obtained. It must be noted that the selectivity points should be changed by small increments in the case of polar compounds (irregular triangle) if the regular step sizes cause a large change in resolution. Furthermore, in such cases the solvent strength is changed when the selectivity points are, and the solvent strength should be adjusted to maintain the separation in the optimum R_f range.

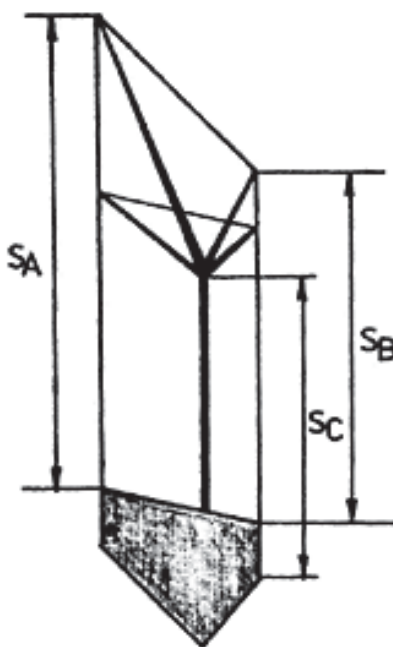


Figure 3 The PRISMA model for solvent system optimization.

There is vertical and horizontal correlation between the hR_f values of nonpolar compounds and the selectivity points (P_s) at different constant levels of the solvent strength (S_T) in saturated chambers. These correlations, given by the following equations, are described by Nyiredy and Fater (51):

$$\ln hR_f = d(S_T) + e \quad (31)$$

$$hR_f = a(P_s)^2 + b(P_s) + c \quad (32)$$

Pelander et al. (52) studied the retardation behavior of cyanobacterial hepatoxins in the irregular part of the prism, and they concluded that the horizontal correlation (Eq. 32) can also be applied in the cases of polar compound separations ($r^2=0.9860$).

The PRISMA method is rather simple and can be used to describe all binary, ternary, or quaternary mobile phases. The optimum mobile-phase composition can be obtained on the basis of a relatively small number of experiments and with very little error. Some degree of chromatographic experience is required because of the possibility of making errors in the determination of the solvent strength.

Pelander et al. (53) applied this method and used regression equations to optimize the mobile phase for the separation of some cyclic heptapeptides by HPTLC and for the separation of some phenolic compounds by RP-HPTLC. Cimpoiu et al. (54), after optimization of the mobile-phase composition used for the separation of the 1,4-benzodiazepines, concluded that for the polar compounds the mobile-phase composition could not be modified more precisely even if good separation is obtained.

The PRISMA method represents a useful approach for the optimization of mobile phases, especially in the cases of complex samples containing a great number of components (55, 56). The time to evaluate each solvent system composition is short because several different compositions can be studied simultaneously.

D. Overlapping Resolution Mapping Scheme

One of the most important additions to the literature of mobile-phase optimization is the introduction of the overlapping resolution mapping (ORM) scheme by Glajch et al. in 1980 (57). This method includes the determination of coefficients for a quadratic equation containing six to 10 terms that allows the generation of a chromatographic response surface for a multicomponent mobile phase. Also, this method is an interpretive optimization method in which the extent of chromatographic separation is predicted indirectly from the retention of the individual compound (58). The solvents are selected according to the Snyder selectivity triangle, and for optimization of the mobile phase only seven experiments are required.

ORM involves eight steps (59):

1. Set criteria.
2. Select an appropriate mobile-phase strength.
3. Define the vertices of the solvent selectivity triangle.
4. Perform a set of seven experiments.
5. Calculate the resolution for each peak pair.
6. Determine coefficients for the second-order polynomial equation.
7. Plot diagrams for each peak pair.
8. Superimpose individual diagrams to obtain the ORM diagram.

Generally, two criteria are used: The resolutions of all peak pairs in an optimum chromatogram are higher than 1.5, and the capacity factors, k' , are in the range 0–20 so that the analysis time is adequate. Of course, other criteria can be used in accordance with the separation purpose.

The experimental values of resolution are fitted into a second-order polynomial:

$$R_s = a_1x_1 + a_2x_2 + a_3x_3 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 + a_{123}x_1x_2x_3 \quad (33)$$

where a_i are coefficients and x_i the volume fractions of the solvents. It is possible to use a logarithmic equation to increase the nonlinearity of the response model when modeling the minimum resolution criteria (60):

$$\ln(1+R_{s,\min}) = b_0 + b_1 \ln(x_1) + b_2 \ln(x_2) + b_3 \ln(x_3) + b_{12} \ln(x_1)\ln(x_2) + b_{13} \ln(x_1)\ln(x_3) + b_{23} \ln(x_2)\ln(x_3) + b_{123} \ln(x_1)\ln(x_2)\ln(x_3) \quad (34)$$

Resolution values calculated with Eq. 33 were used to construct the individual diagrams for all peak pairs, and the areas corresponding to $R_s < 1.5$ are shaded. The individual resolution plots are then superimposed to give an ORM diagram (Fig. 4). In Fig. 4 the region marked with # represents the optimum area. Moreover, a three-dimensional diagram should be obtained by superimposing individual resolution plots (Fig. 5), and the highest point represents the optimum mobile-phase composition.

The ORM scheme is a rapid and versatile method, and the optimum mobile-phase composition can be achieved without much difficulty even when quaternary mobile phases are considered. Only seven different mobile phases need to be examined in order to obtain the resolution plots.

This method was applied for the optimization of the mobile phase for the HPTLC separation of 1,4-benzodiazepine mixtures (61). The R_s values for all mobile phase compositions within the solvent triangle were used to calculate the quality factor Q for all the pairs of peaks by the equation

$$Q = \min(R_{si}, i=1, \dots, n-1) \quad (35)$$

The individual quality factor plots were then superimposed, and the optimum mobile-phase composition was given by the maximum of the obtained surface.

In addition, many researchers report the use of the previously discussed chromatographic response functions instead of the resolution function. The relations between these functions and the mobile-phase composition are given by the same formula (Eq. 33); a plot of the CRF versus the experimental variables is generated, and the optimum conditions are those that correspond to the optimum value of the CRF found on the plot. These optimization techniques are often called response surface modeling. As reported by Cimpoi et al. (62), if the global CRF (Eq. 23) is used, the final result is more reliable due to the introduction of a great number of factors. The chromatographic separation achieved in this case is better than the separation performed with a mobile-phase composition determined by using resolution as the criterion function (Fig. 6).

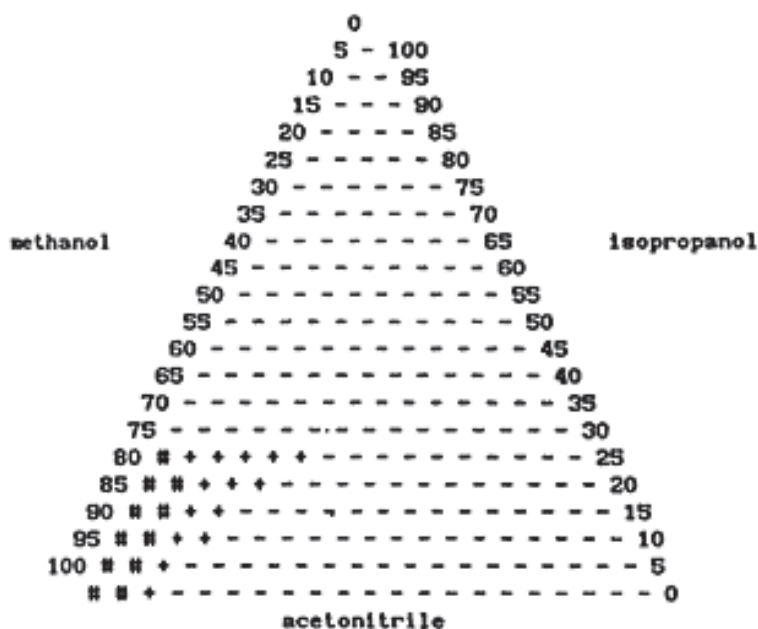


Figure 4 Superimposing of resolution plots for nine peak pairs: (–) $R_s \leq 0.5$; (+) $0.5 < R_s < 1.0$; (#) $R_s \geq 1.0$. (From Ref. 59.)

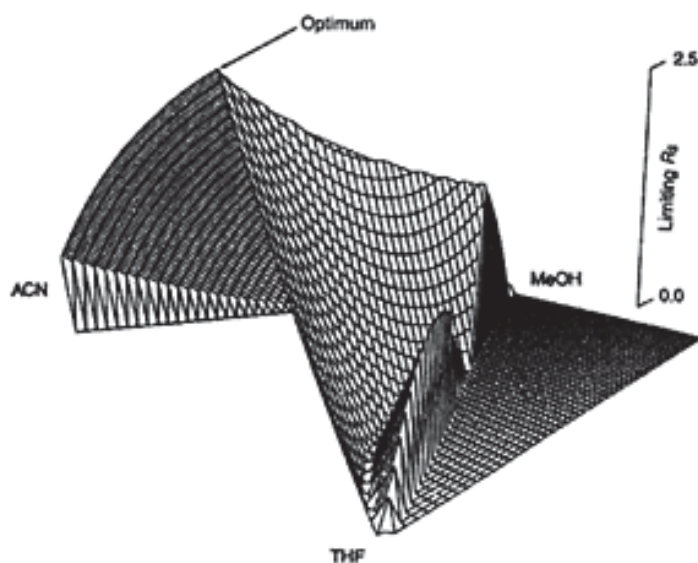


Figure 5 Three-dimensional ORM diagram. (From Ref. 57.)

The ORM and response surface modeling methods have the advantage of being able to be implemented with a computer program, allowing automation of the optimization process.

E. Numerical Taxonomy

The numerical taxonomy method was used originally in biological research and allows classification of the organisms according to their relationship or resemblance. Taxonomy is an art rather than a science because this technique is somewhat intuitive and tends to be subjective.

Numerical taxonomy was developed relatively recently as a quantitative approach. This method uses a variety of mathematical techniques to classify the elements into groups or individual groups into larger groups. Each classified unit is generally called an "operational taxonomic unit" (OTU), and in TLC the OTU is the solvent-stationary phase system (63, 64).

The essential idea of numerical taxonomy is to attach numerical values to a number of characteristics for each of the OTUs and compare these values to discover similarities so that the OTUs can be classified according to their resemblance. The characteristics that can be used in chromatographic systems will be the retention parameters, Kovacs indices, etc. for standard substances or for compounds that have to be separated.

Generally, the numerical taxonomy technique consists of three steps. In the first step, the data X_{ij} are recorded in an $(i \times j)$ matrix with $i=n$ characteristics and $j=j_{\max}$ OTUs. In chromatography, n is limited to the total number of components for which the separation is investigated. The second step is to compare each OTU with each other OTU and record this comparison as similarity values. Many kinds of similarity values have been proposed in numerical taxonomy, but the most common is a measure of distance. The taxonomic distance for two OTUs with $j=k$ and $j=l$ is given by the equation

$$\Delta_{kl} = \left[\sum_{i=1}^n \frac{(x_{ik} - x_{il})^2}{n} \right]^{1/2} \quad (36)$$

The division by n represents normalization and allows the inclusion of OTUs for which not all n characteristics are known. When m is the number of missing values, the denominator of Eq. 36 becomes $n-m$. A symmetrical $(j_{\max} \times j_{\max})$ resemblance matrix is constructed with the Δ_{kl} values.

The final step of this method consists of grouping together the OTUs with the largest similarity (the smallest distance). The procedure is as follows: The smallest Δ_{kl} value is sought in the matrix and found to be Δ_{qp} . The resemblance matrix is thereby reduced to $(j_{\max} - 1) \times (j_{\max} -$

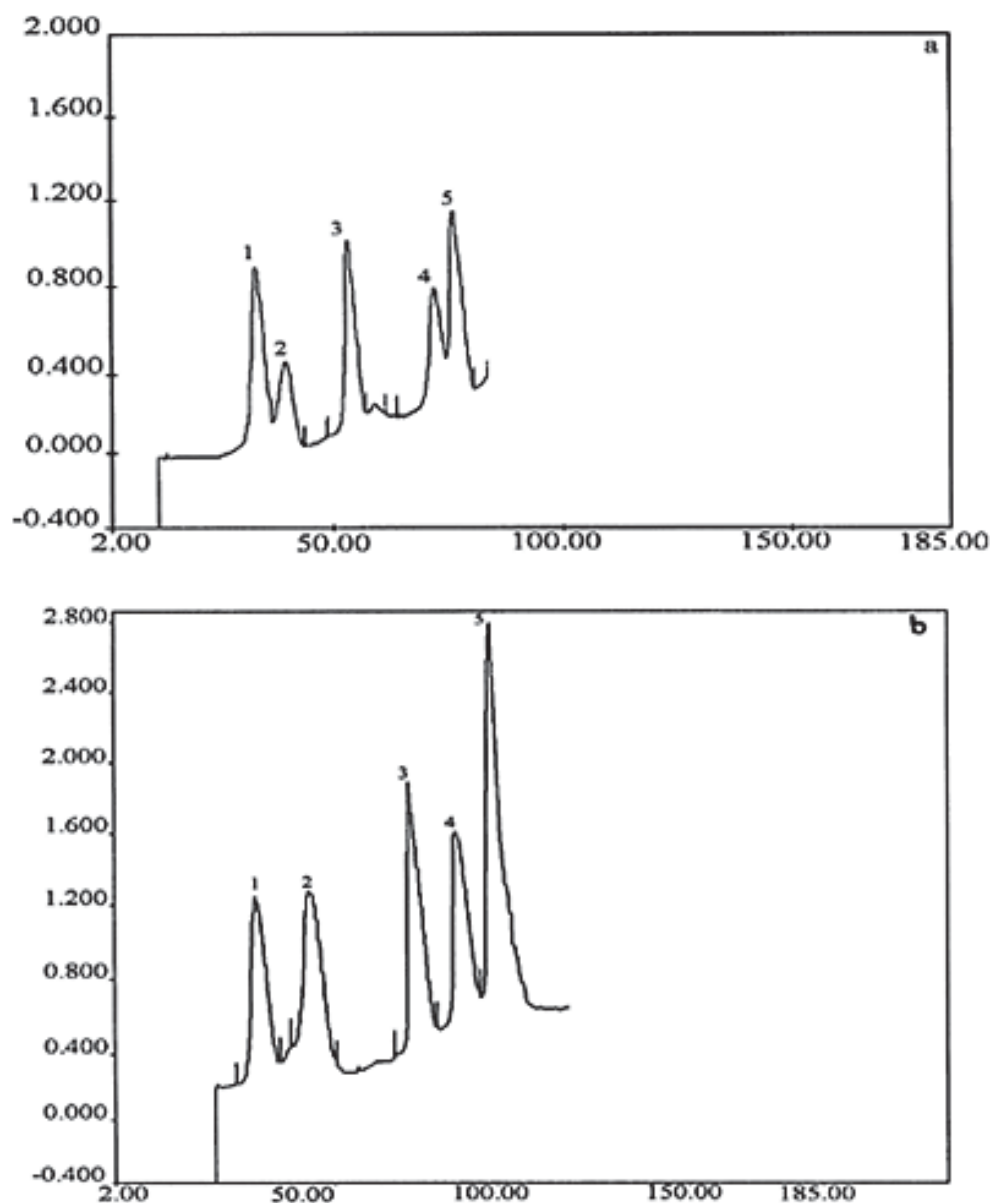


Figure 6 Chromatographic separation obtained by (a) the “quality factor” method and (b) the response surface modeling method. (From Refs. 61 and 62.)

1) because the OTUs with $j=q$ and $j=p$ are the most similar systems, and they form a new combined OTU, p' . In the reduced matrix, the $\Delta jp'$ is given by the equation

$$\Delta jp' = (\Delta jp + \Delta jq) / 2$$

(37)

and all the other Δkl values remain unchanged. This process is repeated until all OTUs are brought together in one classification system that consists of a hierarchy of nonoverlapping groups and subgroups. Moreover, this can be visualized by designing a dendrogram (Fig. 7).

The combination of numerical taxonomy classification and calculation of the information quantity (Eq. 13) and the discriminating power (Eq. 38) is an example of trends in analytical

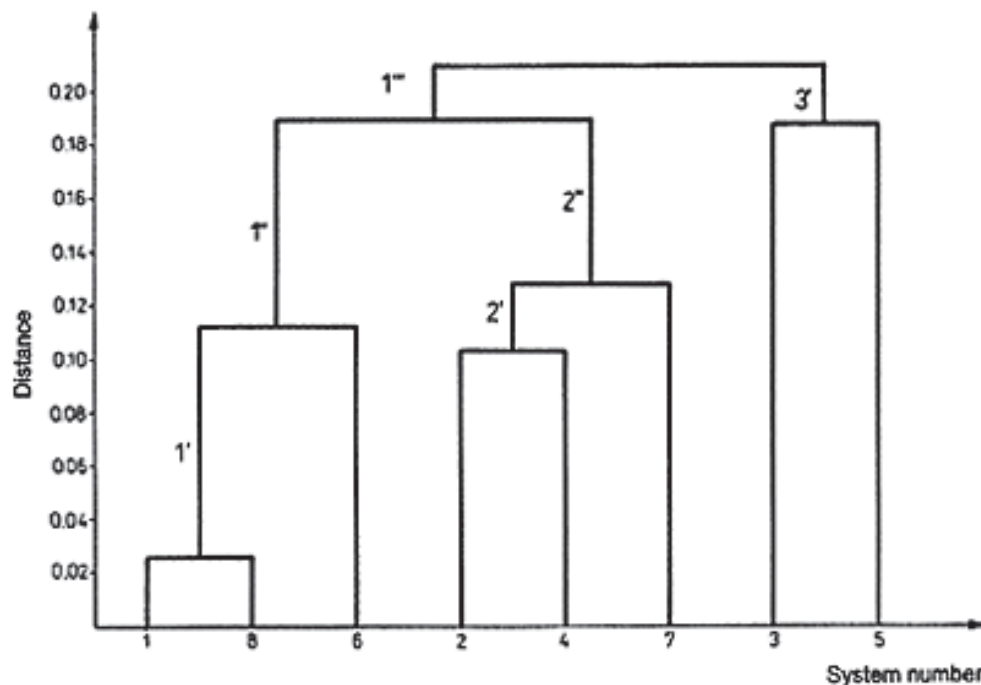


Figure 7 Dendrogram for eight solvent systems. (From Ref. 68.)

chemistry. Discriminating power (DP) is used as a measure of the effectiveness of the chromatographic system and is the probability of a random selection of chromatographically similar pairs from the total number of matching pairs (M) (65).

$$DP = 1 - \frac{2M}{N(N-1)} \quad (38)$$

For evaluation of the separation power of a chromatographic system or for the rational selection of the mobile-phase system, the information quantity or the discriminating power is used as the selection criterion in the groups obtained by numerical taxonomy (66, 67). Another rational and logical choice of the optimum solvent system can be accomplished by using the information quantity and objective function (Eq. 23) as a selection criterion (68). This method was found to be a rapid and efficient tool in the choice of optimum solvent system.

F. Principal Components Analysis

The main application of principal components analysis (PCA) is to reduce the number of variables that describe an experimental situation. Therefore, PCA is applied as a data reduction method, and it can generally be used to describe the data set with maximum possible information (69). This method is now used more frequently due to computer accessibility and the availability of computing programs (70–72). New axes (indices) are defined as linear combinations of the original variables to reduce the number of variables. These indices, representing the directions of maximum variance, are called principal components; they are uncorrelated variables and contain most of the information provided by initial data. The PCA assumes that all the variability in an item should be used in the analysis.

The PCA method starts by coding the variables $x_1, x_2, \dots, x_n$ that represent the characteristics of objects (samples). Then the data matrix X is constructed (Eq. 39) in which each line-vector represents a point in n -dimensional space and each column-vector represents a point in m -dimensional space.

$$X = \begin{bmatrix} x_{11} & x_{12} & \cdots & x_{1n} \\ x_{21} & x_{22} & \cdots & x_{2n} \\ \cdots & \cdots & \cdots & \cdots \\ x_{m1} & x_{m2} & \cdots & x_{mn} \end{bmatrix} \quad (39)$$

Basically, the extraction of principal components amounts to a variance-maximizing rotation of the original space. This type of rotation is said to be variance-maximizing because the criterion for the rotation is to maximize the variance (variability) of the new variable. After the line is found on which the variance is maximal, there remains some variability around this line, and for this reason, after the first factor has been extracted, another line is defined that maximizes the remaining variability, and so on. With this end in view, the general transformation used to obtain the new variable is given by the equations

$$\begin{aligned} P_1 &= a_{11}x_1 + a_{12}x_2 + \cdots + a_{1n}x_n \\ P_2 &= a_{21}x_1 + a_{22}x_2 + \cdots + a_{2n}x_n \end{aligned} \quad (40)$$

$$\vdots$$

$$P_n = a_{n1}x_1 + a_{n2}x_2 + \cdots + a_{nn}x_n$$

$$P = AX$$

$$(41)$$

The matrix of coefficients, A , represents the matrix of orthogonal vectors of the covariance matrix (Eq. 42).

$$C = \begin{bmatrix} c_{11} & c_{12} & \cdots & c_{1n} \\ c_{21} & c_{22} & \cdots & c_{2n} \\ \cdots & \cdots & \cdots & \cdots \\ c_{n1} & c_{n2} & \cdots & c_{nn} \end{bmatrix} \quad (42)$$

The elements of matrix C can be calculated by using the equation

$$c_{kl} = \frac{1}{m-1} \sum_{i=1}^m (x_{ik} - \bar{x}_k)(x_{il} - \bar{x}_l); \quad \bar{x} = \frac{1}{m} \sum_{i=1}^m x_i \quad (43)$$

For each element of the covariance matrix, a correlation coefficient (Eq. 44) can be calculated so that the covariance matrix can be transformed into a correlation matrix, R , where

$$r_{kl} = \frac{c_{kl}}{s_k s_l} \quad (44)$$

s_k and s_l in Eq. 44 represent the standard deviations of variables k and l , respectively. Use of the correlation matrix is necessary to prevent the variables from having a strong influence on the principal components.

The maximization problem is equivalent to

$$Ca_i = \lambda_i a_i \quad (45)$$

The vector a_i found by solving Eq. 45 is called an eigenvector of the variance—covariance matrix C , and λ_i is called an eigenvalue. The eigenvalues represent the variances extracted by the factors, and they are calculated by a least squares procedure. The sum of the eigenvalues is equal to the sum of the diagonal elements of the covariance or correlation matrix that is analyzed.

The first principal component is the variable P_1 , which has the maximum variance (λ_1 maximum) and is an uncorrelated linear function of the original variables. The coefficients of the original variables for a principal component are the coordinates of the corresponding eigenvector. The loading of a variable for a principal component is defined as this coordinate multiplied by the square root of the eigenvalue of the principal component. The loadings can be interpreted as correlations between the variables and the components. The value taken by an object for a principal component is called the score of the object for this principal component. The scores for the first principal component are the maximum variance values. The scores for the second principal component are uncorrelated with those for the first principal components. The variance of the third principal component is smaller than those of the first two principal components, but it is higher than the variances corresponding to the next components.

It is theoretically possible to determine n principal components. The question is, how many factors do we want to extract? Because they are obtained in order of decreasing contribution to the total variance and they account for less and less variability, it is usually sufficient to consider the first few principal components that still retain most of the variance. The decision as to when to stop basically depends on when there is only a very little random variability left. This decision is arbitrary, but several methods have been proposed for making it. One much used method is to select the first p principal components in such a way that they account for at least 80–90% of the total variance. Another criterion often used to select principal components is to keep eigen-values that exceed 1. In practice, two or three principal components usually account for an important part of the variance.

The loadings corresponding to the principal components are plotted (Figs. 8 and 9), with each variable represented as a point. From this plot, it can be seen which of the

initial variables have the greatest shares in the variance of particular principal components. Furthermore, scores plots are very useful as a display tool for examining the relationships between objects and looking for trends, groupings, or outliers (73).

An example of the application of PCA to the choice of optimum solvent system is the paper of Bota (74), who used this method to find the optimum mobile phase for the separation of seven polycyclic aromatic hydrocarbons. They concluded that the PCA enables rational selection of a restricted set from nine available mobile-phase systems and is a useful graphical tool.

IV. AUTOMATED MULTIPLE DEVELOPMENT

Complex mixtures containing components with a wide range of polarities or molecular structures cannot be separated by isocratic TLC. Low-strength solvents will separate the compounds weakly retained on the stationary phase (high R_f values), while the compounds strongly retained on the layer (low R_f values) will migrate short distances. On the other hand, strong solvents cannot separate the poorly retained compounds, which migrate as a single spot or as unresolved spots. Automated multiple development (AMD) (75–77) is used to solve these problems so that optimum separation will be achieved. AMD is a sequential, programmed, incremental, multiple development technique using, for silica gel, a gradient of the mobile phase starting with a very polar solvent, decreasing the polarity of the mobile phase with a solvent of medium polarity, and ending with a nonpolar solvent.

The mobile-phase gradients are generated step by step using as many solvents as necessary to realize the desired separation. The number of steps is kept as low as possible to optimize the

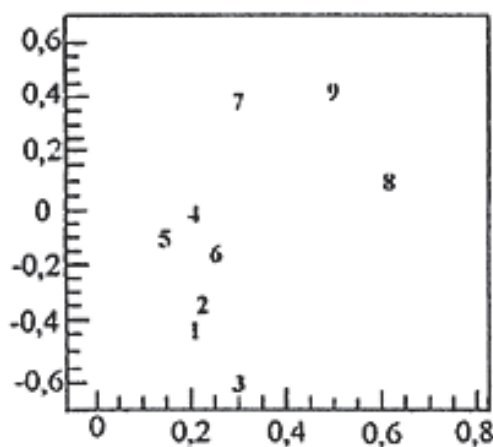


Figure 8 Plot of the first two loading vectors (A_1 and A_2). (From Ref. 74.)

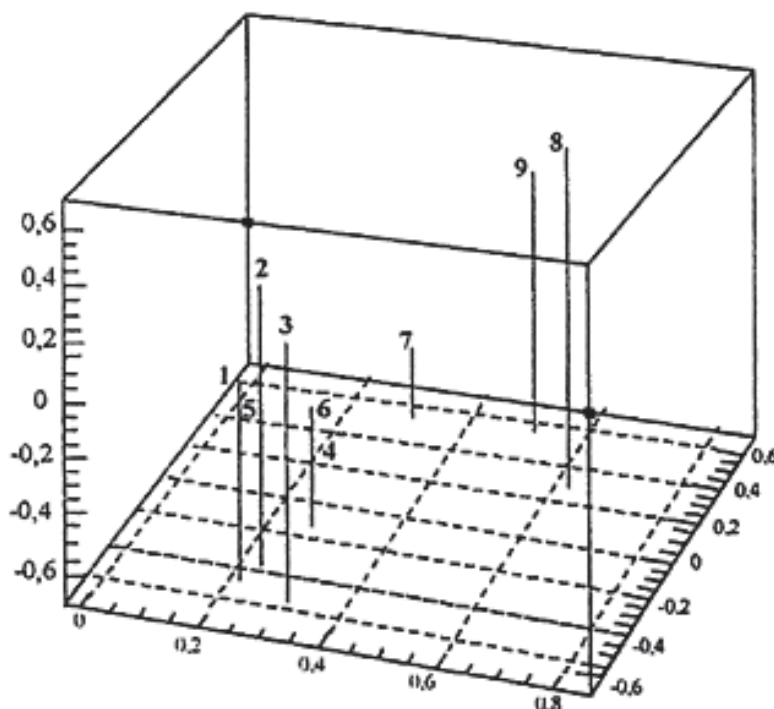


Figure 9 Plot of the first three loading vectors (A_1 , A_2 , and A_3). (From Ref. 74.)

separation time. Usually, one AMD run consists of 10–30 separate development steps, each 1–3 mm longer than the previous step. Between two developments, the plates are dried in vacuum to remove the solvent, and the mobile phase is removed also from the chromatographic chamber. These steps are repeated until the entire developing program is completed. In each chromatographic run, the bottom part of the spot starts to migrate while the top part does not move, so the spot is reconcentrated and the diffusion effect that usually controls the chromatographic separation is strongly decreased. Thus, the spots will be focused as bands of 0.1–1 mm width, depending on the compound characteristics.

The optimum AMD separation is that in which all components are separated from each other and the spots are distributed along the length of the layer. The peak positions on the final chromatogram depend on the choice of mobile-phase composition and the shape of the gradient, and correct adjustment of the several instrumental settings of the AMD equipment is required. Various solvent compositions can be used to form the AMD gradient, and the best choice is usually achieved by empirical experimentation. The gradients used in AMD can be universal gradients that contain a sudden change in the solvent strength or linear gradients that provide a linear change in the solvent strength.

Many authors compared isocratic TLC with AMD, concluding that the number of separated compounds is greater with AMD than with isocratic TLC and that chromatographic separation is optimized with AMD (78, 79). Because AMD is an instrumental technique, it can be coupled online with other chromatographic methods, and this represents a new trend in chromatographic analysis. For example, Stan and

Schwarzer (80) realized the on-line coupling of reversed-phase HPLC with AMD on a normal-phase layer. This coupling represents a very promising technique because it allows the combination of two different separation principles.

AMD is suitable for the separation of multicomponent mixtures in TLC and is a useful tool that provides more powerful screening than conventional TLC methods. This technique provides large spot capacities because the reconcentration effect is caused by multiple development as well as by the accommodation of many spots on the same chromatographic plate due to gradient development. Moreover, reproducibility, separation quality, and the possibility to obtain accurate and reproducible quantitative determination have been significantly improved by using the AMD technique.

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4

Sorbents and Precoated Layers in Thin-Layer Chromatography

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I. INTRODUCTION

The scientific work of Friedlieb Ferdinand Runge can be regarded as the beginning of thin-layer chromatography.* In 1850 he described the separation of mixtures of dyestuffs by means of a type of capillary force during development on paper (1). The further development of chromatography was due to the work of the Russian botanist and biochemist Michael S. Tswett, who realized the potential of chromatography for analytical and preparative separations. At the beginning of the twentieth century, Tswett was engaged in the separation of plant pigments in columns containing stationary phases such as calcium carbonate (2), and he assigned the term "chromatography" after the Greek words for "color writing." For many years after, chromatography fell entirely into disuse. It was revived again in the mid-1950s by Egon Stahl, who was the driving force behind thin-layer chromatography (TLC) becoming an important analytical method in modern chemical laboratories. This was achieved by Stahl's fundamental work in developing sorbent materials and equipment for thin-layer chromatography. It culminated in his standard handbook (3), which is still considered a "bible" of silica gel TLC work. Stahl's contacts with the chemical industry resulted in the development of a silica gel with standardized and reproducible properties for homemade thin layers in 1956. The introduction of commercial precoated layers in the mid-1960s was first described by Halpaap (4).

These advances were followed by continued development of thin layers with unique selectivity and improved separation efficiency. Examples include

- Precoated layers suitable for high-performance thin-layer chromatography (HPTLC)

- Combinations of different sorbents on a single precoated layer

- Hydrophilic and hydrophobic modifications of bulk TLC sorbents and precoated layers

With this brief historical introduction, the sorbents that are commonly used today in thin-layer chromatography are characterized in terms of their physical and chemical parameters as well as by their resulting chromatographic properties in the following sections.

* In present linguistic usage the expression “thin-layer chromatography” is used as a generic term for this analytical technique. Here one must distinguish among preparative layer chromatography (PLC), conventional thin-layer chromatography (TLC), and high-performance thin-layer chromatography (HPTLC).

II. SORBENTS AND PRECOATED LAYERS WITHOUT MODIFICATION

From the beginning, most thin-layer chromatography has been performed using sorbents without chemically modified surfaces. Few users today have ever made their own TLC plates, but their predecessors did just that before manufacturers made precoated layers available. Like any physical process, the preparation was not difficult, but it did take practice to do it well. In addition to glass plates, plastic and aluminum sheets are offered as supports for precoated layers. The advantages of these are discussed in Section VII. To stabilize the precoated layers mechanically, special binders are added that do not interfere (or interfere only minimally) with the chromatographic properties. These binders are discussed in Section VI. To increase the possibility of detection, indicators can be mixed homogeneously with sorbents during plate preparation. Various types of silica gel are by far the most versatile and therefore the most frequently used stationary phases in the case of bulk TLC sorbents as well as for application to precoated layers.

A. Silica Gels

Silica gels used in thin-layer chromatography are porous, synthesized materials. Because the chromatographic behavior of silica gels is determined by their chemical and physical properties, it is essential to standardize these parameters for the industrial production of efficient and reproducible thin-layer plates.

Investigation over a manufacturing period of 5 years showed that in the case of TLC plates precoated with silica gel 60, the retention data for a chosen test system have maximum relative standard deviations of 2.8%, and separation efficiency data show relative standard deviations of 4.1% (5). Both values are evidence of the very good reproducibility obtained in the manufacture of plates used in modern thin-layer chromatography.

1. Physical and Chemical Properties

From a chemical point of view, all silica gels are silicon dioxides. Each silicon atom is surrounded by four oxygen atoms in the form of a tetrahedron. At the surface of the silica gel, the free valences of the oxygen are connected either with hydrogen (Si—O—H, silanol groups) or with another silicon atom (Si—O—Si, siloxane groups) (Fig. 1). All

silica gels have uniform density of their silanol groups of about $8 \mu\text{mole}/\text{m}^2$ (6). The silanol groups represent adsorption-active surface centers that are able to interact with sample molecules. This is the main reason silica gels are suitable as stationary phases in chromatography. The ability of the silanol groups to react chemically with appropriate reagents is also used to effect surface modifications (see Sec. III.A).

Chromatographic behavior of any TLC sorbent is determined mainly by physical parameters to be discussed. Silica gels used in thin-layer chromatography are porous matrices. This is an important prerequisite for suitability as a carrier in chromatography, because all solute-exchange processes, which are responsible for chromatographic separation, take place at the surface or on the surfaces within the pores. The parameters that serve for the characterization of the pore structure are pore diameter, specific pore volume, and specific surface area.

Pore diameter. The pore diameter (D) for a specified silica gel shows a certain distribution. To characterize a defined type of silica gel, the mean pore diameter is indicated. The silica

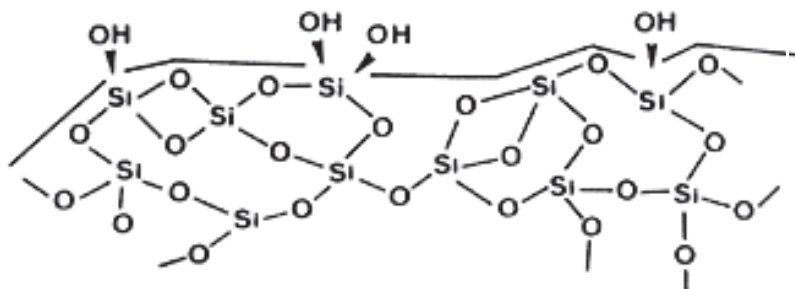


Figure 1 Chemical structure of silica gel.

gels most frequently used in thin-layer chromatography have mean pore diameters of 4, 6, 8, and 10 nm. The 40, 60, 80, and 100 designations used for these types of silica gels are based on angstrom units, which were customary in former times. The mean pore diameters as well as the pore size distribution can be determined by measurement with a mercury porosimeter (7).

Specific pore volume. This parameter gives information about the maximum possible load-ability of the silica gel with a liquid stationary phase. Filling of the pores of a chromatographic sorbent with a liquid stationary phase in whole or in part is a prerequisite for liquid-liquid chromatography (partition chromatography, Sec. III.A.3). The measuring unit of the specific pore volume V_p is milliliters per gram of sorbent. The specific pore volume of silica gels used in thin-layer chromatography ranges from 0.5 to 2.0 mL/g. A possible method of determining V_p is the titration method according to Fisher and Mottlau (8).

Specific surface area. Because of the constant density of the silanol groups, the specific area is a direct indicator of the adsorption capacity of

a silica gel in chromatography (Sec. II.A.2). The specific surface area S_{BET} of silica gel in thin-layer chromatography ranges from 200 to 800 m²/g. A possible method of determination of S_{BET} is based on the measurement of nitrogen adsorption isotherms (9).

These three physical parameters that characterize pore structure are mutually dependent. The correlation of these data is specified by Wheeler (10):

$$D[\text{\AA}] = \frac{4V_p \times 10^4}{S_{\text{BET}}}$$

In combination with the respective chemical properties, the three primary physical parameters determine chromatographic selectivity of the different types of silica gel. To characterize packing structure and separation efficiency of a stationary phase in a chromatographic system, further parameters are necessary. These “secondary” physical parameters are the particle size distribution and the mean particle diameter. Note, too, that the pore diameter (D) and the specific surface area (S_{BET}) are inversely related. As the pore diameter increases, the specific surface area decreases, as shown in Table 1. This means that there are fewer silanols for interaction or bonding. Although a whole range of pore diameters are found with HPLC packings (60, 100, 300, 500, 1000 Å), the most used TLC silicas are those with 60 Å pores, with some 40 Å or 100 Å silicas also used.

Particle size distribution. Silica gels for bulk packing (for column chromatography) as well as for precoated layers are produced by (a) grinding rather large granules or (b) impacting these particles against one another. Either method results in irregular particles with a wide particle size distribution. In a chromatographic system, permeability is influenced negatively by proportions of fines, and separation efficiency deteriorates if coarse particles are present. Therefore, the quality of sorbent materials in thin-layer chromatography depends on a narrow particle size distribution, and it is necessary to size the material obtained in the grinding process.

Mean particle size. Aside from particle size distribution, the separation efficiency of a chromatographic system is determined mainly by the mean particle size of the stationary phase. If the width of the particle size distribution is comparable, then separation efficiency increases with decreasing mean particle diameter. However, in this case flow properties of a thin-layer chromatographic system deteriorate by slowing down. As a consequence of the facts mentioned, a mean particle size of about 5–6 μm has proved optimal. This has been realized in the form of the now widespread HPTLC precoated layers. The different mean

Table 1 Typical Silica Gel Pore Sizes and Surface Areas

Pore size, Å	40	60	100	150	200	300	500	800	1000
Surface area, m ² /g	600	480	270	175	130	90	55	45	30

particle sizes and particle size distributions of the silica gels used for TLC, HPTLC, and preparative layer chromatography (PLC) precoated layers are shown in Fig. 2. Methods for determining particle sizes include counting particles, sedimentation, sieve analysis, sifting, and diffraction of light (11, 12).

In addition to the performance reasons for a particular particle size distribution, the distribution can be changed by manufacturers to give a more easily made thicker or thinner layer. With these special particle size distributions (along with the correct binder and its concentration), an evenly coated, reproducible layer of a given thickness can be produced that will not crack or distort after being manufactured or during use. A scanning electron micrograph of a cross section of a typical thin-layer chromatographic plate is shown in Fig. 3. An HPTLC plate would look much the same, but the particles would be smaller and the layer would be thinner. Most TLC plates used for analytical work are made with a layer thickness of 0.25 mm. Analytical HPTLC plates are made with layer thicknesses of 0.2 or 0.1 mm, depending on their application.

2. Adsorption Chromatography

In the case of unmodified silica gels, adsorption of the test substances by the stationary phase is the decisive retention mechanism for chromatographic separation. Selective interactions of the sample molecules to be separated take place at the active surface centers of the silica gel. Forces that affect interactions include hydrogen bonding, dipole—dipole, and electrostatic interactions. The intensity of these forces depends on three factors:

1. The number of effective silanol groups. The intensity of adsorptive interactions is directly proportional to the specific surface area because the density of the silanol groups is constant for all types of silica gels. Therefore, silica gel with 40 and 60 Å pores, with very high specific surface areas as discussed above, are particularly suitable for adsorption chromatography. In this connection, the influence of humidity on the behavior of silica gels in adsorption chromatography has to be mentioned (see Sec. II.B.2).

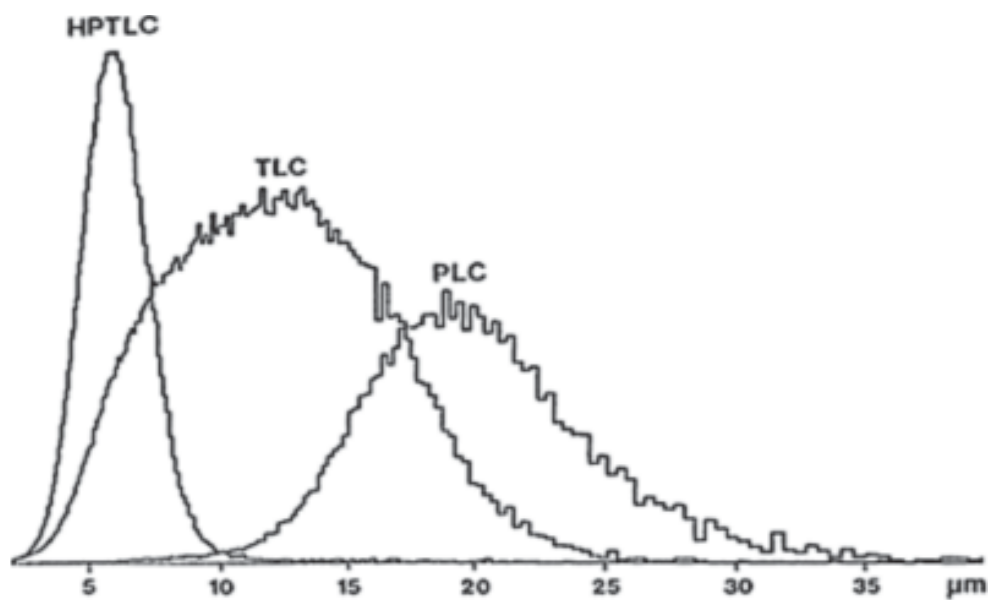


Figure 2 Typical particle size distributions of silica gels in thin-layer chromatography determined with a Coulter Multisizer AccuComp.

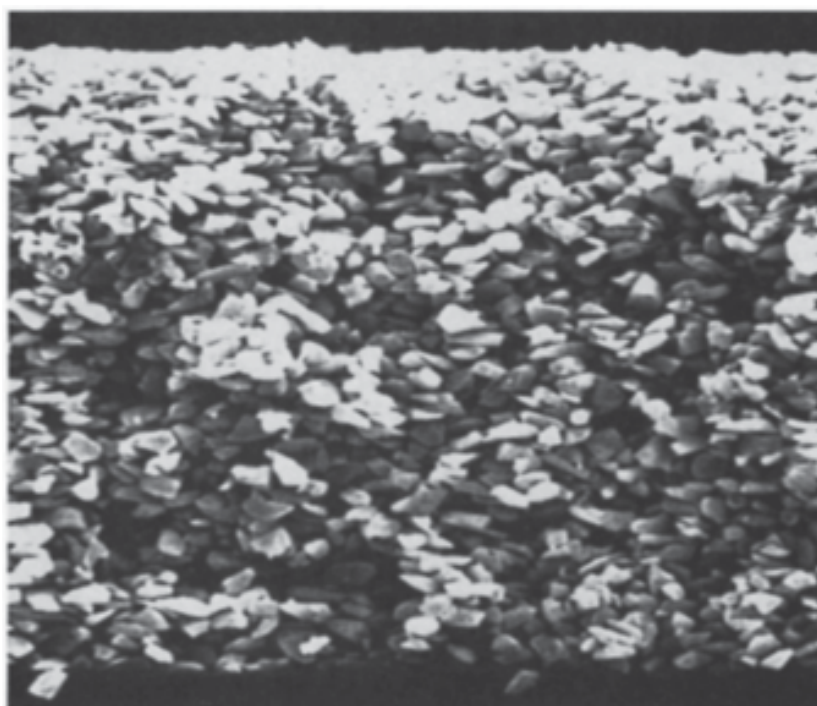


Figure 3 Scanning electron micrograph of a cross section of a

typical thin-layer chromatographic plate.

- 2 The chemical structure of the sample molecules to be separated. Polar functional groups or groups that can be polarized lead to increased interaction with the active surface, resulting in increased retention. The more polar these groups are, the greater the retention. In the absorption mode, the compounds that have greater retention always have a greater polarity. Likewise, the metabolites of drugs (which are oxidized during metabolism) are always retained longer than the parent compound in the absorption mode when a silica gel plate is used.
- 3 The elution strength of the mobile phase. Retention decreases with increasing solubility of sample molecules in the mobile phase. Halpaap (13) arranged the organic solvents most frequently used in thin-layer chromatography according to increasing elution strength with reference to silica gel as the stationary phase. The polarity of the mobile phases used is low compared with the polarity of the surface-active silanol groups. A large number of different substance classes have been separated in thin-layer chromatography by means of adsorption chromatography. A selection of some important representatives of these substance groups is listed in Table 2.

3. Partition Chromatography

Silica gel also can act as a support for a liquid stationary phase. In this liquid-liquid or partition chromatography, selective retention of the sample molecules to be separated results from their differential solubility in the liquid acting as stationary or mobile phase (see Sec. II.A.1). Retention of sample substances in the ideal case of partition chromatography (i.e., no adsorptive interactions with the support) is influenced only by the following factors:

Table 2 Applications on Silica Gel in Adsorption Chromatography

Substance class	Reference
Aflatoxins	14–16
Alkaloids	17
Antibiotics	18, 19
Antihistamines	20
Antihypertensive drugs	21
Antitubercular drugs	22
Antiulcer drugs	23, 24
Benzodiazepines	25, 26
Fatty acids	27
Laxatives	28

Lipids	29–31
Mycotoxins	14, 32
Pesticides	33, 34
Steroids	35, 36
Sulfonamides	37
Vitamins	38

1. The chemical nature of the liquid stationary phase. Retention increases with increasing solubility of sample molecules in this phase.
2. The volume of the stationary phase that is applied into the pores of the support. The maximum possible volume is limited by the specific pore volume of the matrix. Therefore, silica gels 60 and 100, with their large specific pore volumes, are especially suitable as supports for partition chromatography.
3. The chemical structure of the sample molecules. Strength of retention increases with increasing mutual solubility of the sample and liquid stationary phase, that is, with increasing chemical similarity of the two compounds.
4. The composition of the mobile phase. For a given liquid stationary phase, retention decreases with increasing solubility of the sample molecules in the mobile phase. The different probabilities of the sample molecules dissolving in the mobile or stationary phase are expressed by the respective partition coefficients.

Loading of the support with liquid stationary phase can take place in two different ways:

1. By impregnation before chromatographic development. The support is impregnated with a solution of the liquid stationary phase by either dipping or spraying, and subsequently the solvent is evaporated. Dipping has the advantages of exactly defined loading of the support with stationary phase up to complete filling of the pores and of being more reproducible. Furthermore, in this case the composition and the film thickness of the liquid stationary phase are constant over the entire migration distance.
2. By self-adjusting impregnation during chromatographic development. During development with a solvent mixture, a liquid stationary phase is formed within the pores of silica gel, which changes in composition and amount of the liquid stationary phase along the direction of development. In effect, a gradient is formed, with greater amounts at the origin and lesser amounts near the solvent front. The formation of such a gradient is a particularity of thin-layer chromatography, because solvent is being introduced into a dry sorbent matrix. It can be attributed to differences in the affinities of the solvent components for the surface silanols of the silica gel.

Table 3 lists some important substance classes that have been separated on silica gel by partition chromatography.

In reality, pure adsorption or partition retention mechanisms ordinarily do not occur. On the contrary, in many cases a combination of both retention mechanisms is operative. To increase selectivity, adsorption and partition can be applied not only simultaneously but also in a controlled way, one after the other, in what is called “multidimensional chromatography.”

B. Aluminas

The use of aluminas as stationary phases or supports for liquid stationary phases in thin-layer chromatography is of importance for some fields of application, but it is less widespread than the use of silica gels.

1. Physical and Chemical Properties

Aluminas used in thin-layer chromatography have the formula Al_2O_3 . Surface-active centers of these types of alumina are hydroxyl groups and oxide ions (O^{2-}) (60). The average density of hydroxyl groups of the aluminas is about $13 \mu\text{mole/m}^2$ (61). Chromatographic properties of alumina are also influenced by the adjusted pH value. Three ranges of pH values have proved suitable: aluminas with pH values of 9.0–10.0 are designated as “basic”; “neutral” in this connection means pH 7.0–8.0; and “acid” aluminas have pH values of about 4.0–4.5.

A number of physical parameters are necessary to standardize chromatographic properties of aluminas in thin-layer chromatography. Because these aluminas are porous materials, the parameters characterize the pore structure and specific surface area. The values of pore diameters, specific surface areas, and pore volumes of aluminas most frequently used in thin-layer chromatography are listed in Table 4.

As with silica gels, the chromatographic separation efficiency of aluminas is determined by the mean particle size and the particle size distribution. The respective numerical values are of the same order of magnitude as in the case of silica gels for TLC and PLC. Methods of measurement for these parameters are identical with those described in Section III.A.1.

2. Adsorption Chromatography

The majority of applications of aluminas as sorbents in thin-layer chromatography are based on adsorption mechanisms. Aluminas 60 and 90, with their large specific surface areas, are the most

Table 3 Applications on Silica Gel in Partition Chromatography

Substance class	Reference
Aflatoxins	39
Alkaloids	40, 41
Antibiotics	42, 43
Carbohydrates	44–46
Glycosides	47
Lipids	48, 49
Nucleotides	50

Peptides	51
Pesticides	52
Phenols	53
Steroids	54, 55
Sulfonamides	56, 57
Sweeteners	58
Tetracyclines	59

Table 4 Parameters of Alumina Pore Structure

Type of alumina	Pore diameter (nm)	Specific surface area (m ² /g)	Pore volume (mL/g)
60 (Type E)	6	180–200	0.3
90	9	100–130	0.25
150 (Type T)	15	70	0.2

suitable types for this purpose. Retention of sample molecules by adsorption on aluminas is influenced not only by the type of sorbent but also by the effect of humidity in a non-negligible way. Because of the high density of hydroxyl groups, aluminas tend to adsorb water molecules from the surrounding atmosphere and thereby become deactivated. Without due note being taken of this property of aluminas, reproducibility of analytical results can be affected. Some typical applications of aluminas in adsorption thin-layer chromatography are listed in Table 5.

3. Partition Chromatography

Aluminas are not used widely as supports for liquid stationary phases. As with silica gels in partition chromatography, aluminas with larger pores, such as Al₂O₃ 150, are preferred for this purpose. Examples of partition chromatographic mechanisms on alumina are the separations of diterpenes (68) and water-soluble vitamins (69).

C. Inert Silicon Dioxides

A series of sorbents that are used exclusively in partition chromatography are various wide-pore silicas. They are distinguished by having a very low specific surface area. Therefore, in partition chromatography almost no adsorption interactions contribute to the selective retention of the solutes. Natural products (diatomaceous earth, commonly known as kieselguhr) as well as synthetic silicon dioxides (silica 50,000) are employed to prepare these TLC phases.

1. Diatomaceous Earth

Diatomaceous earth (kieselguhr) is found in natural deposits. It consists mainly of the skeletons of dead diatoms. The composition of diatomaceous earth is dependent on its origin and on the cleaning process carried out before its use in chromatography. An average of 90% of the diatomaceous earth matrix consists of SiO_2 . The remaining 10% consists of Al_2O_3 , Fe_2O_3 , MgO , Na_2O , K_2O , CaO , and TiO_2 in various proportions. Depending on the batch, secondary by-products may influence the chromatographic behavior of the diatomaceous earths. This means that the reproducibility of the results obtained on such materials cannot be guaranteed in all cases. Because diatomaceous earths have a natural origin, parameters determining the chromatographic properties can be declared only as ranges: medium pore size varies from 1000 to 10,000 nm (very large pores), and an average pore volume of 1–3 mL/g demonstrates the high porosity of the system.

Table 5 Applications on Layers of Alumina in Adsorption Chromatography

Substance class	Reference
Alkaloids	62, 63
Carbohydrates	64
Flavonoids	65
Inorganic ions	66
Pesticides	67

Surface areas in the range of 1–5 m^2/g show that the diatomaceous earths are materials with a very low surface activity. Diatomaceous earths are used, for example, for the separation of anthraquinone derivatives (70), herbicides (71), phenolic compounds (72), tetracyclines (73), and vitamins (74) in a partition chromatographic mode.

Diatomaceous earths in thin-layer chromatography are not used only in their pure form; mixtures with surface-active silicas are also available. These mixed layers have a smaller adsorption capacity than pure surface-active silicas. The speed of chromatographic development with these mixed layers is very high. The separation of sugars (75) demonstrates that these layers can also be used successfully in partition chromatography.

2. Silica 50,000

An ideal carrier material for partition chromatography should have the following properties:

1. The sorbent must be only the support for the liquid stationary phase. There should be no retention of the samples by interaction with the carrier material.

2. The chemical composition and the physical parameters describing the structure have to be defined clearly and manufactured in a reproducible way.

Diatomaceous earth found in natural deposits fulfills these requirements only to some extent (see Sec. III.C.1). In particular, with regard to reproducibility and optimization of the structure parameters, it is obviously desirable to produce a synthetic material that is comparable with diatomaceous earths. Therefore, the development of a silicon dioxide named silica 50,000 was undertaken. This material consists of 100% SiO₂ with a mean pore size of 5000 nm, a pore volume of around 0.6 mL/g, and a specific surface area of approximately 0.5 m²/g. Silica 50,000 is available commercially as a precoated layer. The mean particle size and the particle size distribution correspond to HPTLC quality. Typical applications of this wide-pore material in partition chromatography are separations of amino acids (76), carbohydrates (77–79), and digitalis glycosides (80).

Diatomaceous earths and silica 50,000 are used not only in thin-layer chromatography as carriers for the partition chromatographic process, but also as inert sorbents for the so-called concentrating zone in front of the separation layer (see Sec. IV).

D. Celluloses

Celluloses are used in paper and in thin-layer chromatography as organic stationary phases. In contrast to paper chromatography, where cellulose is applied as a self-supporting layer, in thin-layer chromatography the cellulose particles are classified and spread as layers on glass, aluminum, or plastic supports. As a result, cellulose layers can be produced in different qualities up to precoated layers for HPTLC. In general, celluloses used for chromatography are composed of long chains of β -glucopyranose units, which are connected to one another at the 1,4 positions.

In thin-layer chromatography two types of celluloses are distinguished (81):

1. Native cellulose has a degree of polymerization of 400–500 glucose units and a fibrous structure. The length of the fibers is in the range of 2–20 μ m, and the specific surface area measures around 2 m²/g.
2. Microcrystalline cellulose consists of an average of 40–200 glucose units.

The lower degree of polymerization of microcrystalline cellulose compared with that of native cellulose results from the process of synthesis: The amorphous parts of highly pure native cellulose are dissolved by acid hydrolysis. After this cleaning process, the residual cellulose forms rod-shaped crystalline aggregates. The specific surface area is comparable to that of native cellulose.

Like silica gel, microcrystalline cellulose is available not only as bulk TLC material for self-coating plates but also as industrially produced precoated layers for conventional thin-layer chromatography, high-performance thin-layer chromatography, and preparative layer chromatography. With regard to the different morphologies of the particles, particle size distributions and mean particle sizes are in ranges comparable to those of silica. Because both types of cellulose used in thin-layer chromatography have a low specific surface area, they are applied mainly in partition chromatography, especially for the separation of relatively polar compounds.

Often cellulose thin layers need no binders because of the strong hydrogen bonding of the cellulose hydroxyl groups with the supports used. Care must be exercised in the

preparation of cellulose layers, because the slurry needs to be mixed carefully so as not to break the fibers, which would give a much more slowly running TLC plate.

Separations on cellulose of some important substance classes are listed in Table 6.

E. Polyamides

Another organic sorption material for thin-layer chromatography is polyamide. In contrast to celluloses, polyamides are synthetic organic resins. Two types of polyamides are used: polyamide 6 and polyamide 11. Polyamide 6 consists of a polymeric caprolactam, whereas polyamide 11 is a polyundecanamide. Polyamides are synthesized as coarse granules. To get a particle size distribution suitable for thin-layer chromatography, two different techniques are applied: (a) grinding at low temperature and (b) temperature-programmed precipitation after dissolution of the granules.

Both types of polyamides for thin-layer chromatography are available as bulk TLC materials and as precoated layers on various supports (glass, plastic, aluminum). The particle sizes are in the same ranges as those of other sorbents. Polyamides are applied for the separation of polar compounds, which are able to interact with the amide group by hydrogen bonding because of their molecular structure. This is why substance groups such as amino acids and derivatives (96, 97), benzodiazepines (98), carboxylic acids (99), cyclodextrins (100), fatty acids (101), flavonoids (65), food preservatives (102), and peptides (103) can be separated on polyamide TLC layers. A special application for polyamide layers is the separation of isomeric compounds with the addition of cyclodextrins to the eluent (104).

F. Sephadex

Sephadex materials used in thin-layer chromatography are cross-linked, polymeric dextran gels. Some physical and chromatographic properties of these Sephadex gels are listed in Table 7.

Sephadex gels are available in four particle size distributions:

Coarse	100–300 μm
Medium	50–150 μm
Fine	20–80 μm
Superfine	10–40 μm

These data refer to the dry gel. Only the superfine fraction is suitable for application in thin-layer chromatography. The hydrophilic Sephadex gels can be applied only in a totally swollen condition as chromatographic sorbents. Because they are used only in size-exclusion chromatography, Sephadex materials in thin-layer chromatography have to be applied with the aid of continuous development techniques. A typical application of size-exclusion thin-layer chromatography on Sephadex gels is the fast and simple determination of molecular weights of proteins (105).

III. MODIFIED SORBENTS AND PRECOATED PLATES

A. Chemically Modified Sorbents

One of the most important factors in achieving a successful separation is the correct combination of solvent and sorbent. Innumerable solvent combinations are possible in TLC, but the sorbent need not only be silica gel. For many years, silica gel, and to a lesser extent aluminum oxide and cellulose, were the only sorbents available for making a TLC plate. However, an ever-expanding choice of sorbents and their unique selectivities became available when modifications began to

Table 6 Applications on Cellulose Layers in Partition Chromatography

Substance class	Reference
Amines	82, 83
Amino acids	84, 85
Antibiotics	86, 87
Artificial sweeteners	88
Carbohydrates	89, 90
Catechols	91
Flavonoids	92
Peptides	93, 94
Polyaromatic hydrocarbons	95

be made on silica gel by way of siloxane bonding. The advantages of these chemical derivatizations are

1. Phase stability (no bleeding of the stationary phase during the chromatographic process, which is a problem with coated phases)
2. The possibility of applying other retention mechanisms to the chromatographic separation process

In recent years, the importance of surface-modified sorbents in thin-layer chromatography has increased continuously, although their market share cannot be compared with that of the corresponding packings in column liquid chromatography. The reason for this is most probably that most people are not developing new TLC methods but are only using existing ones that were developed on plain silica gel layers.

1. Hydrophobic Modified Phases (RP Phases)

The unmodified sorbents discussed thus far exhibit polar surface characteristics. However, many chromatographic separation problems can be solved by using

hydrophobic interactions of a stationary phase with compounds of appropriate molecular structure. Sorbents that are suitable for this task are the so-called reversed-phase (RP) materials. In this connection, “reversed phase” means that the relative polarities of the stationary and mobile phase are reversed compared with the situation in adsorption chromatography described earlier; i.e., the stationary reversed phase is less polar than the mobile phase. The specific properties of hydrophobic modified sorbents in thin-layer chromatography can be adjusted by two parameters: (a) the character of the alkyl or

Table 7 Types of Sephadex® Used in Thin-Layer Chromatography and Their Properties

Type	Capacity for adsorption of water (mL/g)	Fractionating range for dextrans: molecular weight (Da)
G-25	2.5±0.2	100–5,000
G-50	5.0±0.3	500–10,000
G-75	7.5±0.5	1,000–50,000
G-100	10.0±1.0	5,000–100,000
G-200	20.0±2.0	5,000–200,000

Source: Pharmacia, Uppsala, Sweden.

aryl residue chemically bonded to the silanol (Si—O—H) groups within the silica gel matrix and (b) the degree of modification.

The most common matrix for hydrophobic modified sorbents used in thin-layer chromatography is porous silica. The most commonly used material is silica gel with 6 nm pores. Reversed-phase TLC sorbents are available both in bulk and as precoated layers with various mean particle sizes and particle size distributions for quantitative (high-performance), qualitative, and preparative layer chromatography. The most popular organofunctional groups are methyl (RP-2), octyl (RP-8), dodecyl (RP-12), octadecyl (RP-18), and phenyl residues. Chemical bonding to the silica gel matrix occurs when the accessible silanol groups react with silanes that contain the hydrophobic substituent to form new siloxane groups. The hydrophobic character of these alkyl groups increases from RP-2 to RP-18. In this series, too, as the chain length increases, fewer silanols are bonded because of steric hindrance. The percent carbon by weight increases from RP-2 to RP-18, but the coverage of the silanols decreases.

The hydrophobic character of an RP-TLC sorbent is determined not only by the type of hydrophobic residues but also by their surface density. With identical substituents, the hydrophobic character of RP materials increases with increasing degree of modification. The extent of hydrophobicity plays an important role in thin-layer chromatography because

1. Mainly aqueous mobile phases are used.
2. The transport of mobile phase in thin-layer chromatography occurs by capillary forces.

3. The capillary forces can act only when the surface of the capillaries is wetted by the mobile phase.
4. If the hydrophobic character of the stationary phase is strong and if the repulsive forces are higher than the capillary forces, transport of mobile phases with high water content is hindered greatly or, in the extreme, is not possible in the layer.

To overcome the repulsive forces and to enforce the transport of eluent in thin-layer chromatography, an external force (pressure), similar to that in HPLC, can be applied. The corresponding technique is called overpressured TLC (OPLC) (106). To carry out RP thin-layer chromatography with solvent systems containing high amounts of water without requiring expensive OPLC apparatus, another way to solve this problem is possible: A compromise between the great hydrophobicity of a totally modified reversed phase and the strong hydrophilic character of unmodified silica must be found. Such a material has to show clear RP characteristics, but development even with pure water as the mobile phase should be possible. This can be accomplished by partial modification of the silica and retention of a residual number of silanol groups. Figure 3 demonstrates the dependence of the migration characteristics on the water content of the eluent in the case of HPTLC RP-18 precoated plates with high or partial modification.

Figure 4 shows that highly modified RP layers can be developed with eluents consisting of acetone and water up to a maximum water content of approximately 60% by volume. In contrast to these plates, partially modified RP layers can be used in this phase system with all eluent compositions, including pure water. The times of development of the partially modified plates pass through a maximum at an eluent composition in the range of 40% acetone. The explanation of this phenomenon is that the binder fixing the sorbent on the glass plates shows an exceptionally strong swelling at this eluent composition. The eluent system acetone-water has a maximum of viscosity in this range of composition.

In addition to these differences in migration characteristics, reversed phases with different degrees of modification show different retention properties using identical mobile phases. Figure 4 shows the separation of some stilbestrol derivatives on totally and partially modified RP-18 precoated layers.

Figure 5 demonstrates in an impressive way that the retention of the partially modified RP-18 layer is less pronounced (Fig. 5a) than that of the totally modified layer (Fig. 5b). Because of the hydrophobic interactions that effect the separation in an RP system, this separation mechanism is suitable for sample molecules that are relatively nonpolar or possess hydrophobic molecular segments. Typical applications in this field are illustrated in Table 8. A few manufacturers make these plates commercially, usually designating them with a "W" or "Aqua" somewhere in the

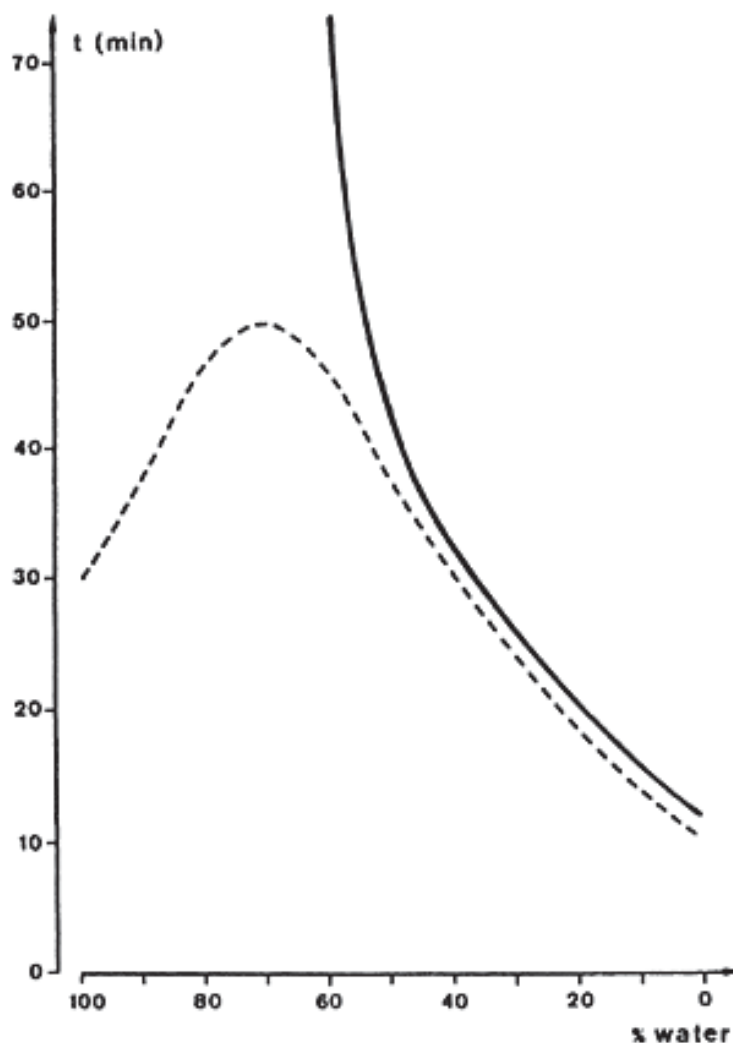


Figure 4 Dependence of the migration times of RP precoated plates on different degrees of modification. (—) HPTLC precoated plate RP-18 F254s; (---) HPTLC precoated plate RP-18W F254s. Eluent: Acetone-water (0:100) to (100:0). Migration distance 7 cm. Normal chamber without saturation.

name or description to describe their compatibility with high or pure water mobile phases (developing solvents).

An area into which many TLC users have wanted to go is the separation of ionic species in the reversed phase as is done often in HPLC. To accomplish this, because the bonded phases absorb un-ionized species best to give more perfectly shaped spots, ion formation must be suppressed. Thus, if the compounds are ionized carboxylic acids, then the developing solvent has to be acidified with acetic or phosphoric acid (only 1–2% by

volume in the developing solvent is necessary). Conversely, if the compounds to be separated are amines, then the developing solvent has to be made basic with ammonium hydroxide. Everyone who performs TLC is familiar with adding a small amount of glacial acetic acid or ammonium hydroxide if tailing is seen. This tailing is the result of the ionization of the ionic groups as discussed above.

If the ionic groups on the compounds are strong, then simple ion suppression does not work. With the aid of so-called ion-pair chromatography, it is possible to selectively retain these more polar ionizing compounds. According to this mechanism, charged polar sample molecules form salts with oppositely charged reaction partners (ions) containing hydrophobic substituents. Because of their nonpolar character, the ion pair formed can interact in a selective way with reversed phases. Applications for ion-pair chromatography in reversed-phase thin-layer chromatography

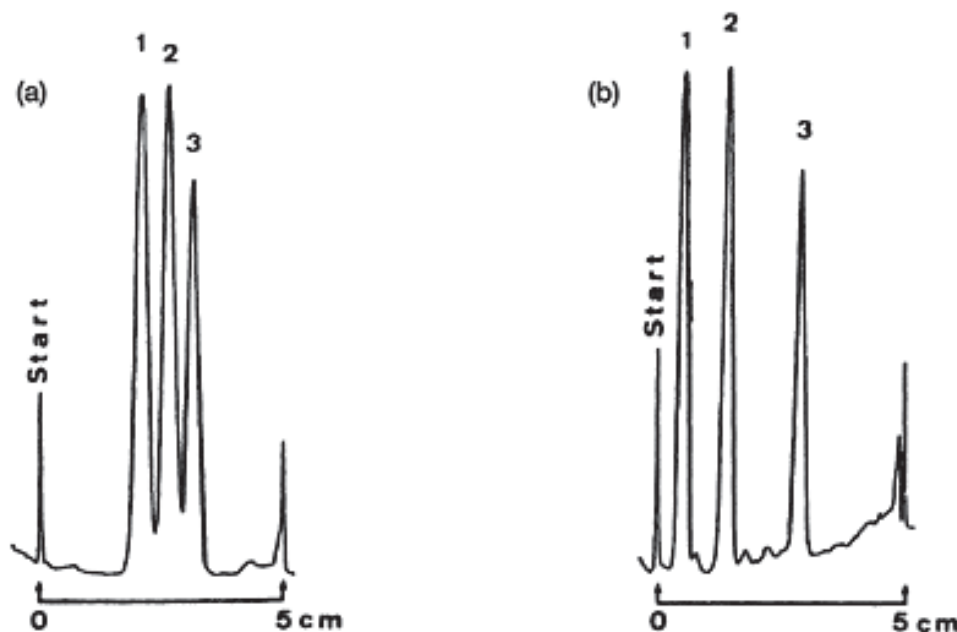


Figure 5 Separation of stilbestrol derivatives on RP precoated plates with different degrees of modification, (a) HPTLC precoated plate with RP-18W F254s; (b) HPTLC precoated plate with RP-18 F254s. Eluent: Methanol-water (80:20). Migration distance 5 cm. Normal chamber without saturation. Compounds: 1, Diethylstilbestrol-dimethyl ether; 2, diethylstilbestrol-monomethyl ether; 3, diethylstilbestrol (all 0.1%). Application volume 200 nL. Detection

by in situ evaluation with TLC/HPTLC scanner (Camag) at 254 nm.

include the separation of antibiotics (132), antihistamines (133), antiarrhythmics (134), and pharmaceuticals (135, 136).

The use of this newer TLC technique is the topic of a number of papers (137–140). These should be consulted to learn the important details of carrying out ion-pair reversed-phase TLC separations.

Table 8 Applications on Reversed-Phase Precoated Layers

Substance class	Reference
Amides	107, 108
Amines	109
Amino acids	110, 111
Antibiotics	112, 113
Antioxidants	114, 115
Fatty acids	116, 117
Peptides	118
Pharmaceuticals	119–121
Phenols	122, 123
PAHs	124–126
Pesticides	127
Steroids	128–130
Vitamins	131

2. Hydrophilic Modified Precoated Layers

The gap of selectivity between the extremely hydrophilic unmodified silica and the nonpolar RP materials is bridged by the hydrophilic modified silicas. These phases show many advantages in their application:

- Extended range of selectivity
- Graduated surface polarity
- Possibility of using different retention mechanisms
- Less influence of the vapor phase on retention behavior and therefore better reproducibility

The hydrophilic modified stationary phases developed so far for thin-layer chromatography possess amino, cyano, and diol residues as functional groups. In each case, polar functional groups are bonded via short-chain nonpolar spacers to the silica matrix. Because of this mixed character of the bonded phase, often both a straight- or normal-phase and a reversed-phase retention mechanism can be invoked on such stationary phases by simple modification of the mobile-phase components.

a. Amino-Modified Precoated Silica Layers. In the case of the NH_2 -modified precoated layers, the amino group is bonded via a propyl group as a spacer onto the silica gel. Besides their use in normal- and reversed-phase retention mechanisms, a further possibility is application in ion-exchange chromatography. This special use is described in Section III.A.3. Typical applications with amino-modified precoated layers used in normal- or reversed-phase mechanisms are, among others, separations of alkaloids (141), antibiotics (142), basic drugs (143), cannabinoids (144), carbohydrates (145), pesticides (146), phenols (147, 148), and steroids (149).

A special feature of the application of NH_2 -precoated layers is the fact that a large number of sample substances (such as carbohydrates and catecholamines) can be converted into stable fluorescing compounds without the need to apply a detection reagent. After development, this is accomplished by simply heating these types of compounds on the NH_2 -bonded plate (150–153).

b. Cyano-Modified Precoated Silica Layers. A further medium-polarity surface modification based on silica is achieved by reacting the silanol groups of the matrix with a cyanopropyl-substituted silane. The cyanopropyl group is built up from a nonpolar part (alkyl chain) and a polar residue (cyano group). Therefore, different retention mechanisms can be used on a layer with such a surface modification. For a certain separation problem, the use of a CN-modified layer in both normal-phase and RP systems can be successful. An example confirming this fact is the separation of some progesterones as shown in Fig. 6. Both chromatograms in this figure show clearly that not only a very nonpolar eluent (Fig. 6a), which causes a normal-phase mechanism, but also a very polar mobile phase (Fig. 6b), and therefore an RP system, can be used. With both mechanisms, comparably good separations of the four progesterones are achieved, but the sequence of retention is reversed. A combination of both retention mechanisms in the form of two-dimensional HPTLC on a cyano plate can be used, e.g., for the separation of sulfonamides (56).

Important substance classes that have been separated on cyano plates are listed in Table 9.

c. Diol-Modified Precoated Silica Layers. The latest development in the field of hydrophilic modified silica gel precoated layers is the reaction of the silica matrix with a silane derived from glycerol (which leaves two remaining hydroxyl groups). The functional groups at the surface of the diol plates are alcoholic hydroxyl residues, and in the case of nonmodified silica gels the active sites are silanol groups. Therefore, the chromatographic behavior of the two types of plates show a certain similarity because identical retention mechanisms occur but with different selectivities. A further difference between the silanol groups and the diol modification results in a differing affinity for water.

In chromatographic practice, this is the reason for a clearly stronger influence of the relative humidity of the vapor phase on the retention in the case of silica compared to a

diol phase. Figure 7 shows the differences in retention at two different relative humidities in the separation of some oligophenylenes using diol and silica gel precoated layers as stationary phases. The same substance sequence of the *m*-oligophenylenes in both cases is evidence of the occurrence of identical reten-

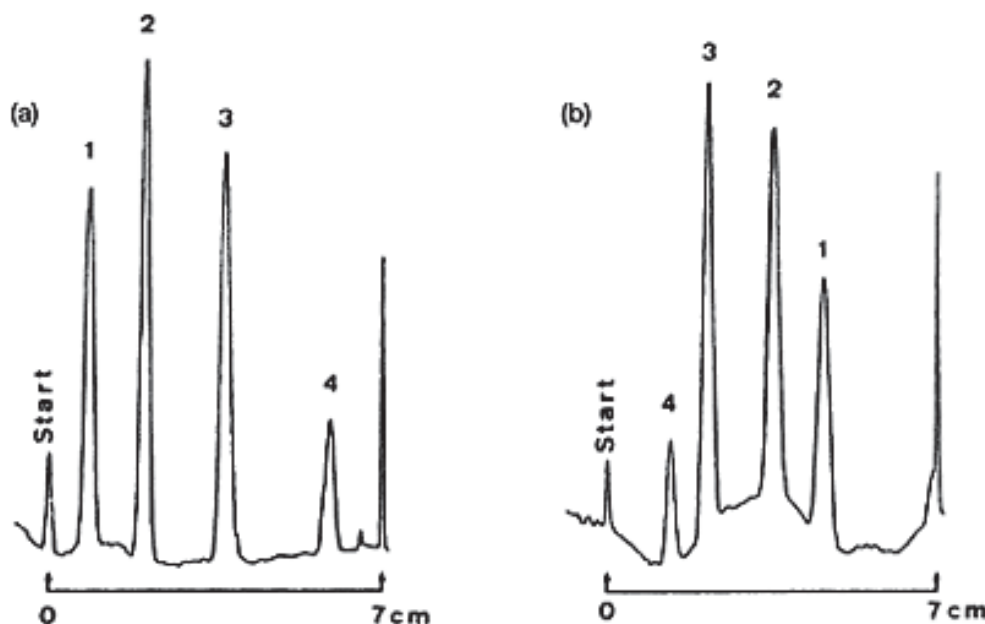


Figure 6 Separation of progesterones. HPTLC precoated plate CN F254s. (a) Normal-phase system. Petroleum ether (40–60°C)-acetone (80:20). (b) reversed-phase system: Acetone-water (60:40). Migration distance 7 cm. Normal chamber without saturation. Compounds: 1, 16-Methyleneprednisolone; 2, 11 α -hydroxypregesterone; 3, progesterone; 4, pregnadienolone acetate (all 0.1%). Application volume 200 nL. Detection by in situ evaluation with TLC/HPTLC scanner (Camag) at 254 nm.

tion mechanisms. For the two humidities investigated, the retention on silica gel is more pronounced than on the diol phase. These differences in retention are caused by different activities of the surface centers. Moreover, it follows that differences of water content in the vapor phase influence the position of substances in the chromatogram to a greater extent in the case of silica gel layers.

An especially pronounced selectivity of the diol-modified precoated layers exists for steroids. An example of this is the separation of some anabolic agents as shown in Fig. 8. Vicinal diol groups are fixed to the silica gel matrix by a quite nonpolar spacer. Therefore, an RP mechanism

Table 9 Applications on Cyano-Modified Layers

Substance class	Reference
Anilines/phenols	148
Analgesics	154
Carotenoids	155
Flavonoids/flavones	156
Nitrosamines	157
Nucleotides/nucleobases	158
Pesticides	159
Phenols	160, 161
Plant extracts	162
Quinolones	163

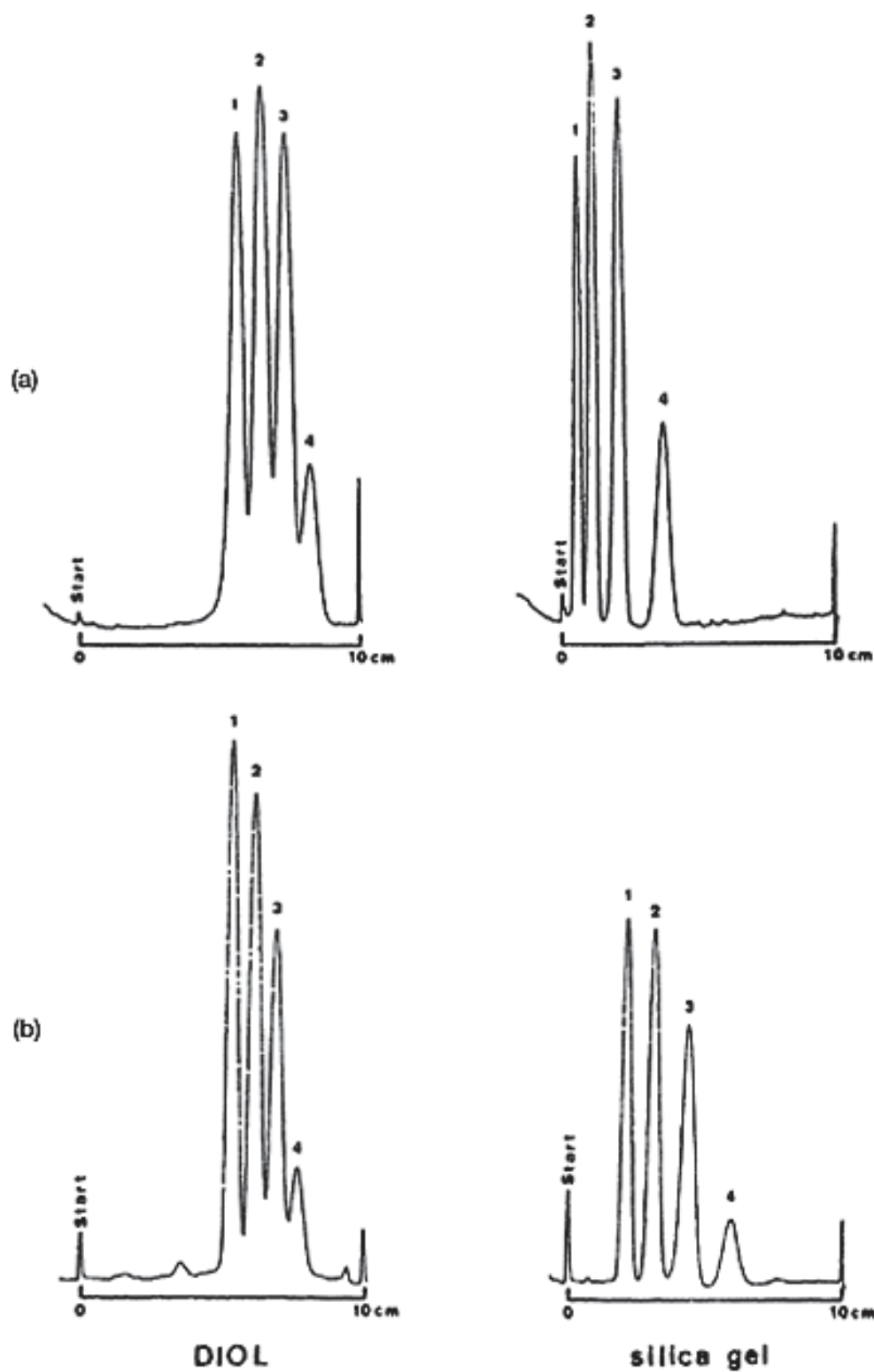


Figure 7 Influences of relative humidity on the retention and resolution of some *m*-oligophenylenes on silica gel and diol precoated plates.

Plates: HPTLC precoated plate diol F254s; HPTLC precoated plate silica gel 60 F254s. Eluent cyclohexane. Preconditioning at (a) 20% relative humidity; (b) 80% relative humidity. Camag Vario KS chamber. Migration distance 10 cm. Compounds: *1,m*-Quinque-phenyl; 2, *m*-quaterphenyl; 3, *m*-terphenyl; 4, biphenyl (all 0.1%). Application volume 600 nL. Detection by in situ evaluation with TLC/HPTLC scanner (Camag) at 254 nm.

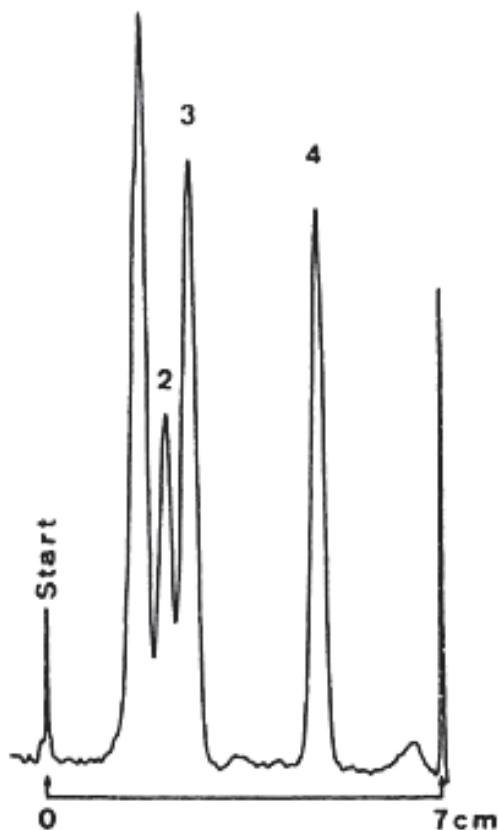


Figure 8 Separation of anabolic compounds. Plate: HPTLC precoated plate diol F254s. Eluent: Di-isopropyl ether—glacial acetic acid (100:1). Migration distance 7 cm. Normal chamber without saturation. Compounds: 1, 19-Nortestosterone; 2,

medroxyprogesterone; 3, progesterone; 4, α -dienestrol (all 0.1%). Application volume 300 nL. Detection by spray reagent MnCl_2 -sulfuric acid with heating to 100°C for 5 min; in situ evaluation with TLC/HPTLC scanner (Camag) at 366 nm.

on diol-precoated layers is also possible when polar solvent systems are used. Further fields of application of the diol-precoated layers are listed in Table 10.

The order of polarity of the hydrophilic surface modifications discussed in the previous section is illustrated in Fig. 9. From the separation of steroids shown, it is obvious that in normal-phase chromatography (Fig. 9a) as well as with an RP mechanism (Fig. 9b), the polarity decreases from the amino to diol to cyano modification.

Table 10 Applications on Diol-Modified Layers

Substance class	Reference
Analgesics	164
Carbohydrates	165
Conjugates	166
Flavors/spices	167, 168
Phenolic acids	147, 169
Phenols	148
Plant extracts	170

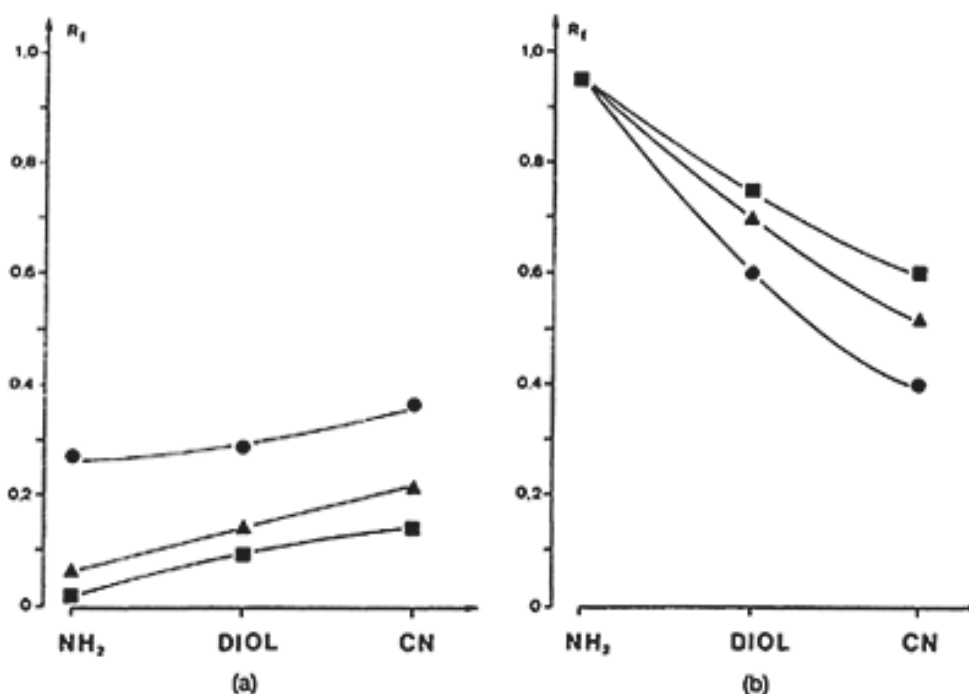


Figure 9 The influence of different hydrophilic modifications on R_f values of steroids. Plates: HPTLC precoated plate NH₂ F254s, diol F254s, CN F254s. Eluents: (a) Normal-phase system, petroleum ether (40–60°C)-acetone (80:20). (b) Reversed-phase system, acetone-water (60:40). Migration distance 7 cm. Normal chamber without saturation. Compounds: (■) Cortisone; (▲) corticosterone; (●) cortexone. Detection at 254 nm.

3. Sorbents and Precoated Layers for Ion-Exchange Chromatography

The ion-exchange mechanism has only minor importance in TLC. With the advent of genetic engineering, the possibility of a re-evaluation of this mode seems possible. At present, silica gels, celluloses, and organic polymers are used as matrices for functional groups suitable for performing ion-exchange separations.

a. Amino-Modified Precoated Silica Layers. The amino-modified precoated layer discussed in Section III.A.2 is not only suitable in normal-phase and RP chromatography, it can also act as a weakly basic anion exchanger. In this special case, the functional groups of the stationary phase, present in the $-\text{NH}_3^+$ form, show interactions that are

different in strength with differently charged anions. Therefore, it is possible to influence the intensity of retention in a definite way by varying the concentration of an added salt, i.e., varying the ionic strength of the mobile phase.

A typical example of the use of an NH_2 -modified precoated layer in ion-exchange chromatography is shown in Fig. 10. In this example, adenosine triphosphate (ATP) with a charge of -4 has the greatest retention (lowest R_f value). Adenosine diphosphate (ADP) (-3) and adenosine monophosphate (AMP) (-2) show increasingly higher R_f values. The noncharged nucleoside adenosine is eluted with the solvent front. Further areas of application besides the nucleotides include, e.g., carboxylic and sulfonic acids (171).

b. Modified Celluloses. Cellulose, described in Section I.D, was the first sorbent in thin-layer chromatography to be used for ion-exchange mechanisms after suitable modifications or impregnations (172). Functional groups used for chemical modification of celluloses are the following:

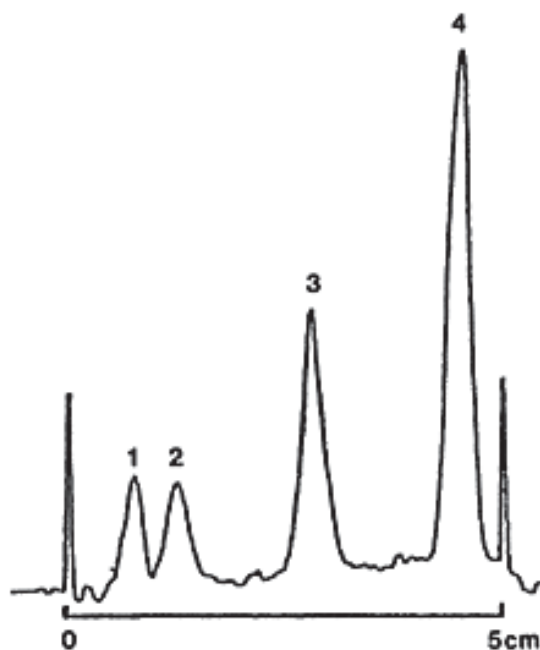


Figure 10 Separation of adenosine phosphates. Plate: HPTLC precoated plate NH_2 F254s. Eluent: Methanol–water (30:70) with addition of 0.2 mol/L NaCl. Migration distance 5 cm. Normal chamber without saturation. Compounds: 1, ATP; 2, ADP; 3, AMP; 4, adenosine (all 0.1%). Application volume 200 nL. Detection by in situ evaluation with TLC/HPTLC scanner (Camag) at 254 nm.

- AE (aminoethyl)
- CM (carboxymethyl)
- DEAE (diethylaminoethyl)
- ECTEOLA (product from reaction of epichlorohydrin, triethanolamine, and alkali cellulose)
- P (phosphate)
- PAB (4-aminobenzyl)

Besides these chemically bonded residues, it is possible to form stationary phases for ion-exchange chromatography based on cellulose by impregnation. Examples of this are the polyethylene imine (PEI) and the polyphosphate (poly-P) celluloses. The cellulose exchangers discussed here have to be distinguished on the basis of their use for an anion- or cation-exchange mechanism. Suitable for the separation of negatively charged ions are the basic AE, DEAE, ECTEOLA, PAB, and PEI celluloses. The acidic CM, P, and poly-P celluloses are used for the resolution of cations.

Some typical applications of cellulose ion exchangers in thin-layer chromatography are listed in Table 11.

c. Polymer-Based Ion Exchangers. A typical matrix for ion exchangers based on organic resins is polystyrene cross-linked with divinylbenzene. In thin-layer chromatography, Fixion 2X8, Dowex I-X8, and Ionex-25 S Bac are used as strong basic anion exchangers. Suitable strong acidic cation exchangers containing a sulfonic acid residue are, e.g., Fixion 50X8, Dowex 50W-X8, and Ionex-25 SA-Na. For improvement of the mechanical and chromatographic properties of the precoated layers, silica gel or cellulose is added. For higher stability, the polymer-based ion exchangers are delivered in their Na^+ or acetate form. Before they are used in thin-layer chromatography, the exchangers can be converted into the H^+ or OH^- form by a suitable equilibration step.

Some examples of charged substances separated with the aid of polymer-based ion exchangers in thin-layer chromatography are amino acids (188), amino sugars (189), antibiotics (190), inorganic ions (191), nucleotides (192), organic acids (193), and pharmaceuticals (194).

Table 11 Applications on Cellulose Ion Exchangers

Substance class	Type of ion exchanger	Reference
DNA adducts	PEI	173–176
DNA and RNA fragments	ECTEOLA	177
Dyes for foods	DEAE	178
Inorganic ions	DEAE, P, PEI	179–183
Nucleotide adducts	PEI	184–186
Steroids	DEAE	187

The mobile phases used for all of these ion exchangers are aqueous buffers. Care is given to ensure that the buffers are made correctly and of suitable concentration to prevent pH drift (and irreproducible results). Sometimes up to 10% of an alcohol can be added to

improve spot quality and separation or to decrease viscosity to speed the development times.

B. Impregnated Layers

Besides the possibility of changing the selectivity of sorbents by chemical modification, improvement of selectivity can also be achieved by impregnating the matrix with suitable organic or inorganic substances (physisorption). The two possible methods for impregnating the sorbent (already described in Sec. II.A.3) are (a) prechromatographic impregnation of the porous matrix and (b) formation of a liquid stationary phase during the chromatographic development (with a suitable multicomponent system). Only the first of these methods ensures that the stationary phase will be well defined with respect to both qualitative and quantitative composition. This is true both for adding the impregnating agent to the suspension before plate preparation and for impregnating the precoated layer with an appropriate solution containing the liquid stationary phase. Impregnating agents frequently used in thin-layer chromatography can be divided into the following groups, depending on the nature of the interaction with the substances to be separated:

1. Nonpolar liquids that are able to form a liquid stationary phase for a partition chromatographic RP system that is independent of the matrix used. For this purpose, saturated and unsaturated hydrocarbons (paraffins, squalene), silicon oils, and plant oils have most often been used. Characteristic fields of applications of such hydrophobic impregnated layers are listed in Table 12.

2. Impregnating agents that are able to form complexes with the sample molecules to be separated. Examples include organic substances that are able to act as ligands in a complex-formation process, such as EDTA (ethylenediaminetetraacetic acid). These substances can be used

Table 12 Applications on Nonpolar Impregnated Layers

Substance class	Reference
Antibiotics	195–198
Nitrophenols	199
Peptides	200
Pesticides	201, 202
Phenols	203
Pigments	204
Steroids	205

to separate antibiotics (206, 207), metal ions (208, 209), and phospholipids (210). A variation of this method is the impregnation of layers with metal ions that act as central atoms. For example, thin-layer plates impregnated with cadmium, copper, zinc, or

manganese salts have been used to separate amino acids (211), aromatic amines (212), humic acids (213), peptides (214), phenolics (215), and sulfonamides (216). Also, thin-layer plates can be impregnated with various organic compounds such as salicylic acid, syringic acid, o-phthalic acid, and phenolic acids to separate various metal ions such as Cu^{2+} , Fe^{3+} , Hg^+ , Pb^+ , and Ni^+ (217–219). Impregnation with silver nitrate is especially important in this connection. The Ag^+ ions are able to form complexes with π systems. In this way, selectivity is achieved with respect to the number, position, and geometry of double bonds. This property is used to separate fatty acid derivatives (220, 221), lipids (222–224), and steroids (225–227).

3. Impregnating agents that are able to form charge transfer complexes. An example is HPTLC precoated silica gel 60 plates impregnated with caffeine, which was introduced in 1994. This stationary phase is especially suitable for the separation of polycyclic aromatic hydrocarbons (228–231).

4. Substances that lead to the adjustment of pH values. In general, acidified carriers are very useful for the separation of aromatic amines (232), aromatic compounds (233), and phenolics (234). Sorbents with alkaline pH values can be used for separations of basic compounds and amines (235, 236).

5. Impregnating agents that lead to a defined change in the solubility of the analytes in the liquid stationary phase. For this purpose, formamide and ammonium sulfate are frequently used for directed modification of partition coefficients. Impregnation with formamide has been described, e.g., for the separation of alkaloids (237), digitalis glycosides (238), and nitrophenols (239). A typical field of application of ammonium sulfate-treated layers is the separation of lipids, and, above all, of phospholipids (240–242).

The impregnating agents mentioned are only a few of the possibilities for easily and inexpensively adjusting selectivity in a thin-layer chromatographic system.

C. Precoated Layers for Enantiomeric Separation

Only one or two bonded chiral stationary phases for TLC enantiomeric separation have been developed and are commercially available. This is unlike the many bonded chiral stationary phases now available in HPLC columns. The difference is that the HPLC columns can be used for hundreds of samples before having to be replaced. Such bonding for one-time-use TLC prepared plates would be prohibitively costly.

Most chiral separations need maximum efficiency for distinct resolution. To achieve such efficiency and high resolution with TLC, HPTLC plates have to be used. Separation is enhanced, too, with sample banding (so sample streaks are obtained, not spots) and multiple development (redeveloping the dried TLC plate for a second or third time—the TLC version of recycle chromatography).

The separation principle used for the only commercial chiral TLC plate is based on a ligand-exchange mechanism. The plates consist of optimized RP carriers impregnated with copper salts and chiral selectors based on amino acids (such as L-proline). Typical applications of this separation mechanism are mainly amino acids and their derivatives (243, 244) and hydroxy carboxylic acids (245). Figure 11 shows an example of the application to separation of a racemic mixture of phenylalanine. Similar separations can be accomplished by impregnation with the chiral selectors or using them in the mobile

phase. Separations done with the addition of L-amino acids to the plate or mobile phase include alkaloids (246), amino acids (247), an analgesic (248), and antiarrhythmics (249).

One other fact to remember is that diastereomers can be made from the enantiomers by various derivatization methods. These species differ in chemical characteristics and can be separated by traditional silica gel or bonded phase TLC methods. Although this is an extra step in the analytical process, it may result in the most expedient and least expensive method.

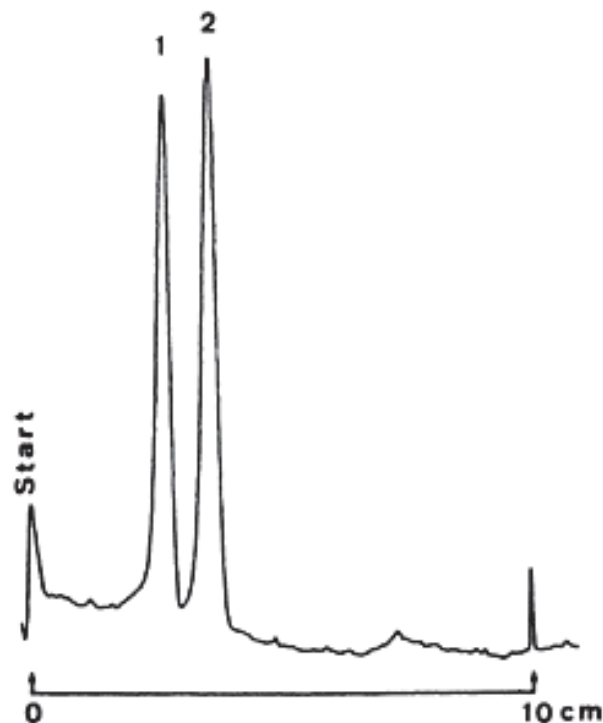


Figure 11 Separation of DL-phenylalanine. Plate: HPTLC precoated plate CHIR. Eluent: Methanol– water-acetonitrile (50:50:30). Migration distance 10 cm. Normal chamber with saturation. Compounds: 1, D-Phenylalanine; 2, L-phenylalanine (both 0.01%). Application volume 5 μ m. Detection: Plate dipped in 0.5% ninhydrin in ethanol-glacial acetic acid (98:2) and heated to 120°C for 5 min; in situ evaluation with TLC/HPTLC scanner (Camag) at 254 nm.

D. Enantiomeric Separation with Impregnated Layers

Not only chemically bonded stationary phases but also impregnated layers are able to separate optically active compounds. With this version of a chiral separation, cost is not a factor. Most often the chiral selector (β -cyclodextran, protein, antibiotic) is simply added to the mobile phase used for development. If limited resolution is seen, note the comments in the previous paragraph about using sample banding and multiple development. Some typical separations using this technique are beta-blocking drugs (250–252), amino acids, and others (253–255).

IV. PRECOATED LAYERS WITH CONCENTRATING ZONE

All the thin-layer plates discussed so far consist of a uniform sorbent layer. Precoated layers introduced in this section are combinations of different types of layers. Specific advantages of these precoated layers with preadsorbent or concentrating zones can be summarized as falling into three categories:

1. Simplification of sample application
2. Improvement of separation efficiency in the case of large-sample volumes
3. Possible decrease in number of sample preparation steps

The mode of operation of such a precoated layer is based on the combination of the separation layer with a preceding small inert band of sorbent. At the beginning of the development, sample substances to be separated are transported with the solvent front. Upon reaching the interface of the two layer sections, the sample molecules are retarded and therefore concentrated into small bands. A clearly improved starting position for the subsequent chromatographic separation results, particularly in the case of large sample volumes, leading in turn to significantly improved separation efficiency. Because of their physical and chemical properties, inert silicon dioxides such as kieselguhr and silica 50,000 (see Sec. II.C) are suitable sorbents for the formation of concentrating zones.

Combinations of concentrating zones with a series of different types of separation layers are used. Some of these combinations are listed below, together with examples of applications.

Surface-active silica gel. Silica gel layers with concentrating zones are especially suitable for use in normal-phase systems. Typical fields of application are shown in Table 13.

RP-modified silica gel. The advantages of the concentrating zone can also be utilized by combination with RP layers. Some applications of this type of plate are of aminoalcohols (270), carotene and lutein (271), lipids (272), and sunscreens (273, 274).

V. MIXED LAYERS

Prepared plates are available with both silica gel and RP modified silica gel. These plates allow both normal-phase and reversed-phase separations to be accomplished on a single TLC plate. Two versions are available. In one the bottom 20% is coated with a reversed phase and the remaining 80% with silica gel. This plate allows the RP mode to precede the normal-phase mode. The other is the reverse of this, allowing a normal-phase mode to precede the RP mode. To save time and cost, separate RP and silica gel plates are used for method development. When it has been determined what developing solvents give the best resolution in both modes, the combination plate is used for samples and standards.

VI. PREPARED PREPARATIVE LAYERS

Preparative chromatography is defined as the isolation of a quantity of a substance for further study or characterization. Depending on the need, this quantity may be only a few micrograms, a few milligrams, a few grams, or a kilogram. For most needs where TLC is the method routinely used for preparative chromatography, it is a few milligrams of a component.

It is possible to simply load as much as possible onto typical analytical plates, whose usual thickness is 0.25 mm, run a few of these, and then isolate and extract the component(s) of interest. It is simpler to run one or two preparative thin layers to accomplish this isolation, however.

Preparative or thicker thin layers have long been available from the TLC plate manufacturers. They are available in 0.5, 1.0, and 2.0 mm thicknesses. These allow 2×, 4×, or 8× the scale-up, respectively, compared to the analytical layer.

A note to the users of these plates: Because the layers are thicker, they will give off more heat of solvation when development begins. This will cause the R_f values of the components to

Table 13 Applications on Silica Gel Layers with Concentration Zones

Class substance	Reference
Amines	256
Antiasthmatics	257
Antibacterials	258, 259
Carbohydrates	260
Explosives	261
Flavors	262, 263
Lipids	264–267

Steroids	268
Taxols	269

be generally higher on the plate. Hence, some reoptimization of the developing solvent may be necessary to reduce the migration and possibly restore some of the lost resolution.

A frequently asked question is, how much can be loaded on these plates? The scale-up mentioned above is true, but the absolute amounts have to be experimentally determined. This is done by increasing the amounts spotted (or streaked) on a few preparative plates. Each mixture (amounts of each compound), the resolution (spots well separated or near one another), and the solvent system (which has to successfully dissolve the increased amounts of sample and still resolve the components) all play a role in the final loadability of any preparative plate.

Some preparative TLC applications include azo dyes (275), coumarins (276), plant components (277, 278), and triterpenoids (279).

VII. BINDERS IN PREPARED TLC PLATES

To form a rugged TLC surface—one that can be spotted, developed, and visualized without damage—a binder has to be incorporated into the slurry formulation when the plates are being made. When chromatographers began using TLC plates and had to make their own, the traditional binder used was gypsum (G coding) or calcium sulfate hemihydrate (the very familiar plaster of Paris). It was used in about a 10–15% by weight proportion in the silica gel. After mixing and pouring or casting onto a glass plate, the slurry goes from a shiny wet look to a flat finish. This is the first stage of the drying and setting up of the calcium sulfate to form a dihydrate. Further air drying completes the plate manufacture. Note that the plate appears dry at this stage but still contains a great deal of water associated with the silanols. Heat activation is still necessary to remove the absorbed water.

Although the gypsum helps keep the silica gel on the glass plate, it is a very fragile binder, and such layers were called “soft” layers. Care in all the steps of TLC had to be taken so as not to disturb the layer and cause poor chromatography or loss of some of the components. Often, after visualization, the plates were sprayed with a polymeric fixative (such as a poly vinyl alcohol). Other binders such as silicate solutions and starch have also been used, but these were never as popular as the gypsum binder.

Aware that “soft” layer TLC plates were difficult to ship, various TLC plate manufacturers began experimenting with alternative binders. Most settled on various water-soluble polymeric binders to replace gypsum. The result was a much more durable layer that could be stacked for easy shipment and written on with a soft lead pencil to keep track of samples and TLC conditions. These are often referred to as “hard” layer plates.

Although the binders used are proprietary, they are related to polyvinyl alcohol, polyvinyl pyrrolidone, or similar compounds. The binders and their amounts might be changed, with the sorbent being made into a plate to ensure a better product able to withstand the mobile phases most used with that particular sorbent. When these plates are

made, oven drying (rather than air drying) is routine, so the plates from a newly opened box are fairly active.

One possible complaint with the polymer-bound plates is the softening and swelling that occur with certain solvent combinations. In the worst case, the layer can wrinkle or lift off the support. Often, on questioning people who have experienced this, it is found that they did not activate their plates. Although this is routinely done to dry the TLC plate to give greater reproducibility, it has a second positive effect. The additional drying can also help increase the binder strength. Presumably, this occurs because the heat causes extra cross-linking of the binder and/or the removal of water.

A final solution to the lifting or softening of these layers is to use 1 M sodium chloride in place of the water portion of the mobile phase in polymer-bound RP plates. The salt prevents hydration of the binder so that swelling or buckling is much less likely to occur.

If these additional steps do not help, then it is advisable to change to a plate designed to be used with a particular mobile phase. Many manufacturers have produced TLC plates to be used with highly aqueous developing solvents, because their original prepared layers could be a problem with such developing solvents.

The layers of a polymer-bound plate are somewhat water-resistant. This is an advantage because they are less sensitive to relative humidity (and its inherent nonreproducibility of R_f values) than were the gypsum-bound plates. They develop in the same manner, and results are the same (same separation and retention) in almost all cases. However, differences between hard and soft layer plates, and even between plates of the same type from different manufacturers, can occur because the silica gel and binders used are unique to each manufacturer. When changing from any type of TLC plate, the new plates should be run beside the old ones under identical conditions for a few days to compare results.

Another possible problem with the polymer-bound plate, but one that is easily overcome, is that detection reagents made up only in water will not wet the layers as easily as they do gypsum layers. This is seen when the totally aqueous reagent (originally developed in the age of gypsum-bound plates) is sprayed on a prepared plate. The solution does not penetrate as well, perhaps even running off the silica gel layer if it is sprayed too heavily. The remedy is to add 5% methanol or ethanol to the formulation. This decreases the surface tension of the reagent solution, and then penetration, whether application is by spraying or dipping, is instant.

VIII. TLC SUPPORTS

As mentioned above, suitable supports onto which any sorbent can be coated include glass, plastic, and aluminum. Analytical results on any plate will be identical regardless of the support, especially supports made by the same manufacturer. It should be noted that manufacturers of flexible layers (plastic and aluminum) often apply a thinner layer to these supports. This prevents the layer from cracking should the plate be bent too much.

Any support needs to be perfectly flat and clean to ensure that good, intact layers result. Most people are familiar with glass supports. These can be purchased in many different sizes from 20 × 20 cm to 2.5 × 7.5 cm. Fewer sizes are available in plastic- or aluminum-supported plates, but these are simply cut with scissors or straight edge-sharp

blade combination, of which there are many today, including roller blade cutters (check your local craft store). When cutting with the straight edge and blade, the sorbent surface is laid face down on some clean paper.

Large glass plates that are prescored on the back can be purchased. This allows them to be broken down to a smaller size. This is a convenience and saves wasting a larger TLC plate on a few samples, minimizing the cost of analysis. Care should always be taken in breaking these plates to avoid getting cut by the glass. A special plier-like tool called a “running plier” or “grozier” that can make breaking prescored plates safe and easy is available from glass craft stores. It is a wide-nosed plier with a curved end coated with plastic (Fig. 12). Once the grozier is lined up with a score mark, a simple closing of the handles to apply pressure will snap the plate cleanly.

Glass-backed TLC plates stand up well in any TLC chamber. The plastic- (usually polyphthalate) and aluminum-backed plates need to be placed in a chamber at a sharper angle so they do not bend after being wetted by solvent. One distinct advantage of the flexible supports is that they can be cut to any size needed with scissors, razor blade, or roller cutter. They are usually placed face down on a cutting board or thick paper to be cut on the support side.

After cutting any plate from a larger plate, carefully hold the smaller plate and wipe the sides with a paper towel to remove loose sorbent clinging to the edges. If these random particles are not removed, they will act as wicks and will give crooked solvent fronts.

IX. RECENT TLC DEVELOPMENTS

The newest advances in TLC precoated layers include plates made with small-particle spherical silica gels with 60 Å pores. These are 6–8 μm and are applied to glass (0.2 mm thick layer) and aluminum (0.1 mm thick layer) supports. Because of their particle size, they are high-performance thin-layer plates. Their advantages include even more rapid separations (about 20% faster) and more compact spots compared to HPTLC plates made with irregular particles. Applications include

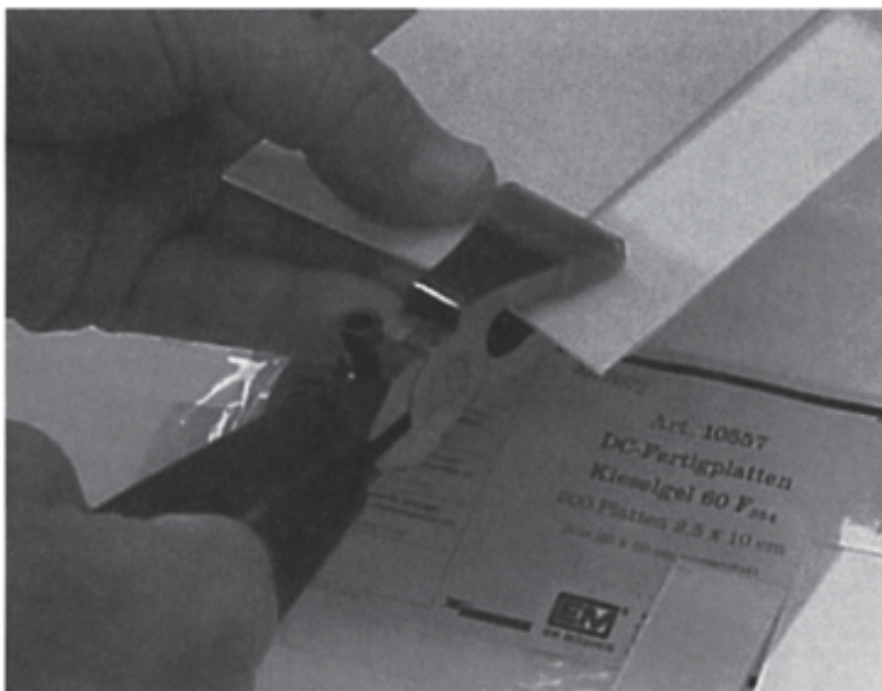


Figure 12 Using a grozier to help break a glass-backed, scored TLC plate.

antifungals (280), coumarins (281), and phenolics (147). A scanning electron microgram of a cross section of this 6–8 μm spherical particle HPTLC plate is shown in Fig. 13.

Another version of the spherical silica gel 60 plate is one made with even smaller particles. This plate has 3–5 μm particles placed on an aluminum support that is 0.1 mm thick. It was made for in situ Raman spectroscopy of separated components. The spherical silica gel allows a tenfold increase in signal intensity compared to a similar layer made with irregular silica gels.

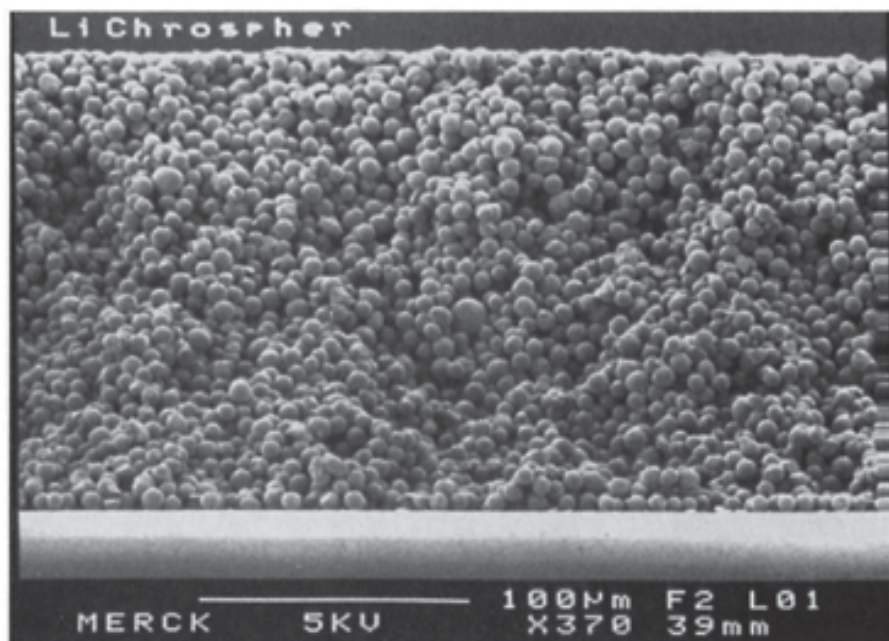


Figure 13 Scanning electron micrograph of a cross section of an HPTLC plate made with spherical 6–8 μm LiChrospher particles.

The aluminum support was chosen for this application so that after separation the spot area could be cut from the plate to be placed into the Raman spectrometer. A scanning electron micrograph of a cross section of this 3–5 μm spherical particle HPTLC plate is shown in Fig. 14. A typical in situ Raman spectrum of trenbolone acetate compared to the pure substance is shown in Fig. 15.

Another small change is a plate made for GLP (good laboratory practice) work, for better record keeping. The silica gel layer is laser etched with three numbers: (a) the catalog number of the plate, (b) the sorbent lot number, and (c) an individual plate number. Thus, no two prepared plates will have identical numbers, aiding in correct analytical assignment of work done on such plates. These are available only for a few top-selling TLC and HPTLC prepared layers. Applications performed on these plates include analgesics (282, 283), antihistamines (284, 285), herbals (286), lipids (287), and a motion sickness drug (288).

X. SUMMARY

Thin-layer chromatography today continues to be a dynamically developing modern analytical method. The areas of progress include an increase in the spectrum of selectivity, improvement of efficiency, and, in certain cases, simplification of handling. The foregoing discussion of bulk sorbents and precoated layers is not a complete enumeration of all possibilities; for example, the different carriers for the layers (glass

plates or aluminum or plastic sheets) are not shown explicitly. In addition, special plates with very restricted applicability are not discussed.

Focal points of recent and expected future developments in thin-layer chromatography are located in the fields of surface modification and in the improvement of the efficiency of precoated layers. Advances in these areas are preconditions for maintaining and extending the importance of TLC as a qualitative and quantitative analytical method in chemical laboratories.

Thin-layer chromatography can be an important part of any analytical laboratory scheme. It is the only chromatographic method that excels at screening large numbers of samples. Likewise, it has to be one of the simplest and least difficult to begin and to use. As with any tool, it can be kept simple or can be expanded in its use with the newest HPTLC plates, special spotting devices, developing chambers, and densitometers.

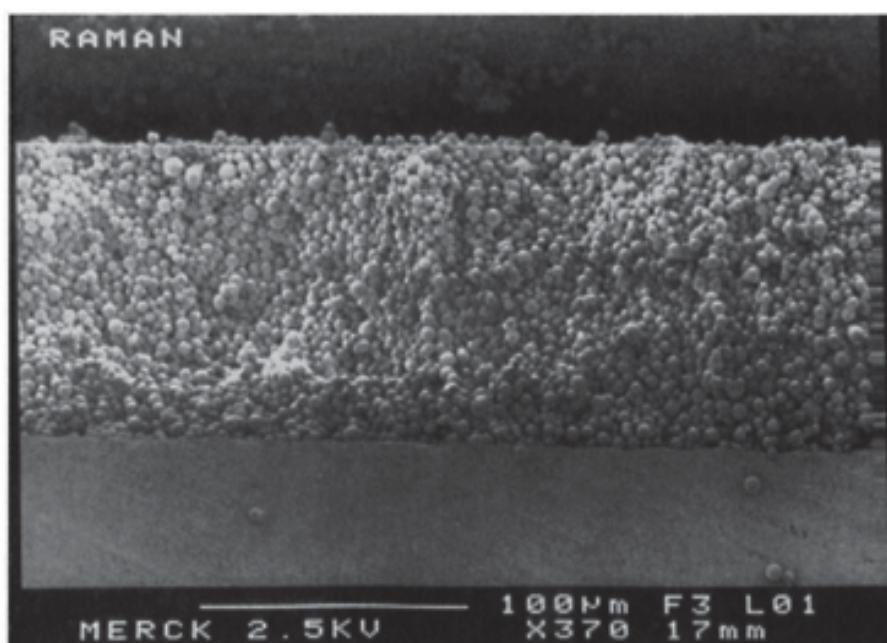


Figure 14 Scanning electron micrograph of a cross section of an HPTLC Raman plate made with spherical 3–5 μm LiChrospher particles.

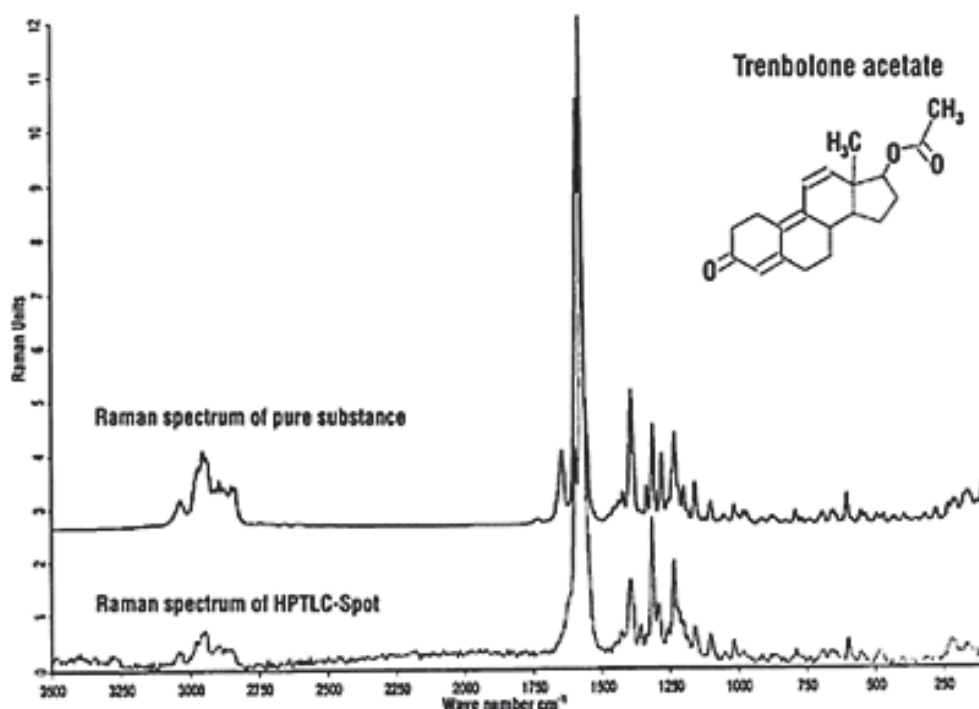


Figure 15 Raman spectra of a pure trenbolone acetate and an in situ measurement of this compound on a LiChrospher Si60 F254s Raman HPTLC plate.

Furthermore, there is a recognizable trend in the direction of coupling TLC with spectroscopic methods (e.g., FTIR, Raman, SERS, and MS) to enlarge the analytical possibilities. Mention was made here of one special plate made for such applications. TLC/MS has been done with the transfer of separated zones from a developed plate to a special matrix (289) and directly on the layer with an overlay of a special graphite solution (290).

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5

Instrumental Thin-Layer Chromatography (Planar Chromatography)

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I. INTRODUCTION

The purpose of this chapter is to present the state of the art in instrumentation for thin-layer Chromatography (TLC), particularly its high-performance version (HPTLC). For each step of the TLC process, the benefits of proper instrumentation are illustrated and guidance is provided for choosing the right instrument for a given task. In addition, a novel concept of an all-inclusive TLC software is presented.

With that in mind one should not ignore the fact that even today most TLC is still done at a level that was introduced by Stahl more than 40 years ago, yet the results seem to be sufficient. In such cases instrumentation could possibly replace manual labor and make the task easier to complete, but the expenditure would hardly be justified.

This chapter is written for the TLC user who has arrived at the point where the results of the classical approach no longer meet the expectation of analytical quality. It will clearly answer the questions about how planar Chromatography should be done to significantly improve its result. Neither historical aspects nor instruments that are no longer available on the market are covered. A detailed discussion is given in Ref. 1. Also not discussed are overpressured layer Chromatography (OPLC) and hyphenated techniques. The reader is referred to the appropriate chapters of this book for these topics.

A. Scope of the Chapter

Although many advantages of TLC can be utilized with very simple or no instruments, it is the availability of modern, usually computer-controlled, equipment that has unlocked the full power of the method and opened new fields for qualitative and quantitative applications of planar chromatography. Work in a regulated environment and demanding issues of quality control for routine analyses have changed TLC from “quick and dirty” to a dependable, sophisticated, and good manufacturing practice (GMP)-compliant analytical technique that has its established place in almost any modern laboratory. Today’s instruments, such as automatic application devices, sophisticated developing

devices, scanning densitometers, and video documentation systems, have complemented the inherent advantages of TLC with increased reliability, better sensitivity, and improved precision and accuracy of the analytical result. The serious analyst can select instruments with different levels of automation without sacrificing the quality of the analysis or losing the immense flexibility of the method.

B. HPTLC—Instrumental TLC

Originally, the term high-performance thin-layer Chromatography (HPTLC) referred mainly to the use of special HPTLC plates as outlined in Chapter 4. Soon it became clear that the potential of the new stationary phase could be fully used only if the chromatogram was miniaturized and all steps of TLC were precisely executed with the help of special instruments. Even though all modern TLC equipment can also be used with conventional plates, it should be understood that only with HPTLC plates is the maximum performance achieved and all advantages of the technique realized. Therefore, HPTLC is often used as a synonym for instrumental TLC. Planar chromatography is, and will probably remain, an off-line technique, even though approaches to fully automate the process have been discussed in the literature (2, 3). The individual automation of all steps, on the other hand, is already possible and is demonstrated in this chapter.

II. SAMPLE APPLICATION

A. General Aspects

Following sample preparation, the first step in TLC—sample application—is very important, because it determines the achievable quality of the chromatographic result. Two basic requirements must be met: (a) The position of the application should be exact and (b), particularly for quantitative analyses, the applied sample volume should be precise and accurate. Furthermore, it is clear that the layer must not be damaged during sample application. This requires careful mechanical actions when the applicator makes contact with the sensitive chromatographic layer.

To maximize the separation power of the chromatographic system, the application zone should be as small as possible in the direction of chromatography: 2–4 mm should not be exceeded on conventional layers, and for HPTLC plates 1.5 mm is the upper limit. This requirement causes restrictions in the volume of samples that can be applied as spots in one stroke. Typically, 0.5–5 μL can be spotted onto TLC plates and 0.1–1 μL onto HPTLC plates. During spot application, the solvent of the sample performs “circular chromatography.” This can cause irregular distribution of the sample components across the spot, and after chromatogram development spots may be broad and not symmetrical. Generally, separation efficiency is decreased. As a rule of thumb, the sample solvent should be as low in solvent strength (nonpolar for normal-phase systems, polar for reversed-phase systems) as possible. This ensures small and compact starting zones. The great advantage of spot application is its simplicity and the very low time consumption. The applied volumes can be very small, and a large number of samples can be applied onto a given plate. Spot application should be chosen when the chromatographic

separation of sample components is not problematic. A typical example is the content uniformity test in pharmaceutical analysis.

It has been shown (4) that resolution and detection limits of a given TLC system can be significantly improved if samples are applied as narrow bands. This statement particularly applies to the spray-on technique, which ensures a homogeneous distribution of the sample over the entire length of the band. Hence, sample application in the form of bands is usually selected for complex or multicomponent samples and whenever precise quantitative results are required. Another great advantage of the spray-on technique is that any chromatography during application can be avoided if the dosage speed is properly selected. This allows the formation of very narrow starting zones, even with samples dissolved in solvents of low volatility and high solvent strength. It is also possible to apply large volumes of samples with low concentration of analyte without losing the quality of the band. Improved resolution by band-shaped separation zones can also be attained by using plates with so-called preconcentration or preadsorbent zones (see Chap. 4) or by other means of focusing samples applied as spots into bands. It should be noted that the sample distribution across "bands" obtained in this way is not as homogeneous as after a proper spray-on application and therefore is not suitable to be quantified by aliquot scanning.

B. Technical Solutions

To meet the requirements of proper sample application, instruments must have the capability of positioning and dosing samples reproducibly. Simple mechanical tools are rulers for manual selection of the application position in the *x*-direction (distance from the left edge of the plate) and *y*-direction (distance from the lower edge of the plate in the direction of chromatography). More sophisticated instruments are computer-controlled and can be programmed to deliver samples automatically to selected positions on the TLC plate.

Volume dosage is achieved either manually, with fixed-volume pipets (capillaries) that are lowered onto the chromatographic layer, or mechanically with motor-driven syringes. Whereas micropipets allow sample transfer only by capillary action, syringes are typically emptied with a selected dosage speed while either in contact with the layer or slightly above it using the spray-on technique. During contact with the layer, samples are usually applied as spots unless several very small spots are applied next to each other to form a band. Spots can also be sprayed on. However, the great advantage of the spray-on technique lies in the possibility of applying narrow bands. During application of bands, either the syringe or the plate is moved in the *x*-direction. While the sample is dispensed from a motor-driven syringe, it is "atomized" by a stream of gas (nitrogen or compressed air) and sprayed onto the layer.

C. Instrumentation

A simple instrument for precise manual sample application as spots is the Nanomat (CAMAG, Muttens, Switzerland) (Fig. 1). It allows 0.5, 1, 2, or 5 μL volumes from capillaries to be applied as spots with a minimum distance of 5 mm in the *x*-direction. The instrument is usually used for quick qualitative work, for initial trials during method

development, and whenever the cost of instrumentation has to be kept very low. When handled with care, the Nanomat is also well suited for quantitative work. Operation of the Nanomat is quite simple:

1. The chromatographic plate lies precisely positioned on the base plate of the Nanomat. The sample application position in the y -direction can be selected from 1 to 33 mm. The first application position in the x -direction is 5 mm, and the last is 195 mm. The positions of all other samples are shifted in multiples of 5 mm in the x -direction. Typical choices are $x_1=15$, $y=8$ mm for HPTLC plates or $x_1=25$, $y=15$ mm for TLC plates.
2. A disposable capillary is conveniently taken with the capillary holder from a dispenser.
3. The sample is taken up by dipping the capillary into the sample solution.
4. The capillary holder is placed on the applicator head, where it is held by means of a magnet.
5. Pushing down the applicator head gently brings the capillary into contact with the TLC plate, and the sample is applied as a spot without damaging the layer.
6. A new capillary is loaded to apply the next sample, thus avoiding cross-contamination.

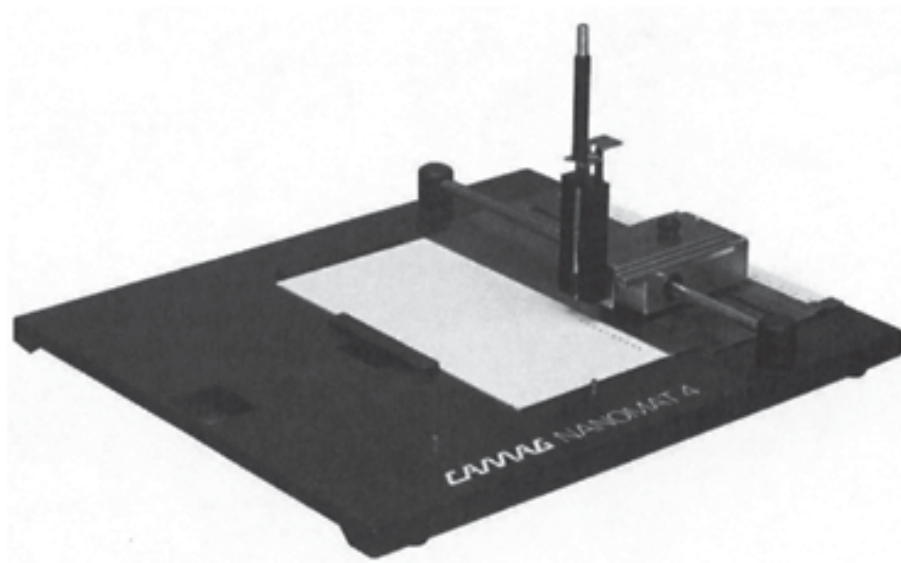


Figure 1 Nanomat 4 (CAMAG, Muttenz, Switzerland).

Automated sample application as spots can be performed with the TLS100 (Baron, Reichenau, Germany). The instrument uses a motor-driven syringe of 1, 10, or 100 μL volume. Via a keypad, application positions and sample volumes are programmed for up to 30 samples and four standards on up to 6 plates of 20 $\times$ 10 cm. The TLS100 can also generate bands of defined length by applying the specified sample volume evenly divided into small spots next to each other. The instrument can store up to 15 methods.

One of the most widely used sample applicators is the Linomat (CAMAG) (5) (Fig. 2), an affordable semiautomatic device that introduced all the advantages of the spray-on technique to planar chromatography. Precise volume dosage and exact positioning

combined with flexibility and convenient handling are among the most important features of the instrument. The user loads the sample manually into a syringe and selects the y-position of the application; the instrument manages all other parameters of the application process. During sample application, the stage with the chromatographic plate moves in the *x*-direction underneath the dosing syringe. The movement is automatically adjusted so that for each band an even number of complete passes is maintained, which ensures fully homogeneous distribution of the samples across each band. This is a prerequisite for aliquot scanning, in which the densitometer measuring slit is set to cover only the central 50–75% portion of the band. If the proper dosage speed is selected, the shape of the applied band is nearly unaffected by the type of solvent used to dissolve the sample, as shown in Fig. 3. The latest Linomat (Model 5) is controlled from a computer running the winCATS software (see Sec. VII). The instrument can also be operated in a stand-alone mode and programmed either via a keypad or by downloading up to 10 methods from a computer. Samples of 100 nL to 2 mL can be applied as bands of 0 (spot) to 195 mm length, which allows sample application for qualitative, quantitative, and even preparative tasks. The unusable portion of the sample solution is extremely small.



Figure 2 Linomat 5 (CAMAG, Muttensz, Switzerland).

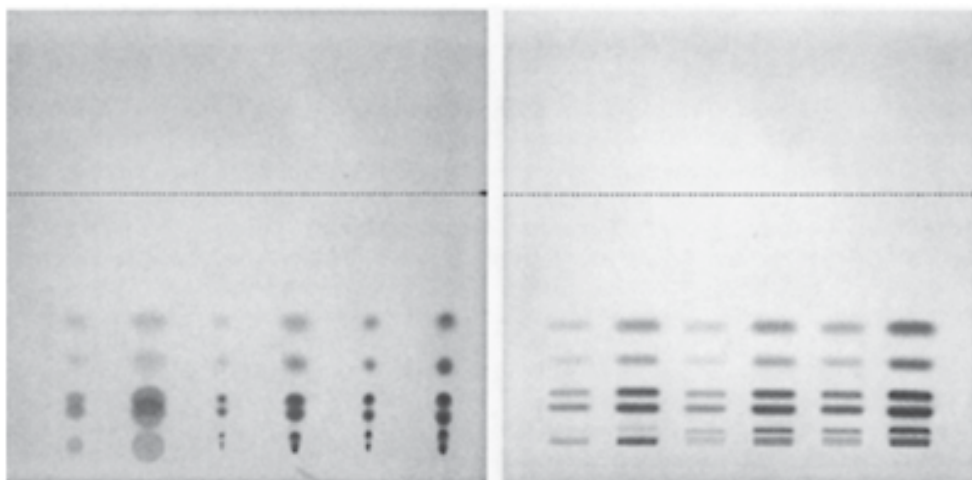


Figure 3 Effects of sample application on the chromatographic result. Left plate: spot application (contact); right plate: band application (spray-on). Test dye mixture on HPTLC silica gel 60 developed with toluene. 1 μ L and 5 μ L of samples dissolved in (a) methanol, (b) toluene, or (c) hexane.

The AS30 (Desaga, Heidelberg, Germany) (Fig. 4) represents a fully automatic software-controlled application device. In combination with a conventional autosampler, it can apply up to 30 samples as spots or bands by using a spray-on technique.

The most advanced, versatile, and powerful system on the market is the Automatic TLC Sampler 4 (ATS 4; CAMAG) (Fig. 5). Up to 66 samples from vials or 96 samples from well plates can be applied fully automatically by using either the spray-on technique of the Linomat or spot application by contact. Any x - and y -positions on the TLC plate can be selected for application. The ATS 4 can also apply samples as rectangles, a feature that is very useful for large quantities of samples that contain the analyte in very low concentration. Prior to chromatography, such rectangles are focused into narrow bands with a solvent of high solvent strength. An optional heated spray nozzle allows the application speed to be increased, which is particularly useful when aqueous solutions are applied. A special feature of both the ATS 4 and the Linomat 5 is “over-spotting,” by which more than one sample can be applied as a spot or band onto a single given



Figure 4 AS 30 (Desaga, Heidelberg, Germany).



Figure 5 Automatic TLC Sampler 4 (CAMAG, Muttenz, Switzerland).

position. Spiking of a sample, application of several reference compounds from different vials onto the same track, or prechromatographic derivatization can easily be accomplished. The ATS 4 is not only the ideal choice for routine analyses but also offers the ultimate flexibility for laboratories facing rapidly changing tasks.

III. CHROMATOGRAM DEVELOPMENT

A. General Aspects

Thin-layer chromatographic plates can be developed in three geometrical modes: linear, circular (radial), and anticircular. Although the latter two modes have merits in certain cases, today linear development is used almost exclusively. Hence, only linear development is discussed here. Planar chromatography differs from all other chromatographic techniques in that a gas phase is present in addition to the stationary and mobile phases. This gas phase can significantly influence the result of the separation.

The “classical” way of development is to place a plate into a developing chamber that contains a sufficient amount of developing solvent. The lower end of the plate should be immersed to a depth of several millimeters. Driven by capillary action, the developing solvent moves up the layer until the desired running distance is reached, and the plate is removed from the mobile phase to interrupt chromatography. The following considerations primarily regard development of silica gel as the stationary phase, which can be described as an adsorption process. Provided that the developing chamber is closed and reasonably tight, four partially competing processes occur (Fig. 6):

1. Phase equilibrium is eventually established between the components of the developing solvent and their vapor phase. Depending on the vapor pressure of the individual components, the composition of the gas phase can differ significantly from that of the developing solvent.

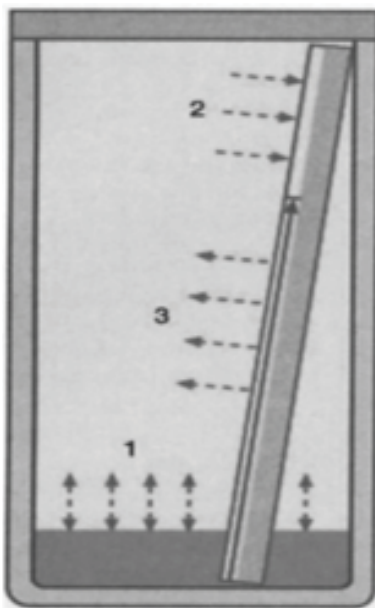


Figure 6 Processes taking place in a chromatographic chamber (see text for details).

2. While still dry, the stationary phase adsorbs molecules from the gas phase. This process also approaches an equilibrium state called adsorptive saturation. In this way, particularly polar components will be withdrawn from the gas phase and loaded onto the surface of the stationary phase.
3. Simultaneously, the part of the layer that is already wetted with mobile phase begins to interact with the gas phase. The less polar components of the liquid are given off into the gas phase preferentially (3). Unlike process 1, this process is governed not so much by vapor pressure as by adsorption forces.
4. During migration, the components of the mobile phase can be separated by the stationary phase, which causes the formation of secondary fronts.

Processes 1 and 2 can be experimentally affected by

Fitting the chamber more or less completely with filter paper that is soaked with developing solvent.

Waiting a certain time between the introduction of developing solvent into the chamber and the beginning of chromatography—chamber saturation.

Allowing the plate to interact with the gas phase without contact with the developing solvent—preconditioning.

Interactions according to processes 2 and 3 can be effectively prevented by placing a counter plate at a distance of one or a few millimeters from the chromatographic layer. This is called a sandwich configuration. The further equilibria 1 and 2 have been established and the less different the components of the mobile phase are in their adsorption behavior, the less pronounced are the secondary fronts resulting from process 4. In well-saturated chambers and on preconditioned layers, they are often not even seen, but in sandwich chambers and particularly in OPLC, secondary fronts are very prominent. During chromatography, components of the developing solvent, which have been loaded via the gas phase onto the dry layer during process 2, are pushed ahead of the true but invisible solvent front. Exceptions are polar substances such as water, methanol, acids, and bases. As a result, R_f values are lower in saturated chambers, particularly on preconditioned layers, than in unsaturated chambers and sandwich configurations.

Planar chromatography in most cases proceeds in a nonequilibrium condition among the stationary, mobile, and gas phases. That is why it is very difficult to correctly describe mathematically the conditions in a developing chamber. Reproducible chromatographic results can be obtained only if all parameters are kept as constant as possible. Chamber form and saturation play a dominant role in this regard. Unfortunately, this means that the chromatographic result is different in each chamber. For illustrations of this statement, see Ref. 6. There are neither “good” nor “bad” chambers. However, in some chambers the parameters can be better controlled or reproduced than in others. Selection of the “proper” chamber is done during method development and generally follows practical considerations such as which chamber is conveniently available, which one is “always” used in the laboratory, or which one is used by a collaborating laboratory. However, attention should also be paid to the economic aspects such as time requirements and solvent consumption.

B. Developing Chambers

The “classical” flat-bottomed chamber is available in many sizes from various manufacturers. When it is lined with filter paper, a stable saturated system can easily be achieved. The biggest disadvantage is the high solvent consumption of such chambers. The large solvent volume makes it unpopular to follow the recommendation to always use fresh solvent to develop a new chromatogram.

Much more economical and also more flexible are the so-called twin trough chambers (TTC) (CAMAG) (Fig. 7), which are among the most widely used chambers. They are available for 10×10 cm, 20×10 cm, and 20×20 cm plates. Only 5 mL of solvent is required per trough for an HPTLC plate in a 10×10 cm chamber. This amount of solvent generates a liquid level of 5 mm. If samples are applied at 8 mm from the lower edge of plate, they will be 3 mm above the solvent level. Twin trough chambers can be operated in the following modes:

Unsaturated. Only the front trough contains developing solvent. After the chamber is charged with developing solvent, the plate is introduced, and chromatogram development starts immediately.

Saturated. Both troughs contain developing solvent. A filter paper wetted with solvent is placed in the rear trough. Prior to introduction of the chromatographic plate, the chamber is left for saturation to be established (typically 20–30 min).

Preconditioned. The plate is positioned in the empty front trough while the rear trough contains conditioning solvent [acid, base, a solution that establishes fixed humidity (7), or

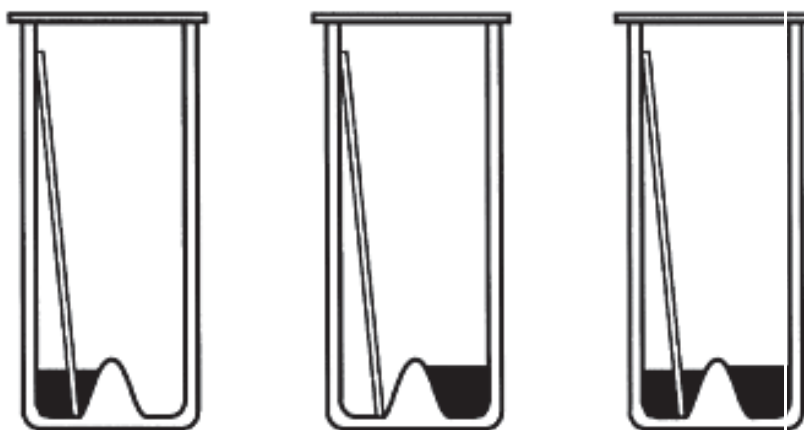


Figure 7 Schematic of Twin Trough Chamber (TTC) (CAMAG) configuration for (a) unsaturated mode, (b) preconditioning, and (c) saturated mode.

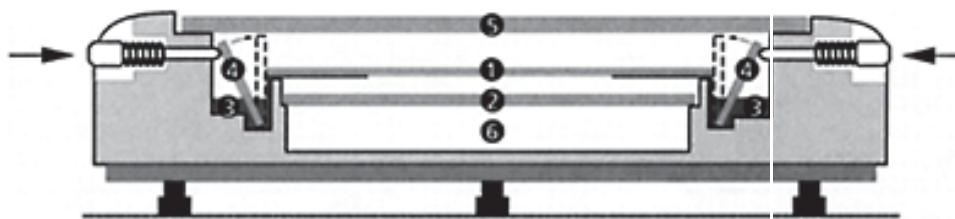


Figure 8 Schematic of the Horizontal Developing Chamber (HDC) (CAMAG, Muttenz, Switzerland). 1, HPTLC plate (layer facing down); 2, glass plate for sandwich configuration; 3, reservoir for developing solvent; 4, glass strip; 5, cover plate; 6, conditioning tray. The HPTLC plate is placed into the chamber with the layer facing down. The reservoir (3) is charged with developing solvent. The plate can be developed horizontally either from one side only or from opposite sides simultaneously, in this way doubling the number of samples per plate. Chromatography is started when the glass strip (4) is brought into a vertical position. In the unsaturated configuration, the conditioning tray (6) is empty; the glass plate (2) is removed. In the saturated configuration, the conditioning tray (6) contains developing solvent; the glass plate (2) is removed. For preconditioning, the conditioning tray (6) contains conditioning liquid; the glass plate (2) is removed. Development is started after preconditioning is completed. In the sandwich configuration, the conditioning tray (6) is empty; the glass plate (2) is in place.

developing solvent]. After a certain conditioning time, developing solvent is introduced into the front trough that contains the plate.

The ultimate versatility is achieved with the horizontal developing chamber of CAMAG (Fig. 8), which is designed for either 10×10 cm or 20×10 cm HPTLC plates. Not only are several configurations (saturated, unsaturated, preconditioned, sandwich) possible, but also development of samples from opposite sides of the plate. Applied as spots, up to 72 samples can be simultaneously chromatographed on a 20×10 cm plate. By using the center tray of the chamber for conditioning, the relative humidity during chromatographic separation can be controlled.

For method development and optimization of chromatographic parameters, the HPTLC-Vario chamber (CAMAG) is the ideal tool. Up to six different solvents or six different conditions can be used simultaneously on 10×10 cm HPTLC plates that have to be scored for this purpose with a special device. The optimized system can easily be transferred to a horizontal developing chamber.

C. Automated Multiple Development

Automated multiple development (AMD) a step-gradient technique derived by Burger (8), achieves the maximum resolution feasible within the limited separation distance available on an HPTLC plate. In terms of peak capacity, it compares with HPLC while retaining the inherent benefits of planar chromatography. Unlike a gradient in column chromatography, an AMD gradient starts with the solvent having the strongest elution power. In successive runs the solvent is varied toward decreasing elution power, and each run proceeds to a higher migration distance than the previous one. Typical distance increments are 3 mm or less for a 20–25-step gradient. Between developments, the solvent is completely removed from the chamber and the layer is dried under vacuum. Preconditioning through the gas phase prior to development is possible (9). “Universal gradient” is the term for an AMD gradient that starts with a very polar solvent and is varied via a solvent of medium polarity to a nonpolar solvent. Depending on solubility considerations, methanol or acetonitrile is typically used as the most polar component. The central or “base” solvent and, to a certain extent, the nonpolar solvent determine the selectivity of the separation. A solvent such as dichloromethane or *t*-butyl methyl ether is used as the base solvent in most AMD applications. Solvents for AMD must meet two requirements: They must be suitable for being dried off by vacuum, and they must be pure.

During the AMD procedure, fractions are focused into narrow bands with a typical peak width of about 1 mm. This allows the separation of multicomponent mixtures that had no chance of being separated by TLC in the past (10). The fully computer controlled AMD 2 instrument (CAMAG) (Fig. 9) features five bottles from which solvents can be drawn by syringe action to form the gradient. A charge-coupled device (CCD) monitors the migration distance of the mobile phase, and the drying time can be varied for each development step. AMD is a very reproducible technique. Typical fields of application include analysis of pesticides (11) and lipids (12) and screening for biological activity (13).

IV. DERIVATIZATION

A. General Aspects

It is an inherent advantage of planar chromatography that fractions are stored on the plate and can readily be derivatized after chromatography in order to be rendered detectable, improve detection limits, or selectively change properties of sample components. Substances that are not responding to white or UV light after chromatography need to be reacted with chromogenic or fluorogenic reagents. There are two general considerations for reproducible results: (1) transfer of the reagent must be controlled and homogeneous, and (2) if a heating step is part of the derivatization, the entire plate must be heated uniformly.



Figure 9 AMD 2 (CAMAG).

B. Instrumentation

Prechromatographic derivatization can be helpful for improving the chromatographic behavior of the desired sample compound. An interesting example of prechromatographic

derivatization directly on the TLC plate is the reaction of fatty acids in picomole amounts to fluorescing mono-dansylpiperazine and -cadaverine compounds (14). With a Linomat or an ATS 4, one reagent (monodansylpiperazine) is sprayed onto the starting zone and oversprayed with the analyte, followed by overspraying with the second reagent (dicyclohexylcarbodiimide). The reaction occurs spontaneously, without heating.

For the purpose of postchromatographic derivatization, liquid derivatizing reagents can be transferred onto the plate by spraying or dipping. Provided the reagent is suitable, dipping is the preferred technique. CAMAG's Chromatogram Immersion Device (Fig. 10) is an example of an instrument that allows proper execution of the dipping technique. The chromatographic plate must be immersed and withdrawn at a uniform speed to avoid tidemarks, which could interfere with densitometric evaluation. By maintaining a defined immersion time, derivatization conditions can be standardized. Spraying cannot usually be circumvented when two reagent solutions have to be applied in sequence without intermediate drying. Diazotization followed by coupling is an example. There are several sprayers on the market, from simple laboratory atomizers to electropneumatic TLC sprayers. A sophisticated instrument for derivatization by spraying is the Chromajet (Desaga), which allows computer-controlled application of defined amounts of reagents onto the individual tracks of the chromatogram. Whenever reagents are sprayed onto a plate, an efficient dust- and mist-removing device should be used to protect laboratory personnel against poisonous or irritating sprays and solvent vapors. The TLC Spray Cabinet (CAMAG) ensures the complete removal of excess spray from the atomizer and spray particles rebounding from the TLC plate. There is no deflection of the spray jet before it reaches the chromatogram, an effect often occurring in a normal laboratory fume hood.

In most cases the derivatization reaction has to be completed by heat treatment. Heating the chromatographic plate uniformly and reproducibly at the desired temperature can be accomplished with a plate heater specifically designed for this purpose. For more details on derivatization, see Chapter 8 of this book.

V. CHROMATOGRAM EVALUATION

A. General Aspects

In planar chromatography, chromatograms are usually evaluated densitometrically. During classical (scanning) densitometry, the separation tracks on the plate are scanned with a light beam in the form of a slit selectable in length and width. The photosensor of the densitometer measures diffusely reflected light. The difference between the optical signal from the sample-free background and that from a sample zone (fraction) is correlated with the amounts of the respective fractions of calibration standards chromatographed on the same plate. Densitometric measurements of planar chromatograms can be made by absorbance or fluorescence. The majority of

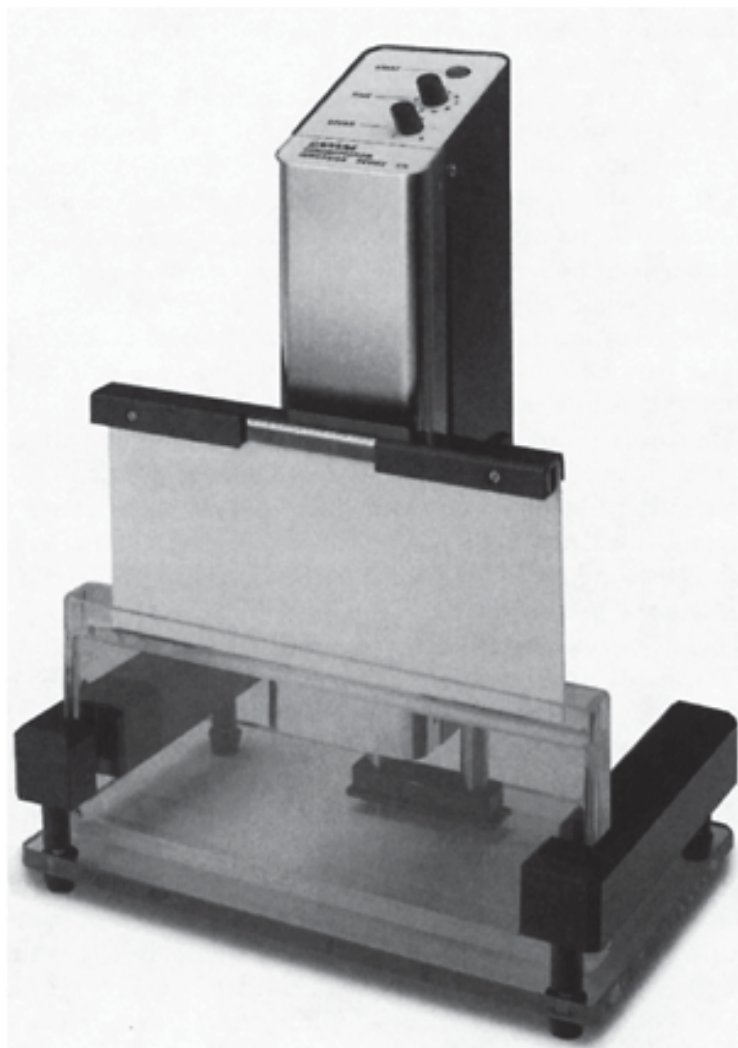


Figure 10 Chromatogram Immersion Device (CAMAG).

densitometric measurements of thin-layer chromatograms are carried out in the absorbance mode. The low UV range from 300 nm to 190 nm is the most useful.

Due to light scattering at the particles of the layer, a simple mathematically well-defined relationship between light signal and amount of substance in the layer has not yet been found. A fair approximation for measurements on particulate surfaces by absorbance is given by the Kubelka—Munk equation (15), which can be suitably derived for TLC (16). Absorbance measurements typically give data that are best fitted with nonlinear calibration functions. However, over smaller concentration ranges linear functions can be employed. For more information about the theoretical foundations of densitometry, see Chapter 10 of this book.

For scanning by fluorescence, the substances are excited by UV light, most often at 366 nm. A photosensor measures the emitted light, which is always of longer wavelength. A cutoff filter positioned between the sample and the photosensor eliminates diffusely reflected light of the excitation wavelength. Accordingly, the measured light is directly proportional to the amount of the fluorescing substance. Measurements of fluorescence

are more sensitive than absorption measurements by a factor of 10–1000. Calibration functions are often linear over a comparatively wide concentration range. For these reasons, substances with inherent fluorescence should always be scanned in this mode. For nonfluorescent compounds, pre- or postchromatographic derivatization to render them fluorescent should always be considered.

For convenient visual evaluation, TLC layers usually contain a so-called UV indicator (F254), which is excited by 254 nm light and fluoresces green or blue. The emission of the indicator is reduced in places where substance zones are located that absorb at about 254 nm. Such substances therefore appear as dark zones on a fluorescent background. It is a common misconception that fluorescence quenching is measured if plates containing a fluorescence indicator are scanned in reflectance mode at 254 nm. In fact, the emitted fluorescence light is so low in energy compared to the UV light used for excitation that the difference in quenching is barely measurable. However, the decrease of diffuse reflectance due to absorbance of the substance at the selected wavelength creates the signal, as described under absorbance measurements. Therefore, the monochromator should always be set at the wavelength of maximum absorption of the substance, whether the layer contains fluorescence indicator F254 or not. To truly measure fluorescence quenching, the excitation wavelength of 254 nm must be blocked by a cutoff filter before it can reach the photomultiplier set to reflectance mode. Then the emitted light from the indicator will be treated as the baseline.



Figure 11 TLC Scanner 3 (CAMAG).

As an alternative to classical densitometry, an electronic image of the planar chromatogram can be evaluated by video densitometry. The advantages of this technique are speed, easy and intuitive operation, and the fact that evaluation is done on “visible” chromatograms, unlike in scanning densitometry where the entire process takes place in a “black box.” However, because video technology functions only in the visible range, the UV region, which is exceptionally productive for planar chromatography, is only indirectly accessible through the use of a UV indicator embedded in the layer and in cases where samples fluoresce. In this respect, video technology parallels the human eye. The limitation of image processing to visible light is not caused by the video camera but

by the fact that no solution has yet been found for uniformly illuminating a plate with monochromatic light of a selected wavelength. Spectral selectivity, a strong point of the classical densitometer, is not accessible with a video system. The greater the absorbance of the analyte at or near the excitation maximum of the UV indicator (254 nm), the higher the sensitivity and accuracy of video quantification. In certain cases, it may even become comparable to that of classical densitometry. In the fluorescence mode, video and classical densitometry are comparable in respect to detection of emissions in the visible region caused by longwave UV light (366 nm) excitation. However, video technology lacks the variable-wave-length-excitation-based selectivity of classical densitometry.

B. Instruments for Scanning Densitometry*

Modern densitometers such as the CAMAG TLC Scanner 3 (Fig. 11), Desaga CD 60, and the CS9000 series of Shimadzu Corp., Tokyo, are slit-scanning, single-beam, single-wavelength instruments with powerful software for evaluation after scanning. They consist of an electronic part, a compartment for plate positioning, and the optical system, which is the most important feature of the scanner setup. Figure 12 illustrates the principal design of the TLC Scanner 3 (18). One of the three light sources—mercury vapor lamp, deuterium lamp, or tungsten halogen lamp—is positioned in the light path by motor drive. The deuterium and tungsten halogen lamps are continuum lamps, i.e., they emit light over a wide wavelength range. The deuterium lamp is used in the UV range of 190–400 nm, and the tungsten lamp in the visible region, i.e., 400–800 nm. The third, a high-pressure mercury vapor lamp, provides high energy at definite wavelengths. This lamp is used mainly for scanning by fluorescence, but it can also be used for absorption measurements if it offers an emission line at the wavelength needed.

* This section is based on Ref. 17.

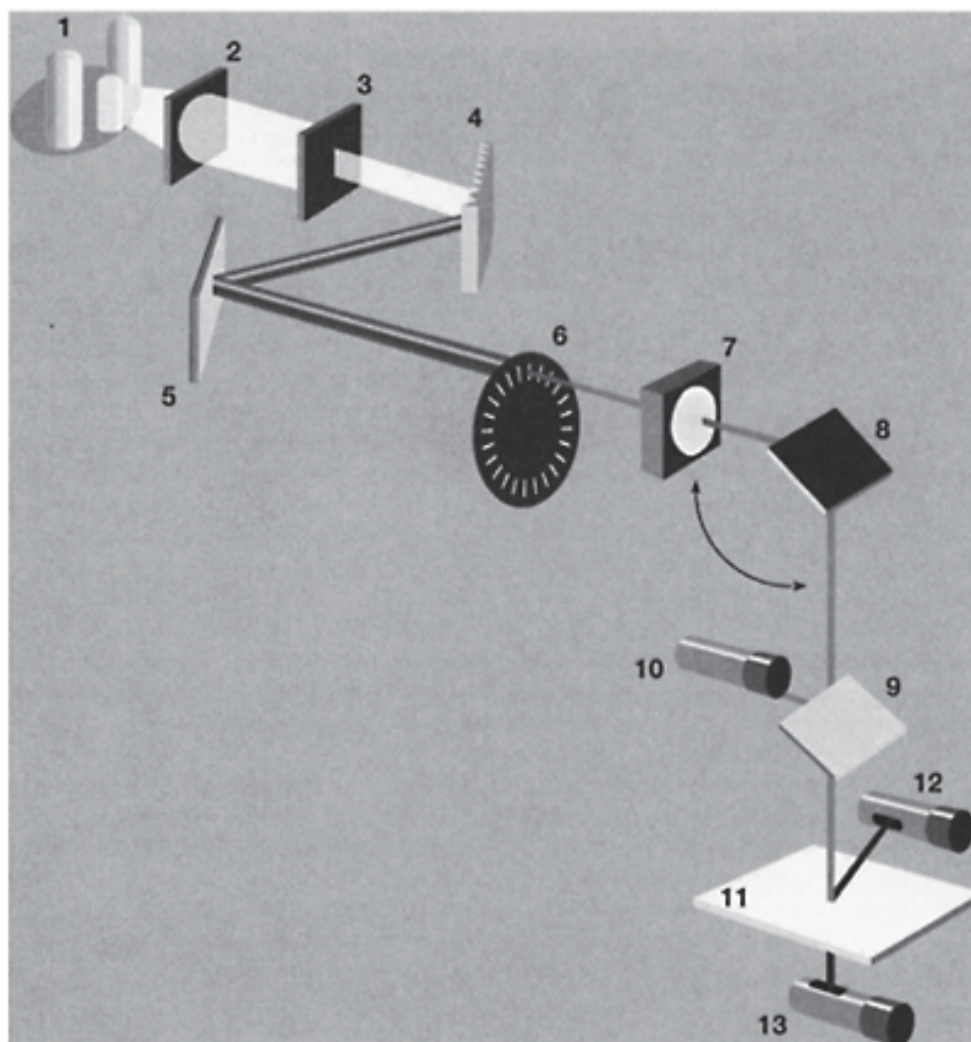


Figure 12 Light path diagram of the TLC Scanner 3. 1, Lamp selector; 2, entrance lens system; 3, monochromator entry slit; 4, monochromator grating; 5, mirror; 6, slit aperture disk; 7, lens system; 8, mirror; 9, beam splitter; 10, reference photomultiplier; 11, scanning object; 12, measuring photo-multiplier; 13, photodiode (transmission).

The emitted light passes through a lens system and the monochromator, i.e., a concave holographic grating that selects light of a certain bandwidth (5 or 20 nm). The light passes a revolving disk with 20 fixed slit apertures and then a lens system for positioning for micro and macro slit sizes. Thus, slit lengths of 0.5-12 mm and slit widths of 0.025–1.2 mm can be selected. Part of the light beam is directed to a reference photomultiplier

by a beam splitter to compensate for lamp aging and short-time fluctuations and to reduce the warm-up time required to reach lamp stabilization. The light beam of defined wavelength range, bandwidth, and slit size strikes the TLC plate at a right angle. The photomultiplier for reflectance scanning is aligned at an angle of 30° to the normal. For scanning in the transmission mode, a photodiode mounted below the object is used as the detector. This feature is useful for evaluation of electrophoresis gels.

Plates up to 20×20 cm are placed on a stage that is mechanically operated in the *x*- and *y*-directions. The scanning speed is variable to a maximum of 100 mm/s. The chromatogram has to be scanned in the direction of chromatographic development or against this direction; it should never be scanned perpendicular to the direction of chromatography (19). If a substance applied as a spot is scanned with a slit scanner, the slit length has to be larger than the diameter of the spot. Samples applied as bands may be scanned by the aliquot method. Instead of scanning a chromatogram track with a fixed slit, it is possible to have the light spot *zigzag* or meander over the sample zones, with the swing corresponding to the length of the slit. This feature, offered by the CD 60 densitometer and also by the CS 9000 series scanner, is claimed to correct chromatogram distortions. Disadvantages are the lower spatial resolution, particularly in the case of HPTLC layers, and unfavorable error propagation when sampling point data from different positions are averaged.

C. Video Densitometry

Video densitometry does not require any hardware. It is performed on digital images of the planar chromatogram with the help of a special software package. Software such as VideoScan (CAMAG) and ProResult (Desaga) is available as an option for video or digital TLC documentation systems. The software groups the pixels of the digital image according to the user-selected tracks of the chromatogram. Within these tracks, the average intensity on a 256-level gray scale of the pixels in each line is used to generate an analog curve of the chromatogram, which can be quantitatively evaluated after integration. The mathematical details of video densitometry are discussed in detail by Henkel (20).

VI. CHROMATOGRAM DOCUMENTATION

A. General Aspects

A unique advantage of TLC over all other chromatographic techniques is that in most cases the entire chromatogram is or can be made visible to the eye. Particularly following derivatization, the image of a TLC plate may contain a wealth of qualitative and semiquantitative information that can be easily communicated without requiring extensive description or tables of data. All samples on the plate can be viewed and compared simultaneously. During multiple detection (fluorescence quenching at 254 nm, fluorescence at 366 nm, and colors under white light following derivatization), several images of the same plate can be generated. Although this is one of the greatest assets of planar chromatography, it can be fully utilized only if properly documented. In the recent

past, photography was the most used documentation tool. Today, digital technology offers the advantage of immediate and, most of all, durable results, which are independent of film or paper quality and photographic laboratories.

B. Instrumentation

Modern video documentation systems such as CAMAG's Reprostar 3 with VideoStore and Desaga's VD 30 with ProViDOC feature a light box for illumination of the TLC plate under 254 nm, 366 nm, and white light; a high-resolution three CCD color video camera; and a digitizer that converts the analog signal of the camera into digital information. The documentation process is extremely rapid and intuitive and is fully compatible with GMP requirements.

The VideoStore 2 software, for example, operates with a configuration that includes all electronic camera settings and settings of the frame grabber (digitizer). Different configurations are used for different illumination modes. The information on the configuration is always stored as part of the image and can be printed as part of the image report, which also includes a computer-generated image ID, information about the user, and date and time of image capture. Raw data are stored in a secure file format (cpf) that cannot be manipulated. Video densitometry is performed in the same file format with the VideoScan software. For use with other software, images can be exported in various open formats (tif, bmp, jpg, etc.). Standardized configuration and mechanical settings (zoom, aperture, plate position) are required if reproducible images are to be obtained and plate-to-plate comparison of data is desired. The principal drawback of video systems is their price.

Currently, less expensive documentation systems based on high-resolution digital cameras (≥ 5 megapixel) are entering the market. With the availability of suitable GMP-compliant software for complete control of such cameras under reproducible conditions, video documentation will soon be replaced by digital methods.

VII. TLC SOFTWARE

A. General Aspects

Thin-layer chromatography is an off-line technique, i.e., the individual steps are separated in time and location. Therefore, traditional software has been developed to control the individual instruments designed to automate those steps. Although it was possible in some cases to generate data files that could be used with more than one instrument—software combination by the same manufacturer (for instance sample application and densitometry), a complete treatment of the information pertaining to an analysis was not possible until now.

B. winCATS—Planar Chromatography Manager

The winCATS method is a completely new concept for planar chromatography. One program communicates with all instruments involved in the TLC process via so-called EquiLinks. A winCATS method can include the following information:

- The stationary phase and its pretreatment
 - Samples and their components
 - Standards and their preparation, including calibration modes
 - All parameters concerned with manual or automatic sample application
 - Prechromatographic derivatization
 - All parameters concerned with development in a glass tank or automatic (multiple) development chamber
 - Postchromatographic derivatization
 - All parameters concerned with densitometric detection, including spectra recording, background subtraction, multiwavelength scanning, and track optimization
 - Qualitative and quantitative chromatogram evaluation, including single- and multilevel calibration and subcomponent analysis
 - Parameters concerned with documentation with a digital camera

The user can select the steps necessary for a particular analytical task. While a method is being executed, an analysis file is generated. Each step that is performed by an instrument is automatically recorded in the analysis log. After transferring the plate to the next instrument, for instance from the chamber to the scanner, the user is prompted to start the next step of the analysis. After completion of the analysis, a report is available that includes all information. For GMP compliance, all data are maintained in a secure format. All changes performed by the user, such as manual integration, are automatically recorded. winCATS runs under Windows 2000 and is ready for use in an environment that complies with U.S. FDA CFR 21 rule 11.

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6

Gradient Development in Thin-Layer Chromatography

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I. INTRODUCTION

The separation of multicomponent mixtures by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) under fixed experimental conditions is often complicated by large differences in the polarity of the various components. To deal with this problem, eluents of low strength are needed to separate the less strongly retained solutes, whereas the strongly retained components of the mixtures can be separated by eluents of high strength. This is referred to as the general elution problem (1), and in TLC it can be handled in various ways: gradient elution (stepwise or continuous), stationary-phase gradient, polyzonal TLC, or temperature programming. These various techniques are based on different band migration rates of the components of the mixture during the separation process.

Gradient development in liquid chromatography stands in contrast to isocratic elution, in which the conditions of separation are not changed throughout the time required for the sample separation. In gradient development the situation is different: The conditions of separation (mobile-phase concentration, composition of the adsorbent layer, temperature, etc.) are changed during the separation. These continuous or stepwise changes in the separation conditions lead to changes in the relative migration velocity of the components of a sample. For example, if the concentration of the stronger solvent in a binary mobile phase increases, the eluent strength and R_f values of all solutes are also increased. As a result, separate optimization of the R_f values of individual bands is possible.

Gradient development in TLC is a technique that allows one to improve the resolution of a given pair of adjacent bands, to accelerate a separation, to concentrate the sample band and lower the detection limit, and to speed up the search for an optimal chromatographic system.

Successful separations of many complex mixtures by HPLC gradient elution have demonstrated the utility of this technique (1–5). In contrast to HPLC, gradient development in TLC has been applied relatively rarely, owing to the rather complex devices required for the generation of reproducible gradients and the lack of a simple

theory of gradient development. Niederwieser and Honegger (6, 7) systematized many experimental results and outlined some theoretical problems.

Recently, gradient development in TLC has become more popular, as evidenced by papers on theory (6–15), devices for gradient development (16–23), and the preparative mode (24).

The purpose of this chapter is to acquaint the reader with the most popular gradient techniques in TLC, including their characteristics, advantages, and limitations.

A. History of Gradient Development in TLC

The idea of using gradient development in column chromatography is ascribed to work by Tiselius and coworkers in 1952 (25), but as early as 1949, Mitchell et al. (26) used salt and pH gradients for the separation of some enzymes.

Gradient elution was applied in TLC in 1962 by Wieland and Determan (27) and by Rybicka (28, 29). Wieland and Determan (27) used gradient elution to separate LDH isozymes and nucleotides on DEAE-Sephadex. Rybicka (28, 29) used gradient elution to separate glycerides and pentaerythritol esters. Later, Niederwieser and coworkers (6, 7, 30, 31) worked intensively to improve this technique.

Gradients in the stationary phase made slower progress, probably owing to the difficulties with devices for spreading the adsorbent layer. Berger et al. (32) used a modified spreader usually used for normal TLC. Later, improved devices for spreading layers were described by Stahl (33, 34) and Warren (35).

The use of a temperature gradient was introduced in 1961 by Liteanu and Gocan (36), whereas Turina et al. (37) described an adapter for evaporation of the solvent during development of a plate.

Geiss et al. (38, 39) and De Zeeuw (40) described a special chromatographic chamber for impregnation of adsorbent layer with vapors of various solvents. These resulted in the formation of an activity gradient of the adsorbent layer.

B. Nomenclature in Gradient Development

In TLC, in contrast with column chromatography, it is possible to apply a gradient in a direction other than the direction of flow of the eluent.

Niederwieser (31) introduced a rational system for full description of gradients. According to the definition given by that system (31), the arrow of gradient direction points to the chromatogram region where the sample components show their greatest mobility. In the case of an adsorbent gradient, the arrow points to the region of lowest activity. In the case of a mobile-phase gradient, the arrow points in the direction of greatest solvent strength.

Each separation process using a gradient development is based on a combination of two vectors that define the gradient direction and solvent flow direction (Fig. 1). When the gradient direction is congruent with the solvent flow direction, the gradient arrangement is termed parallel (p); in the reverse case, when the solvent flow direction is opposite to the gradient direction, the gradient arrangement is said to be antiparallel (ap). The stationary-phase gradient can exist either parallel to the solvent direction flow or at

right angles to the solvent flow. In the latter case, the term orthogonal (o) gradient is used.

Definitions of gradient directions (31) are illustrated in Fig. 1.

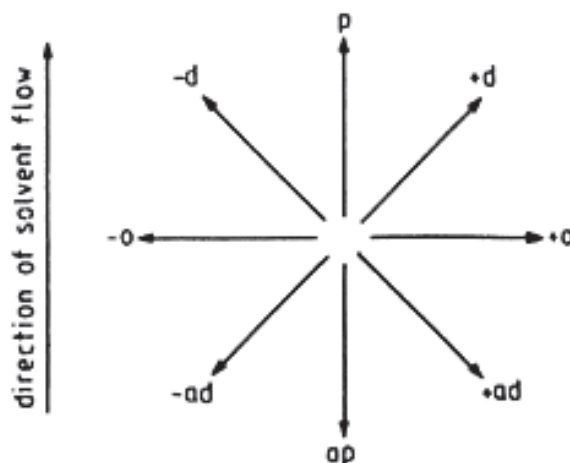


Figure 1 Nomenclature of gradient arrangement related to the direction of solvent flow. For definition of the gradient direction, see the text:
 p=parallel, d=diagonal, o=orthogonal,
 ad=antidiagonal, ap = antiparallel.
 (Reprinted from Ref. 31 with permission.)

C. Classification of Gradients According to Their Shape

According to Niederwieser's (31) definition, gradient TLC is "a chromatographic technique using within the separation area locally different separation conditions." Separation conditions can vary in both the stationary and mobile phases. Taking into account these variations, chromatographic gradient techniques can be classified (3) as follows:

Mobile-phase gradients

Composition

pH

Ionic strength

Stationary-phase gradients

Composition

Impregnation

Activity

Gradients connected with change

Temperature

Flow rate
Vapor pressure

The greatest possibilities of achieving gradients are offered by changing the mobile-phase concentration. Some examples of different shapes of gradients are presented in Fig. 2. The concentration of the more efficient solvent in the mobile phase can vary linearly (Figs. 2b and 2e) or curvilinearly (Figs. 2a, 2c, 2d, 2f). In practice, a continuous gradient is preferred (1, 4, 5), but stepwise gradients are much easier to obtain. It should be emphasized that if several steps are used in a stepwise gradient, then the gradient obtained is almost identical with a continuous gradient (41, 42).

II. APPARATUS FOR GRADIENT DEVELOPMENT

Which device is used for generating the gradient depends on the type of gradient desired. The greatest number of devices have been described for generating mobile-phase gradients. Some of the most typical devices are presented here; however, so far there is no single best one.

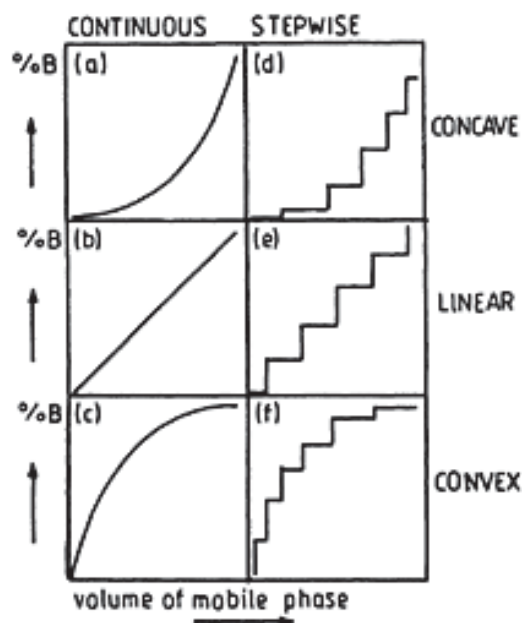


Figure 2 Classification of gradients according to their shape.

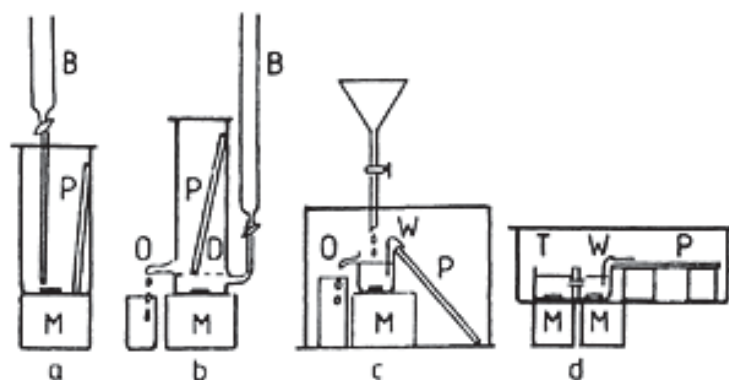


Figure 3 Devices for gradient elution in TLC. (Reprinted from Ref. 7 with permission.)

A. Devices for Achieving a Mobile-Phase Gradient

In gradient elution, devices for generating both continuous and stepwise gradients are used. Details related to the devices are described by Liteanu and Gocan (3) and by Niederwieser and Honegger (6). Some devices for generating continuous gradients are presented in Fig. 3.

Rybicka (28, 29) employed a normal separation chamber (Fig. 3a) equipped with a magnetic stirrer (M) and a buret (B) containing the stronger solvent. Wieland and Determan (27) used a glass cylinder divided by a filter plate into a 1 cm deep mixing chamber equipped with a magnetic stirrer and an upper separating chamber (Fig. 3b).

Luzatto and Okoye (45) used a descending chromatographic technique (Fig. 3c) and a paper wick (W) as a capillary bridge between the mixing chamber and the chromatographic plate.

In Strickland's (46) device (Fig. 3d), a polyethylene trough (T) is divided along its entire length into two equal compartments filled with different solvents and stirred by magnetic stirrers (M). The partition wall between the compartments has two holes through which solvents are able to mix. The eluent from the trough is delivered to the plate (P) by means of a filter paper strip (W).

The devices described (Fig. 3) have some disadvantages: They produce only one type of gradient profile (mostly a convex gradient, Fig. 2c), and they require magnetic mixing and a considerable excess of solvent.

The delivery of the solvent to the adsorbent layer should be determined by the migration rate of the eluent front; otherwise deformation of the gradient shape will occur (6).

Niederwieser and coworkers (7, 43, 44) described a system that allows free choice of gradient shapes, involves reproducible partial mixing of two neighboring solvents in a capillary tube, and requires only as much solvent as the adsorbent layer can absorb. Their device (Fig. 4) differs

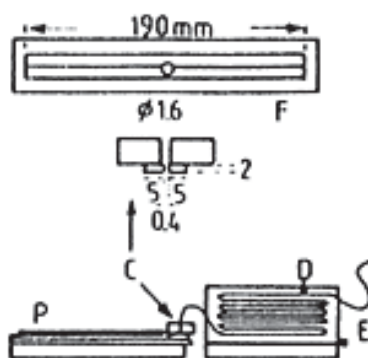


Figure 4 Device for solvent gradient TLC according to Niederwieser et al. (7). (Reprinted from Ref. 7 with permission.)

from the other devices in that a long PTFE capillary tube serves as an eluent reservoir. A PTFE capillary (D), with an inner diameter of approximately 1.5 mm and several meters long, is mounted wavelike on a table (E). The eluent fractions are sucked into the capillary tubing in reverse order. The device (3, 6, 7) basically consists of a chromatographic plate (P) (Fig. 4), covered with glass plate, the all-glass distributor (C), Teflon tubing (D), and the table (E).

Consecutive portions of the eluents, with increasing amounts of the more efficient solvent, are introduced and stored in a length of PTFE tubing. The outlet of the PTFE tubing is put into the distributor hole, and the eluent coming out of the tube is distributed along the lower edge of the adsorbent layer. The stepwise gradient thus obtained is analogous to a continuous gradient because the profile becomes diffuse in the development process.

Sander and Feld (16) used a liquid chromatograph (solvent programmer in conjunction with two pumps) to generate a mobile-phase gradient. The eluent was introduced into the developer trough and distributed across the layer.

Soczewiński and Matysik (21) proposed a simple device, without a magnetic mixer, coupled with a horizontal sandwich chamber. The device consists of two vessels with two solvents, which mix spontaneously owing to density differences and the formation of molecular complexes (e.g., chloroform-ethyl acetate). They also showed (22) that stepwise gradient elution can be easily performed in a sandwich chamber with a glass distributor (41, 47) (Fig. 5). Matysik and Soczewiński (23) also described a device that is a modification of the system introduced by Niederwieser and coworkers (7, 43, 44).

Burger (17) and Jaenchen (18, 19) described a fully automatic machine for multiple development of a plate. An elution gradient is employed in accordance with the gradient program (see also Chap. 5 in this Handbook).

Vajda et al. (20) applied a device originally used for overpressured layer chromatography (OPLC) to multiple step-gradient development. The modified OPLC equipment, with loops filled with the different solvents, can generate a stepwise gradient by switching solvents with a two-position, 10-port valve (for details, see Chap. 7 in this Handbook).

B. Devices for Achieving Stationary-Phase Gradients

Discontinuous gradients in the stationary phase can be conveniently produced using a normal TLC spreader. The spreader cylinder is divided into two (32) or more (31) compartments by the intro-

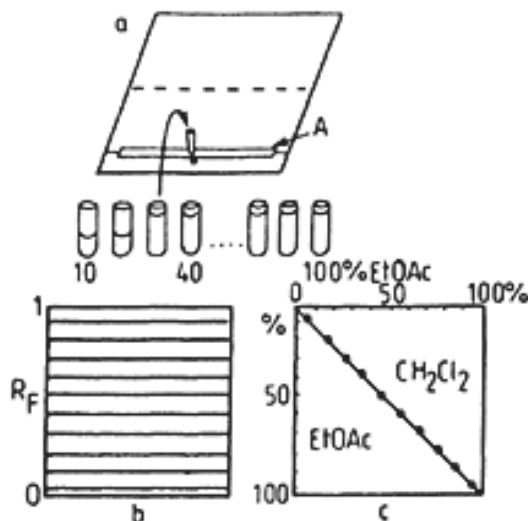


Figure 5 Stepwise gradient elution in a sandwich chamber with a glass distributor (A) of the eluent. (a) 0.4 mL portions of eluents of increasing solvent strength are introduced under the distributor and from the edge of the layer; (b) developed chromatogram with zones of the mobile phase and a stepwise profile of the gradient; (c) corresponding graphical representation of the (approximated) continuous gradient. (Reprinted from Ref. 22 with permission.)

duction of close-fitting pieces of PTFE. The compartments are filled simultaneously to equal height with suspensions of different adsorbents. The plates are coated in the usual way.

Impregnation gradients are usually obtained by immersing a chromatographic plate for a moment in a solution of the impregnation agent or by suspending the adsorbent in a solution of the impregnation agent and simultaneously spreading the different suspensions on the plate (3, 31).

Stahl (33, 34) described an apparatus for obtaining continuous stationary-phase gradients that maintained the basic construction principle of the normal spreader. A

rectangular case divided diagonally into two compartments by a partition wall is filled with two different adsorbent suspensions. When the sliding bottom of the case is opened, the suspensions fall into the spreader cylinder, which is divided into several small compartments, and mix in various proportions. After mixing of the compartments' contents, the plates are coated in the usual way (for details see Refs. 3, 6, 31, 33, and 34).

Activity gradients on adsorbent layers are very convenient (48, 49). The Vario-KS chamber permits preadsorption of vapors on the adsorbent layer, which is placed face down over a tray that contains various solvents. The removable tray consists of many rectangular troughs that can be filled with different solvents or humidity-controlling liquids (details are in Ref. 49). The eluent is in a separate trough and can be delivered to the adsorbent layer by a wick.

III. GRADIENT ELUTION

A. Polyzoal Thin-Layer Chromatography

Polyzoal TLC (6, 7) can be carried out only in a cooled sandwich chamber. Experience has shown that the phenomenon of solvent demixing can take place mainly in sandwich chambers. If a binary mobile phase migrates through an adsorbent layer, e.g., silica gel, the molecules of stronger solvent are preferentially adsorbed, resulting in demixing of the mobile phase. This effect is the basis of frontal analysis and polyzoal chromatography (6, 7). The demixing effect is more pronounced when a cooled sandwich chamber is used (for example, a Brenner-Niederwieser chamber). The demixing effect is also more pronounced if the components of the mobile phase differ strongly in eluent strength.

When the solvent molecules are selectively adsorbed during the separation process and solvent demixing occurs, the α zone, containing only the weak solvent, is formed. Behind the α zone, the β zone, containing in the stationary phase the molecules of the stronger solvent, is formed. The β zone is separated from its predecessor by the β front. Zone and front formation with a ternary mobile phase are illustrated in Fig. 6.

The migration rates of the fronts are different and can be expressed by the retardation factor

$$k_{\beta} = \frac{\text{Distance from immersion line to } \beta \text{ front}}{\text{Distance from immersion line to } \alpha \text{ front}}$$

The k_{β} factor for a given adsorbent and mixed eluent is a function of the concentrations of

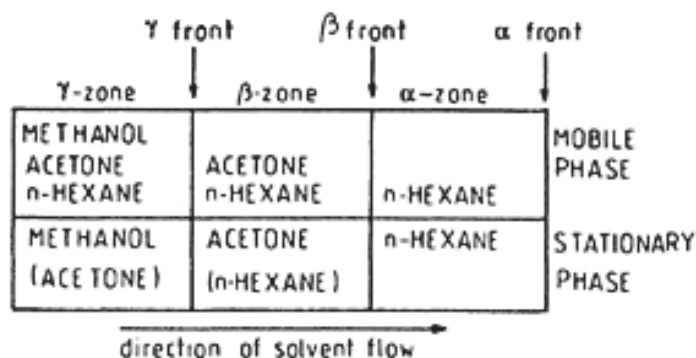


Figure 6 Phase formation with multicomponent solvents (polyzonal TLC) in an unsaturated sandwich chamber.

the individual components in the developing solvent; it increases with an increase in concentration of the stronger solvent. The height and steepness of the gradient and length of the zones depend on the nature and the concentration of the eluent components. [The gradient steepness for linear gradients can be expressed as the percent per minute change in the concentration of solvent B (Fig. 2) or, for nonlinear gradients, as the average percent per minute change in the concentration of solvent B.]

A developing solvent that contains n components will give n zones separated by $n-1$ fronts. In polyzonal TLC, it is of particular interest to vary the distance between the immersion line and the starting point of the mixture. This can be done by applying the mixture solution several times at different distances from the immersion line. Any changes in the mobile and stationary phases during chromatography influence the behavior of the solute, depending on the distance between the starting point and the immersion line. As can be seen in Fig. 7, the complete chromatographic separation of a complex mixture can often be conveniently carried out by the use of two or more different starting points. Spots 4 and 7 from the first starting point (first mixture from the left side) are not separated, although spots 8 and 9 are well separated. The situation is different for the second starting point: Spots 8 and 9 are not separated, in contrast to 4 and 7.

During the chromatographic process, molecules of each new solvent displace the solvent molecules of lower eluent strength and push the demixing front nearer to the α front. Generally, it is advisable to create conditions that allow these fronts to spread in equal proportions over the entire development distance (6).

The greater the differences between the components of the mixture to be separated, the greater must be the range of solvent strengths of the components of the eluent.

In general, mixtures in equimolar amounts of the lower representatives of any homologous series are frequently used. The following mixtures are useful (6):

Chlorinated hydrocarbons: carbon tetrachloride–chloroform–methylene chloride (96:80:64)

Ethers: diisopropyl ether–diethyl ether–dioxane (141:104:85)

Esters: *n*-butyl acetate–*n*-propyl acetate–ethyl acetate–methyl acetate (132:115:98:80)

Ketones: cyclohexanone–diethyl ketone–methyl ketone–acetone (103:106:90:73)

Alcohols: *n*-butanol–*n*-propanol–ethanol–methanol (92:75:58:40)

Polyzonal TLC with a multicomponent mobile phase represents the simplest technique for stepwise gradient elution. A continuous gradient can be realized only if the eluent contains a great number of different components with very small increments in solvent strength. However, because

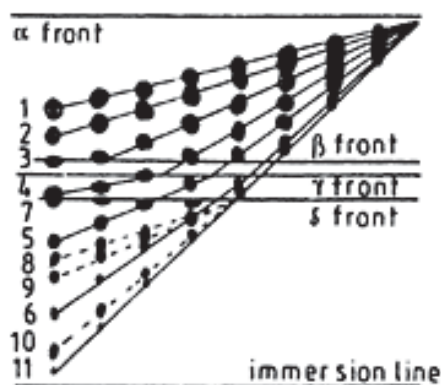


Figure 7 Polyzonal chromatogram of a mixture containing, in $0.5\ \mu\text{L}$, $1\ \mu\text{g}$ each of the 2,4-dinitrophenyl derivatives of the following amines and amino acids: (1) *n*-amylamine, (2) *n*-butylamine, (3) *n*-propylamine, (4) ethylamine, (5) methylamine, (6) tyramine, (7) leucine, (8) methionine, (9) proline, (10) hydroxyproline, and (11) glutamine, with separation starting from eight increasingly higher origins. Silica gel G (Merck), air-dried layer (relative atmospheric humidity, 50%), BN chamber, solvent isopropylether–propionic acid–acetic acid–formic acid (100:0.66:0.66:0.66). (Reprinted from Ref. 30 with permission.)

each solvent has a different elution effect, such a mixture can seldom be realized. For a general discussion of polyzonal TLC, see the review by Niederwieser and Honegger (6).

B. Mobile-Phase Gradient

Complex, multicomponent mixtures containing components with a wide range of R_f values ($0.01 \leq R_f \leq 0.9$) cannot be separated by isocratic elution owing to the general elution problem (1, 50). Eluents of low eluent strength separate the less strongly retained solutes, whereas the strongly retained components are eluted with very low R_f values. On the other hand, strong eluents do not separate weakly retained components, which migrate together and exist on a chromatogram as common or partly resolved spots.

The general elution problem (1) in HPLC is usually solved by application of gradient elution (1–5, 41, 42). The technique can also be applied in TLC (3, 6–16, 21–24, 28–31).

Usually we are concerned with two-component gradients composed of a weak solvent A and a strong solvent B. The concentration of the stronger solvent B can be varied linearly or curvilinearly (convex or concave, Fig. 2), from pure A to pure B (for details, see Ref. 50, pp. 668–686), so the concentration of B in the mobile phase entering the chromatographic plate increases throughout the separation. The eluent is initially weak and becomes progressively stronger as separation proceeds. In this case, the gradient applied is antiparallel.

It is well known that the sample R_f values depend on the concentration of the stronger solvent in a binary mobile phase, so in gradient elution variations in sample retention are achieved almost exclusively by changes in the mobile-phase concentration.

A stepwise gradient, which is more easily achieved in practice and easier to understand, is considered first. In most cases, the stepwise gradients are produced in sandwich chambers equipped with special solvent distributors (6, 7, 9–16, 21–24).

The space under the distributor, a strip of glass (1.3×5×95 mm for a 100×200 mm plate) placed over a margin of the carrier plate cleaned of adsorbent (see illustrations in Refs. 11, 22, and 51), is consecutively filled with up to 0.5 mL of the eluent. The first eluent fraction is, e.g., 10% ethyl acetate in chloroform, the second 20%, and the last is pure ethyl acetate. Each eluent fraction is introduced under the distributor with a micropipet after complete adsorption of the preceding fraction by the layer. If the difference between concentrations in two consecutive steps is relatively small and the gradient is partially smoothed during the separation process, the profile becomes approximately a continuous gradient (51). Five eluent fractions of increasing eluent strength are usually sufficient to avoid marked accumulation of spots on the front between two consecutive zones, as can occur in polyzonal TLC.

C. Optimization Strategy in Gradient Elution

Analysis of the distribution of the spots along a chromatogram enables the formulation of simple quantitative rules of gradient optimization for a particular gradient program. Some of the most important rules were given by Soczewinski (51).

1. Choice of Eluent Strength Range

Soczewinski (51) proposed the following series of solvents for use on silica gel [eluent strength, ε^0 values (52), in parentheses]: heptane (0.0), trichloroethylene, dichloromethane (0.32), diisopropyl ether (0.34), ethyl acetate (0.38), isopropanol.

The gradient program should start from weak solvent A, with which low R_f values are obtained for most components of the sample. With the second solvent B, most components should have high R_f values, and even the strongly retained components should have $R_f > 0$.

The eluent strength range can be chosen more accurately if the R_f values of the sample components in several mixtures of A and B are determined. A plot of R_f versus %B will guide the choice of the optimum range of the gradient. For instance, Fig. 8a shows that a gradient of 10–80% B should be suitable; the mixture in Fig. 8b (51) cannot be separated by a gradient of 10–80% B, because some of the components have R_f values that are too low, even with pure

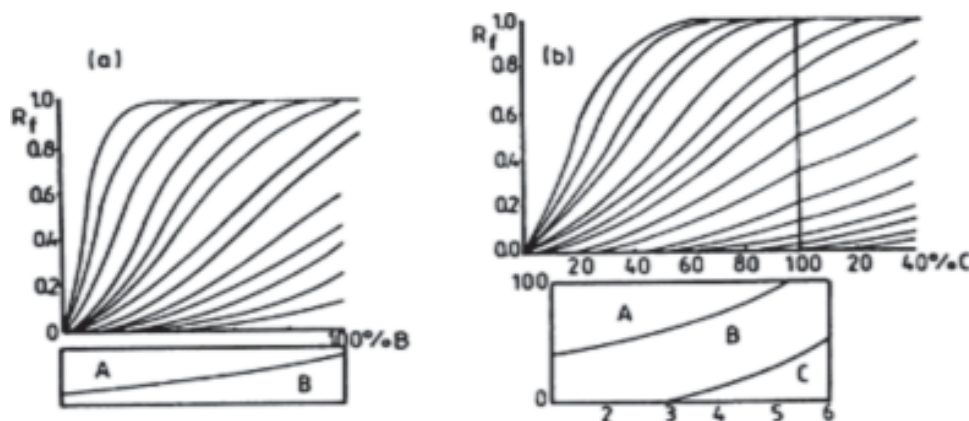


Figure 8 Examples of the relationships between R_f and the modified concentrations for multicomponent samples and the corresponding profiles required for their separation ($\varepsilon_A^0 < \varepsilon_B^0 < \varepsilon_C^0$). (Reprinted from Ref. 51 with permission.)

solvent B. In this case, it is necessary to use a wider eluent strength range by using a three-component mixture, A + B + C ($\varepsilon_A^0 < \varepsilon_B^0 < \varepsilon_C^0$).

2. Gradient Elution and Correction of Gradient Program

With a good gradient elution program, no sample component moves with the solvent front or remains at the starting point.

The gradient program chosen from the preliminary experiments may require correction of eluent strength range and profile. Comparison of the gradient program and the resulting chromatogram (Figs. 9 and 10) shows that changes in gradient shape are required. Two examples of the correction of gradient profiles are given in Figs. 9 and 10.

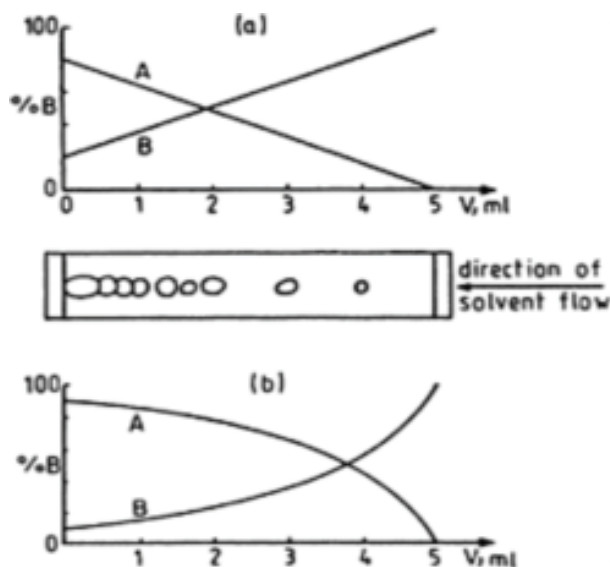


Figure 9 Adjustment of the gradient profile to improve the distribution of spots along a chromatogram (see text).

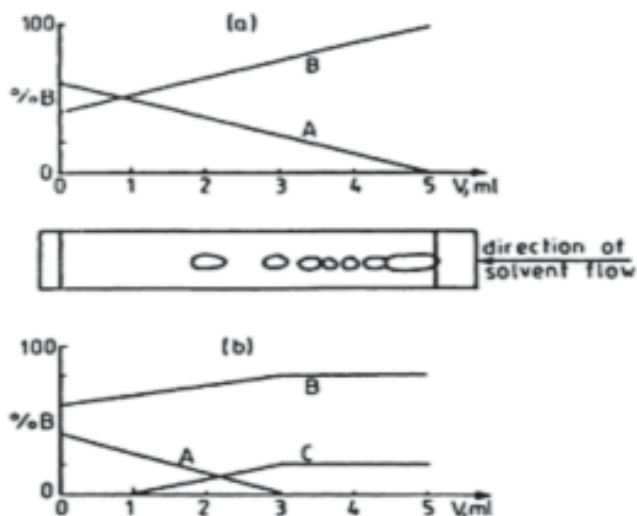


Figure 10 Adjustment of the gradient profile to improve the distribution of spots along a chromatogram (see text).

1. For the linear gradient from, for example, 20% ethyl acetate in methylene chloride to 100% ethyl acetate (Fig. 9a), most of the spots accumulate in the upper part of the

chromatogram. This means that the initial concentration of B and the range of eluent strength were too high. Suggested changes in the gradient profile and initial concentration of B are illustrated in Fig. 9b. Changing the shape of the gradient from linear to concave and lowering the initial concentration of ethyl acetate to 10% should improve the distribution of spots along the plate.

2. Most spots on the chromatogram presented in Fig. 10 accumulate in the lower part. Suggested changes include the use of a stronger solvent C in a mixture of A and B, or a ternary gradient, as shown in Fig. 10b.

For other examples, see Ref. 51.

It should be emphasized that the high efficiency of gradient elution is caused by flattening of the spots due to varying eluent strength and mutual displacement of the sample components. In many cases, it is possible to detect about double the number of spots relative to isocratic elution. It is also possible to vary the R_f values in a poorly separated region of the chromatogram without changing those in the remaining part (51).

The same rules can be applied to continuous gradients, but in this case the situation is more complex. Continuous gradients provide better separation of complex samples, but their applications are relatively scarce because rather complex devices are required to generate reproducible gradients. In many devices, a mixer is used and excess overflowing eluent is discarded, so the user cannot know which section of the elution gradient is responsible for the separation of which fractions.

D. Stationary-Phase Gradient

A stationary-phase gradient in TLC involves a continuous or discontinuous change in the composition or activity of the adsorbent layer along the plate (8). A gradient of the stationary phase can be applied either parallel to the direction of solvent flow or at right angles to it (orthogonal gradient). The latter gradient is equivalent to using several different plates of varying adsorbent composition in searching for the best TLC system.

As was shown in Section II.A, adsorbent gradients can be achieved in several different ways. For example, a strong adsorbent (e.g., silica gel) is mixed with varying proportions of a weak adsorbent (e.g., kieselguhr). As a result, an adsorbent gradient is formed along the plate. In fact, gradients composed of silica gel and kieselguhr have not fulfilled expectations. Greater dilution of the silica gel with kieselguhr (or other adsorbent of low surface area) results in reduced capacity and overloading of the initial part of the plate (31).

Layers containing a discontinuous adsorbent gradient usually consist of a narrow zone of adsorbent A along the lower edge of the plate and an adsorbent B on the remaining part of the plate [layers with five zones of different adsorbents were also proposed (53)]. Discontinuous adsorbent gradients are used for three purposes:

1. To adsorb some interfering components of the sample at the starting point (31, 32).
The adsorbent in zone A strongly retains the unwelcome substances, e.g., an ion-exchange of complexing mechanism, but it does not retain the rest of the components of a mixture.
2. To carry out two-dimensional TLC. In the first direction, isocratic TLC occurs along the zone of adsorbent A. In the second direction, prefractionated sample components

enter the layer of adsorbent B, which differs as much as possible from adsorbent A, for example, in pH or the presence of a complexing agent (31).

3. To concentrate the spot applied on a narrow preconcentration zone of a very weak adsorbent (e.g., kieselguhr). During development by an eluent, the spot is concentrated into a narrow band because the solvent strength is too high for such a weak adsorbent.

Many examples of continuous and discontinuous adsorbent gradients applied in practice are given by Niederwieser (31) and by Liteanu and Gocan (3).

The adsorbent layer can also be exposed to solvent vapors in special sandwich-type chambers that permit various solvent vapors to contact different parts of the plate, resulting in an adsorbent activity gradient along the plate. This technique is called preloading (43) or vapor-programmed gradient TLC (40).

If the chromatographic plate is exposed to the vapors of a strong solvent such as acetone, the adsorbent layer is highly deactivated and high R_f values are obtained. The opposite effect would occur for a weak solvent such as hexane. A vapor-programmed gradient can also be applied either parallel to the direction of solvent flow or at right angles to it (for details, see Ref. 49). This method of gradient generation is relatively simple. However, the actual composition of the adsorbent layer and the gradient shape are virtually unknown.

E. Automated Multiple Development

Perry et al. (54, 55) introduced in 1973 a new technique called programmed multiple development (PMD), in which the TLC plate was repeatedly developed in the same direction with the same solvent. Burger (17) improved this technique but maintained the general principles of PMD. The Burger (17) method is called automated multiple development (AMD). The characteristics of the AMD system are as follows (17–19):

1. A TLC plate is repeatedly developed in the same direction with solvents that differ from one step to the next.
2. Each developing step is longer than the previous one (approximately 3 mm per step).
3. From step to step, the solvent strength is decreased.
4. Gradient elution is used, but, in contrast to HPLC, the gradient starts with the most polar solvent (usually a mixture of methanol and dichloromethane, 50:50) and ends with the weakest solvent (e.g., a mixture of dichloromethane and *n*-hexane).
5. Solvent is completely removed from the plate after each developing step so that the composition of the solvent introduced in the next step is not changed.
6. From 10 to 25 steps are necessary to develop a plate, which corresponds to a total developing time of 0.5–3 h and a total migration distance of 3–10 cm.

A typical gradient in AMD usually consists of three or four solvents: methanol, acetonitrile, dichloromethane, and hexane.

In AMD, the chromatogram is developed under reproducible conditions so the user can compare it or its densitometric scanning curve with the profile of a elution gradient. This is demonstrated in Fig. 11 (19). Such a diagram allows the user to conclude which part of the gradient is ineffective and can be omitted (e.g., steps 1–18 for sample d) and which part should be modified. The samples of PTH amino acids (a), analgesics (b), and barbiturates (e) are resolved sufficiently, but some corrections of the gradient profile and

eluent strength are necessary. Using the methanol-dichloromethane gradient over the full length of all 25 steps would probably improve the separation (19).

The dye mixture (Fig. 11d) migrates through 18 steps as a narrow band and begins to resolve when the hexane-dichloromethane gradient starts, so the first 18 steps should be omitted and a new experiment started with the dichloromethane-hexane gradient.

For mixtures of wide polarity differences, such as the pesticides (56), amino acid derivatives (57), alkaloids (58), or drugs (59), multiple development becomes the obvious choice. It enables the convenient stepwise application of solvent gradients for optimization of the separation of each group of compounds that migrate in a given solvent. Universal (60) solvent gradients are generated in a stepwise fashion, with as many solvents as required being employed to achieve the desired separation.

Universal AMD gradients (60) have found wide application, particularly for the analysis of crop protection agents in surface water (61–63), plant extracts (64), psychopharmaceutical drugs (65), and steroids (66). It was shown in many papers (61, 63, 67, 68) that automation of the multiple development procedure increased the reliability and reproducibility of the method while minimizing operator time and errors.

AMD gradient elution was used for quantitative determination of eight pesticide residues (69) in soil that was considerably contaminated with petroleum derivatives. The excess of the petroleum derivatives was removed by solid-phase extraction. Another application of AMD gradients (70) was for the analysis of pesticide residues in drinking water. This method, elaborated for identification and quantification of active ingredients of plant-protecting agents in drinking and mineral water, has been accepted as standard in Germany.

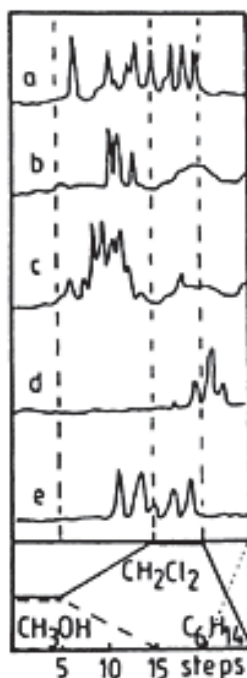


Figure 11 An application of the AMD technique. The denstometric scanning curve is superimposed on the diagram

of the gradient profile. (Reprinted from
Ref. 19 with permission.)

A 25-step gradient based on methanol, diethyl ether, and hexane was used to separate the six major human plantar stratum corneum lipids (71). Peak heights as well as peak areas were used for densitometric quantification of separated lipids.

AMD-HPTLC gradient development enabled the separation and quantification of forskolin and its 10 derivatives (72). These diterpenoids have interesting pharmacological properties.

Multistep gradient elution can also be carried out with modified overpressured TLC equipment (20, 74), described in Chapter 7 of this Handbook. Vajda et al. (20) applied the method to the analysis of the components of total lipid extracts from various human blood samples. Pick (74) used it for the chromatographic separation of membrane gangliosides. The advantage of the procedure consists in the removal of less polar solutes in the first stages of the gradient and separation of the polar gangliosides in the last stages.

IV. OPTIMIZATION OF STEPWISE GRADIENT ELUTION

A. Graphical Method

Consider the elution of a given solute by a two-component mobile phase on a chromatographic plate during stepwise gradient elution (10, 12). The length of the plate is assumed to be unity. The composition of the binary mobile phase is defined in terms of the concentration of the stronger solvent. It is assumed that the composition of the mobile phase changes gradually during elution but is constant in each step. The elution model is presented in Fig. 12 (12).

Assuming a constant mobile-phase flow rate, the straight line $0Y$ shows the migration of the mobile-phase front. The migration rate of compound A is lower than that of the mobile phase, and after one dead volume of eluent has passed through the bed, the R_f value of compound A is 0.2 (point A in Fig. 12). When the front of the mobile phase of 5% concentration reaches the end of the plate ($R_f=1.0$), the concentration of the eluent is changed stepwise. The solvent front is observed by means of a marker (azulene or azobenzene) whose R_f value in the solvent system is close to unity. The line $0'Y'$ in Fig. 12 indicates the migration of the mobile phase of 10% concentration. Obviously, the front of 10% mobile phase will, after some time, overtake spot A , which traveled until then in the mobile phase of 5% concentration (section AA'). From point A' onward, the spot travels in the mobile phase of 10% concentration. It is assumed that the R_f value for compound A in the mobile phase of 10% concentration is 0.3. To find point B , a length $A'C$ corresponding to one dead volume V_m is marked, and a section equal to $0.3 R_f$ unit from point C is measured. Upon connecting points A' , B , B' , the migration of the spot A in the 10% mobile phase and the final R_f value are obtained.

If the R_f values obtained in several isocratic elution steps are known, the program for gradient elution can be constructed (11, 12). Results of stepwise gradient elution of DABS-amino acids are

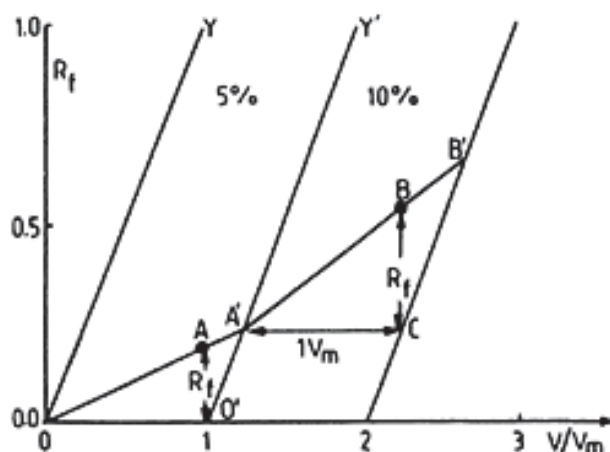


Figure 12 Graphical representation of the movement of sample A during stepwise gradient elution. (Reprinted from Ref. 12 with permission.)

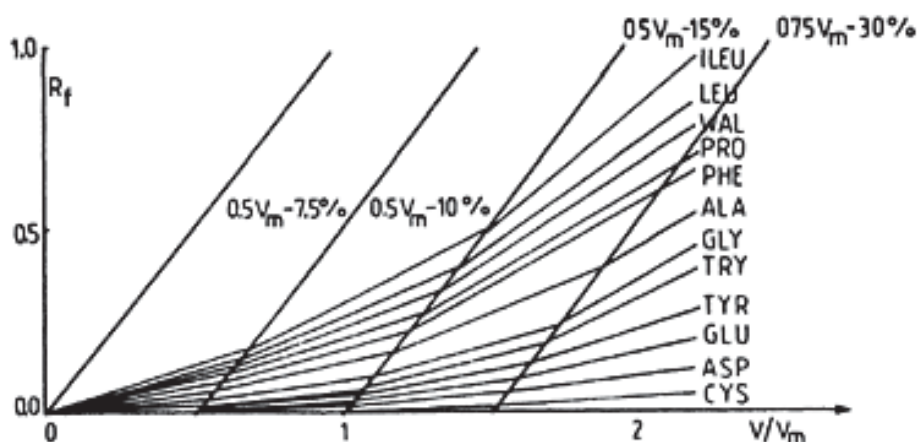


Figure 13 Results of thin-layer chromatography of DABS-amino acid derivatives. (Reprinted from Ref. 12 with permission.)

presented in Fig. 13 (12). The solvent concentration for the first step was chosen from the plot of R_f versus percent of the more efficient solvent (it is still better in normal-phase TLC to use R_m versus log % of the more polar solvent) by assuming that for the first eluted compound the R_f value should be equal to 0.25. In fact, half of the dead volume V_m of the eluent was used, so the R_f value in the first step is equal to $0.25/2=0.125$ (see Fig. 13). Knowing the concentration in which the R_f for the first compound is equal to 0.25, the R_f values for the rest of the compounds were obtained in the same way from a plot of R_f versus %B. In the next steps, $0.5 V_m$ of 10% and $0.5 V_m$ of 15% concentrations were used (12).

If experimental conditions in isocratic and gradient elution are comparable (constant flow rate, temperature, thickness of layer, etc.), the R_f values for gradient elution determined graphically from $R_f=f(\% \text{ concentration})$, or better, $R_m=f(\log \%)$, and also experimentally differ by not more than 0.01–0.03 R_f unit (12). Both the shape of the gradient and the number of dead volumes of the eluents required to ensure that the final R_f values of compounds do not exceed $R_f=1.0$ can be determined. This is particularly important in the separation of colorless compounds.

B. Numerical Method

1. Stepwise Gradient Elution

All recent gradient theories are based on the linear relationship, obtained under isocratic conditions, between $\log k$ (or $R_m=\log 1-R_f/R_f$ in TLC) values and the logarithm of the molar fraction of the more efficient solvent in the binary eluent (in normal-phase chromatography) and between $\log k$ (or R_m in TLC) and the volume fraction of the organic solvent (e.g., methanol or acetonitrile) in an aqueous–organic eluent in reversed-phase chromatography (1, 4, 5, 9, 41, 42).

Soczewinski and coworkers (13, 75) derived an equation for the R_f values of solute chromatographed under stepwise gradient elution. Assuming a definite relationship between the k value and the modifier concentration, the final R_f values of solute j (considering that the last, h th, development step is incomplete) is

$$R'_f = \sum_{i=1}^{h-1} \frac{V_{(i)} R_{f(j,i)}}{1 - R_{f(j,i)}} + R_{f(j,h)} \left[1 - \sum_{i=1}^{h-1} X_{(j,i)} \right] \quad \text{for } h = 1, 2, 3, \dots \quad (1)$$

where

j =the number of the solute (the code)

i =the sequential number of the elution step (eluent fraction)

h =the number of the last step (in which the solute migrates through part of the concentration zone)

$R_{f(j,i)}$ =the R_f value of the solute (isocratic value) in the i th concentration zone

$V_{(j,i)}$ =the volume of eluent introduced in the i th step expressed as a fraction of total eluent volume used in the gradient elution

$X_{(j,i)}$ =the volume of mobile phase corresponding to the migration of solute j through the i th concentration zone

$r_{(j,i)}$ =the fractional distance traveled by solute in the i th step

The volume $X_{(j,i)}$ of mobile phase for sample j in the i th step can be calculated from the equation

$$X_{(j,i)} = \frac{V_{(i)}}{1 - R_{f(j,i)}} \quad (2)$$

As an example of the application of the present method, consider the stepwise gradient elution of a hypothetical sample j . R_f values of solute j in the mobile phase of different concentrations (fraction volume) are as follows:

Volume fraction of solvent B in eluent	0.05	0.1	0.2	0.3	0.4

R_f value	0.09	0.12	0.27	0.48	0.62

Assume that a five-step gradient with equal volumes of mobile phase in each step will be applied, so that $v=0.2$ (one-fifth of the total volume of solvent used for gradient elution). The concentrations expressed as volume fractions in subsequent steps are 0.05, 0.1, 0.2, 0.3, 0.4.

The volume X of mobile phase for sample j in the first step of gradient elution can be calculated by using Eq. 2:

$$X_{(j,1)} = \frac{0.2}{1 - 0.09} = 0.22$$

The volume X in the second step is

$$X_{(j,2)} = \frac{0.2}{1 - 0.12} = 0.23$$

The volume X for the next two steps is

$$X_{(j,3)}=0.27 \text{ and } X_{(j,4)}=0.38$$

The sum of the fractional volumes X is

$$X_{(j,1)}+X_{(j,2)}+X_{(j,3)}+X_{(j,4)}=1.1$$

This is greater than 1.0, which means that solute j migrates through three concentration zones and partly into the fourth zone.

Knowing the R_f value of solute j under isocratic conditions, the value of the fractional distance $r_{(j,i)}$ can be calculated with the help of Eq. 1 (neglecting the second term) as

$$r_{(j,i)} = \frac{V_{(i)} R_{f(j,i)}}{1 - R_{f(j,i)}}$$

Then the fractional distance $r_{(j,1)}$ and $r_{(j,2)}$ values in the first and second steps are

$$r_{(j,1)} = \frac{0.2 \times 0.09}{1 - 0.09} = 0.02 \quad \text{and} \quad r_{(j,2)} = \frac{0.2 \times 0.12}{1 - 0.12} = 0.03$$

and for the third step, $r_{(j,3)}=0.07$.

Now the final R_f value can be calculated for solute j during a four-step gradient:

$$R_f = (0.02+0.03+0.07)+0.48(1-0.22-0.23-0.27)=0.25$$

Markowski et al. (75) elaborated a microcomputer program for the calculation of final R_f values obtained under stepwise gradient conditions. After introduction of R_f values of the sample components obtained for several isocratic runs, the microcomputer calculates R_f values for any gradient program and displays the paths of migration of the spots through the concentration zones. It is thus possible to study by computer simulation the final arrangement of spots for chosen programs of stepwise gradients.

2. Automated Multiple Development

Optimization of gradients in automated multiple development (AMD) can be achieved in three steps:

1. Selection of the “base” solvent (i.e., medium polarity) and at least two modifiers (very polar and nonpolar solvents)
2. Improvement of the separation by development of a final gradient with the appropriate range of eluotropic strengths of the solvent mixtures
3. Development of a suitable slope of a gradient (i.e., rate of change of the eluotropic strength with time)

Solvents with the selectivity necessary for the separation of the mixture are usually selected (57, 58) with the help of the PRISMA model, based on Snyder and Kirkland's (76) solvent selectivity scheme. Selection of the correct base solvent from the different - Snyder classes turned out to be critical to the optimization of selectivity.

The eluotropic strength of the binary solvent mixtures can be calculated using Snyder's equation (77).

When the individual components of the mixture to be analyzed are available, preliminary experiments based on isocratic development may be useful for selection of suitable solvents. The preliminary investigation may be performed as follows (56). The retention behavior of high and medium polarity standards in binary mixtures of strong and “base” solvent is carried out to determine the solvent strength range of the AMD gradient. Successive investigations using binary mixtures composed of the base solvent and (usually) hexane are carried out to optimize the separation of low polarity standards. The isocratic data obtained for different concentrations of binary mixtures are conveniently plotted as the relationship between R_m and solvent composition (9–12). Inspection of these plots gives useful information about the adequate solvent strength and the change in selectivity resulting from the change of base solvent and modifiers.

If the polarity range of an AMD gradient is such that insufficient resolution is obtained, the separation might be optimized by changing the gradient slope. Queckenberg and Frahm (58) stated that, in general, steeper gradients improve peak shape but reduce the resolution, whereas flatter gradients generate broader but better separated peaks.

Two gradient profiles are recommended: universal (56, 61) and linear (58, 59). Some authors (58, 59) prefer a linear gradient because abrupt changes in eluotropic strength occur within the universal gradient (59), and some components of a complex mixture might coelute. The concentration of mobile phase at which the coelution occurred corresponded to an abrupt change in the eluotropic strength, thus explaining the results observed (59).

The optimization procedure is frequently carried out by the trial-and-error method (56–60, 67) owing to the lack of a theoretical model of the multiple development process. Markowski and Soczewinski (78, 79) formulated the physical model for AMD, which is useful for describing the migration of the solute zones and computer analysis of various parameters determining the final optimization of gradient.

Let us consider two-step gradient development (80). After a first development to the distance z_1 , the R_f of the solute is equal to

$$y_1 = z_1 R_{f1}$$

where R_{f1} is the R_f value for the first eluent. The chromatogram is now dried and developed to distance z_2 with the second eluent, for which the solute R_f is equal to R_{f2} . However, the spot does not move until the solvent front overtakes it; thus, the real solute migration distance in the second step is $z_2 - z_1 R_{f1}$. The final R_{fg} value for the two steps of gradient is

$$R_{fg} = \underbrace{z_1 R_{f1}}_{\text{first development}} + \underbrace{(z_2 - z_1 R_{f1}) R_{f2}}_{\text{second development}}$$

Generalizing the situation for an n -step gradient, we can write

$$R_{fg} = \sum_{i=1}^{h=(n-1)} y_i + y_n = \sum_{i=1}^{h=(n-1)} y_i + \left(z_n - \sum_{i=1}^{h=(n-1)} y_i \right) R_{fn}$$

where R_{fg} is the final R_f value after the n -step gradient, $\sum_{i=1}^{h=(n-1)} y_i$ is the sum of the preceding fractional migration distances, y_n is the real R_f value in the last step, z_n is the development distance in the last step, and R_{fn} is the isocratic R_f value of the solute for the solvent used in the last step.

A computer program for the calculation of the final R_{fg} value, taking into account the development distances z_i , compositions of consecutive eluents, and the retention—modifier concentration relationship, was elaborated by Markowski (79).

V. GRADIENT ELUTION IN ANALYTICAL AND PREPARATIVE TLC

As demonstrated in many papers (23, 81–83), much better separation efficiency is obtained for stepwise gradient elution than for continuous elution, especially in the case of plant extracts, owing to enhanced displacement effects.

Matysik and Jusiak (82) used stepwise gradient development for the separation of chelidonium alkaloids in waste industrial fractions. Binary (toulene-methanol) and ternary (toulene—ethyl acetate—methanol) mobile phases were used, and a six-step program was performed. Eight-step stepwise gradient elution was also used for separation of glycosides from *Digitalis* species (83).

Ergot alkaloids (84) and coumarin derivatives (85) were separated on TLC silica plates by using stepwise gradients with different solvents. Stepwise gradients have also been used to separate anthocyanins (86) in the petals of red poppy, furocoumarins (87), and anthraquinones (88).

Marked improvement of the separation of two plant extracts by the use of a modified program of stepwise multiple gradient development was reported (89). Modification lies in the fact that the chromatographic plate was developed over decreasing distances with eluents of increasing eluent strength.

Gradient development combined with densitometry is an efficient method for the analysis of plant extracts, because it eliminates preliminary purification of extract. Examples of such a procedure are presented in some papers, e.g., perstilbene (3, 5-dimethoxy-2-hydroxy-*E*-stilbene) was satisfactorily separated by use of two-step gradient

elution and quantified by densitometric techniques (90). In another work (91), plant extracts containing flavonoids were separated on HPTLC silica plates by two- and three-step gradient elution.

An HPTLC method with densitometric detection was used to determine the convallatoxine content of extracts from flowers, leaves, and underground parts of *Herba convallariae* (92). Plant extracts were separated on HPTLC silica plates by multiple gradient development.

Mycotoxins such as alternariol and alternariol methyl ether, produced by fungi of the genus *Alternaria*, were analyzed by stepwise gradient TLC (93). The obtained chromatograms were well suited for quantitative densitometric determination.

Two-step gradient elution was applied to separate the colored pigments of *Trichoderma har-zianum* fermentation broth (94). The main fractions were identified by instrumental methods (IR, DAD detector, and MS) after gradient reversed-phase TLC. Additionally, multistep gradient elution developed for RP-TLC was successfully used as a pilot method for the rational design of a gradient elution program in RP-HPLC.

Fluorescein, the active component in the French preparation "fluoresceine," was quantitatively determined after gradient HPTLC development (95). Gradient mobile-phase TLC was also applied to the quantitative determination of prednisolone acetate in a Polish preparation "prednisolon" and in the aqueous humor of rabbit eyes (96).

Gradient development has occasionally been employed in preparative TLC chromatography. Soczewinski and coworkers (24, 97) applied an equilibrium sandwich chamber (47) for systematic investigations of the formation of zones and separation selectivity in overloaded preparative liquid chromatography.

The sample solution band (test dye mixture), applied from the edge of the layer, formed a partly separated starting zone (frontal chromatography stage). After adsorption of the sample by the adsorbent layer, the eluent was introduced under the solvent distributor, and the marker (azo-benzene) was spotted. The movements of the marker and the dye zones were recorded on a transparent foil (97). By connecting the points representing the upper and lower boundaries of the zones, a dynamic picture of the movement and separation of the zones could be obtained.

Stepwise gradient elution has been applied to the overloaded zonal preparative TLC of complex, multicomponent plant extracts of the herbal medicines azulan and hemorigen (98) used in therapy. Stepwise gradient elution combined with application of extract from the edge of the layer markedly improved the separation efficiency and purity of fractions, which was revealed by densitometry.

Theoretical and practical problems related to computer-aided optimization of Stepwise gradient development in TLC of plant extracts containing biologically active compounds were reviewed by Matysik and Soczewiński(99).

Figure 14 (24) illustrates the separation of a dye sample during (a) isocratic and (b) Stepwise gradient elution. It can be seen that full separation is obtained only for gradient elution; in isocratic elution, zones of dyes 3 and 4 overlap.

In the case of a Stepwise gradient, the zones, instead of spreading, become narrower and more compact. In consequence, the sample capacity is markedly higher. The improvement of separation in preparative Stepwise gradient elution is caused by two mechanisms: mutual displacement of the components of the mixture to be separated and compression of the zones, described earlier for continuous gradients in HPLC (1, 4, 5).

The compression of the zones results from the fact that the lower edge of a zone is overtaken by the mobile-phase fronts of increasing eluent strength earlier than the upper edge, so that the upper edge of the zone moves in the mobile phase of a lower eluent strength than the lower edge.

VI. CONCLUSIONS

Gradient development can be applied for the following purposes:

Separation of samples that contain many compounds with widely different retention values

Lowering of the detection limit by sharpening of the chromatographic zones

Speeding up the search for a better chromatographic system

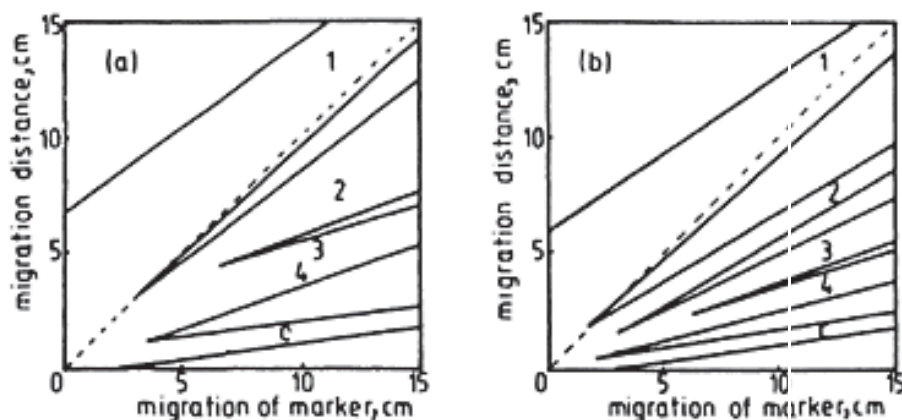


Figure 14 Dynamic representation of the migration of the bands of four test dyes. Sample: 1.5 mL of a 0.4% solution of 4-chlorobenzene-1-azo-1,4(*N,N*)-dimethylaminobenzene (1); disperse blue-Polanildunkelblau 3RT (2); disperse red-Polanilrubid FL (3); and disperse red-Polanilscharlach RP (4); c, contamination of No. 4. The dashed line represents the migration of the marker, azobenzene. (a) Isocratic elution with 30% ethyl acetate in trichloroethylene. (b) Five-step gradient elution, 10–20–30–40–50% of ethyl acetate in trichloroethylene.

(Reprinted from Ref. 24 with permission.)

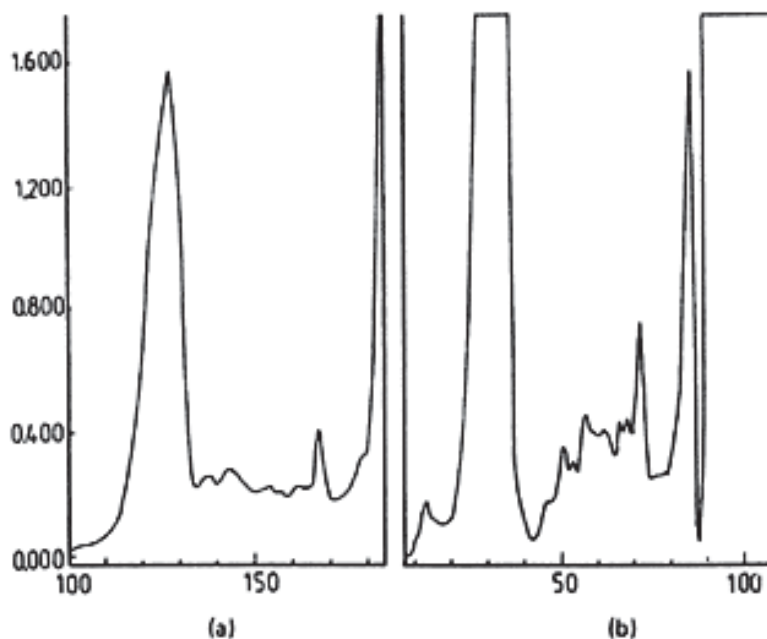


Figure 15 Densitograms (Shimadzu CS-930, 254 nm) of Sebores (plant drug), (a) Isocratic elution, ethyl acetate-chloroform (1:1); (b) stepwise gradient (10–20–30–40–50–70% ethyl acetate in chloroform). (Reprinted from Ref. 100 with permission.)

Increasing the loading capacity of the sample in preparative TLC

Separation of less strongly retained ballast components of the sample in the first gradient steps and chromatographic analysis of the remaining polar compounds in the last steps (69)

It should be noted that not every gradient arrangement is useful in practice. It has been shown (31) that the resolution of neighboring zones is better for antiparallel gradients than for parallel gradients. On the other hand, results of theoretical treatment (8) suggest that the four examined gradient TLC techniques can be arranged in the following order of decreasing resolution: adsorbent gradient layer (best), gradient elution TLC, polyzonal TLC, and vapor-programmed TLC (worst).

In most cases the optimum gradient profile is determined experimentally, but it is always possible to determine the optimum gradient profile, either graphically or numerically, with the help of a microcomputer.

Recently, a device for overpressured TLC and a fully automatic AMD machine for the complete plate-developing process were introduced. Both instruments can be used for gradient development. Gradient development can also be used in preparative TLC. In this case, the sample capacity for full separation of all components of the sample is several times larger for stepwise gradients than for isocratic elution.

In many cases, twice as many spots can be detected in gradient development as in isocratic elution. This is illustrated in Fig. 15, which presents copies of densitometer printouts obtained for Seboren extract (a plant drug) in two elution modes: isocratic and stepwise gradient (100).

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Overpressured Layer Chromatography

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I. INTRODUCTION

A. History of Overpressured Layer Chromatography* and Its Place Among Liquid Chromatographic Techniques

Conventional planar and nonplanar as well as thin- and thick-layer liquid chromatographic techniques require few instruments and are rather simple. Among the planar layer liquid chromatographic techniques, paper chromatography (PC) and its various versions developed in the 1940s by Martin and Synge (1) have to be mentioned first. Thin-layer chromatography (TLC), discovered by Ismailov and Shraiber (2) as well as Békésy (3), improved by Kirchner et al. (4), and standardized and spread by Stahl et al. (5, 6), contributed to the isolation and analysis of many natural and synthetic substances. Today, versions of this classical chromatographic technique are indispensable in various fields of scientific research and practice.

The combination of flame ionization detection (FID) and TLC (TLC/FID) as a nonplanar layer chromatographic technique gives quantitative results without the need to use detection reagents. In this system the thin sorbent layer is, e.g., on a glass rod (open or turned-out column) (7).

Column and layer liquid chromatographic techniques—as supplementary techniques due to their arrangements—have always been characteristically developed in constant mutual interaction. Hence it is not surprising that the intensive development of high-performance column liquid chromatography (HPLC) entailed the need for the fundamental renewal of the most popular planar layer liquid chromatographic technique, TLC. In light of this, it can also be understood that the latest efforts aimed at further

development of layer liquid chromatography are characterized by the desire to introduce sophisticated instrumental techniques similar to those of HPLC (8–10).

Attempts to develop an ultramicro (UM) chamber were first made in the 1960s (11). In this simple chamber, the chromatographic plate is covered by a glass plate in such a way that the end of the cover plate is not immersed in the solvent. This chamber is well suited for modeling classical column chromatographic (CC) separation. Important new instruments were developed

* Sometimes referred to as optimum performance laminar chromatography, for which the same abbreviation (OPLC) is used.

after the UM chamber that were aimed at increasing the efficiency of TLC through improvement of the separation mechanism. Programmed multiple development TLC, as elaborated by Perry (12), combines the techniques of continuous multiple development and evaporation. This technique was improved by Burger (13). In Burger's system, the chromatoplate is developed several times in the same direction with various mobile phases of decreasing elution power. Between developments, the chromatoplate is dried by vacuum. This method is termed automated multiple development (AMD) (14). High-performance TLC (HPTLC) is based on the use of chromatoplates coated with fine particles of a sorbent having a narrow particle size distribution and is carried out with sophisticated instrumentation (15, 16).

Modern methods of column liquid chromatography employ constant flow rates (8–10), although this has not been the case in TLC and HPTLC. The greatly increased developing time on a fine-particle-size sorbent layer (HPTLC chromatoplate) made it necessary to employ forced flow, which is also exploited in centrifugal layer chromatography (CLC) (17) [an alternative term is rotation planar chromatography (RPC) (18)] and in high-speed TLC (HSTLC). The latter used electro-osmosis to force the eluent (19). However, the first successful step to a real planar version of HPLC was the development of a pressurized ultramicro chamber the basic instrument of overpressured layer chromatography (OPLC) (20–22), which used a pump system for application of the eluent. The efficiency-oriented term for the original technique is optimum performance laminar chromatography (22a). The infusion and transfusion (22b) off-line and on-line operating modes in OPLC and their combination (23a), as well as the parallel (23b) and serial coupled (23c) multilayer systems, are basic technical versions of OPLC. The automated OPLC 50 system (23d) provides a user-friendly, automatic, accurate, and sensitive version of the original technique (20–22).

Figure 1 illustrates the place of OPLC techniques among the basic column and layer liquid chromatographic techniques classified according to the mode of transport of the mobile phase and the shape of the sorbent bed.

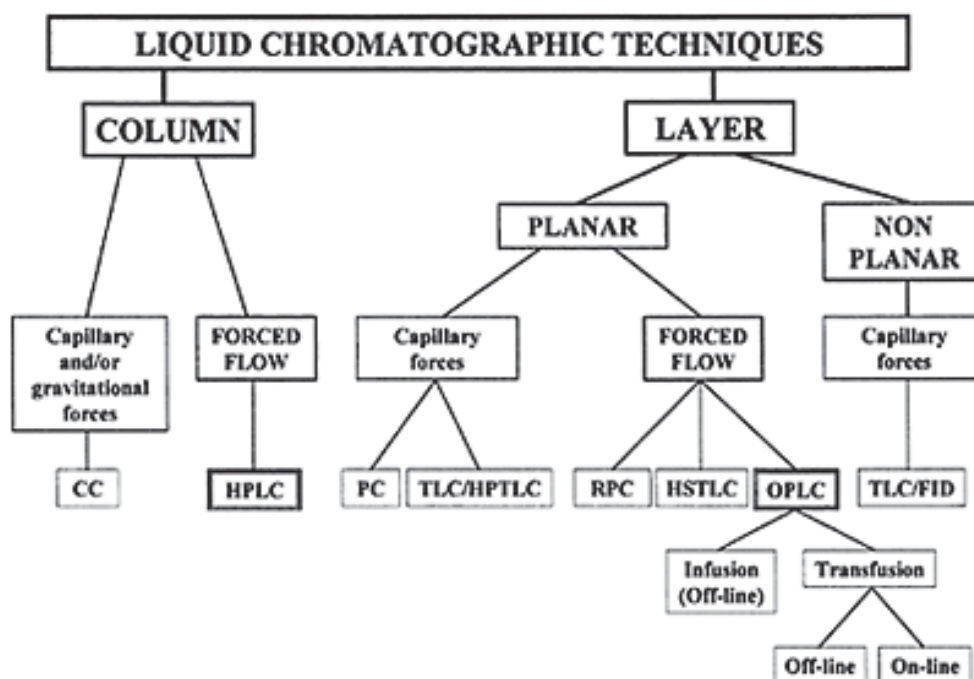


Figure 1 Classification of liquid chromatographic techniques.

B. Basic Elements of OPLC Techniques

Three geometric arrangements are used for chromatographic development in conventional TLC or HPTLC: linear, circular (radial), and triangular (anticircular). Depending on the application, all of these developing modes can be performed in OPLC, and each has its own particular merits. In the linear developing mode, one-directional, two-directional, and two-dimensional development is possible (23, 24). It can also be carried out by infusion and transfusion operations. In the case of the infusion operating mode, there is no existing outlet. The sorbent layer is totally closed, and during development the air of external and internal porosity is continuously compressed at the outlet side of the layer. This backpressure helps in the pore filling of particles, reducing the waviness of the front of total wetness. The infusion process is suitable for off-line development only, and a sorbent layer sealed on four sides should be used.

In the transfusion operating mode, the outlet side of the layer is in the open stage, allowing the outflow of both the air and the mobile phase. In transfusion OPLC, both off-line and on-line operation as well as their combination are possible, corresponding to the classical (original) OPLC technique (22b).

It follows from the principle of OPLC that low (2–5 bar), medium (10–30 bar), and high (50–100 bar or more) operating pressures can be used in this planar layer liquid chromatographic technique (25).

OPLC is an instrumentalized version of planar layer liquid chromatography, and it is suitable for on-line as well as off-line sample application, separation, and detection and their variations (partial off-line methods). In the on-line mode, the solutes are measured in the drained eluent by connecting a flow cell detector to the eluent outlet. In the off-line

mode all the principal steps of the chromatographic process, such as sample application, separation, and quantitative evaluation, are performed off-line (26–28).

A parallel version of overpressured multilayer chromatography (OPMLC) using two or more chromatoplates is very attractive because a large number of samples (50–100 or more) can be separated during one development (23b). Serial coupled OPMLC (called “long-distance” OPLC) can be used to increase both the number of theoretical plates and the resolution (23c). The automated OPLC 50 system generates a controlled separation process (23d).

In OPLC systems, the changes in the composition of eluent provide good possibilities for special separation modes, i.e., isocratic, gradient, and stepwise gradient. The OPLC system permits both analytical and preparative investigations.

II. THEORY

A. Retention and Factors That Influence It

1. Formation and Migration of Eluent Fronts

In conventional layer chromatography [TLC, HPTLC, and preparative layer chromatography (PLC)], the eluent migrates by means of capillary forces, described by the quadratic equation (29–31)

$$z_f^2 = kt$$

where z_f is the distance of the visible α front, t is the time of development, and k is the velocity constant.

In OPLC, the eluent can be forced through (or into in the case of infusion operation) the sorbent bed by means of a pump system by using a chosen flow rate (20). With the eluent fed at constant velocity, the speed of the front depends on the cross-sectional area of the sorbent layer in the direction of the development. Only linear developments are able to result in constant linear velocity; other geometrical shapes of sorbent layers (circular, triangular) are not.

Accordingly, the basic flow rule of linear transfusion OPLC is (32, 33)

$$z_f = ut$$

where Z_f is the migration distance of the eluent front, u is the linear migration velocity of the eluent front, and t is the time of the development. This means that in linear OPLC the velocity is constant along the plate, in contrast to the circular version of OPLC, in which the velocities of fronts and components decrease along the radius during development.

Figure 2a illustrates the basic differences among the conditions of eluent flow in conventional layer chromatography and one- and two-directional linear and circular (transfusion) OPLC at a constant flow rate (34). As can be seen in Fig. 2b, the theoretical line of linear (transfusion) OPLC development intersects the curve of conventional development, and its linear velocity is initially higher than that of OPLC. A starting rapid eluent flash (e.g., the use of a pressurized buffer space system) results in high velocity,

and curve 3 is continuously higher than curve 1. By this means, the straight front line is ensured. The automated OPLC 50 system automatically manages this period, dividing the process into two parts (line 4). The initial rapid period, having a higher constant velocity, ensures the formation of a straight front by quick wetting of the sorbent layer at the trough area. A period of lower velocity of separation follows this high-velocity step. At a certain distance (position 5) the velocity becomes constant, and samples should be applied up to this point. In the case of infusion OPLC, the speed of the alpha front decreases continuously whereas the mobile-phase inlet pressure increases with continuously increasing speed during development (22b).

2. Front of Total Wetness in Fully Off-Line Systems

If a dry porous sorbent bed made of irregular sorbent is used at the beginning of development, two zones can be found that have significant differences in their refractive indices, even if single eluents and conventional or forced-flow layer chromatographic techniques are used (26, 31, 35–37).

In the case of classical, fully off-line transfusion OPLC, in the zone under the α front (F_α), the space between the sorbent particles and within the pores is filled partially with air and eluent. This is the partially wetted zone (z_{pw}), which sometimes disturbs the separation in this narrow range (26, 28, 36). The next zone toward the eluent inlet point is a totally wetted one (Z_{tw}), which is completely filled with the eluent. The border between these zones is the front of total wetness (F_{tw}), which is not straight in most cases, but a sharp zigzag line that arises due to the inhomogeneity of external and internal pore diameters of the sorbent bed.

If the sorbent layer in fully off-line OPLC is “open-ended” (transfusion operation, the opposite side of the eluent inlet is open, with outflow through an eluent outlet tube), F_{tw} and the components migrate proportionally with F_α at a constant flow rate (26, 27) (Fig. 3). F_{tw} changes from a straight line to a *zigzag* one during the separation, and its bandwidth increases with migration distance. This effect is greater on a TLC plate than on an HPTLC plate. In contrast to the transfusion process, infusion yields a continuously decreasing waviness of the front F_{tw} as well as of the sample band shape of that area during development. The air that originally contained the sorbent is continuously compressed, helping to fill the pores with particles (22b). Nyiredy et al. (36) defined a critical pressure that can be related to F_{tw} . The R_f value of F_{tw} (R_{tw}) may vary with the conditions of development.

Valayudhan et al. (37) found that R_{tw} linearly increases with the flow rate but that F_α shows slight nonlinearity at higher flow rates. This phenomenon is independent of the viscosity of applied eluents (methanol, ethanol, and heptane). The pressure drop increases linearly with the migration distance and the time of development (see Fig. 3). It depends on the viscosity of the eluent, the particle size of the sorbent layer, and the external pressure on the layer surface. Within experimental error, their incompressible model is in agreement with experiments, and the velocities of the fronts are (37)

$$U_{F_\alpha} = (1 + a)U_{F_{tw}} \quad \text{and} \quad a = \varepsilon_p/\varepsilon_i$$

where ε_i is the interstitial and ε_p the intraparticulate porosity per total volume of bed.

If the sorbent layer is not wettable by the eluent, e.g., in the case of a reversed-phase sorbent applied in water elution, F_{rw} migrates together with F_α (37).

Along the plate, the sorbent/eluent ratio is not constant, due to the partially filled zone. The front distance always appears to be longer than the one measured at totally filled conditions. This causes the R_f value to be higher than the one calculated from the visible front.

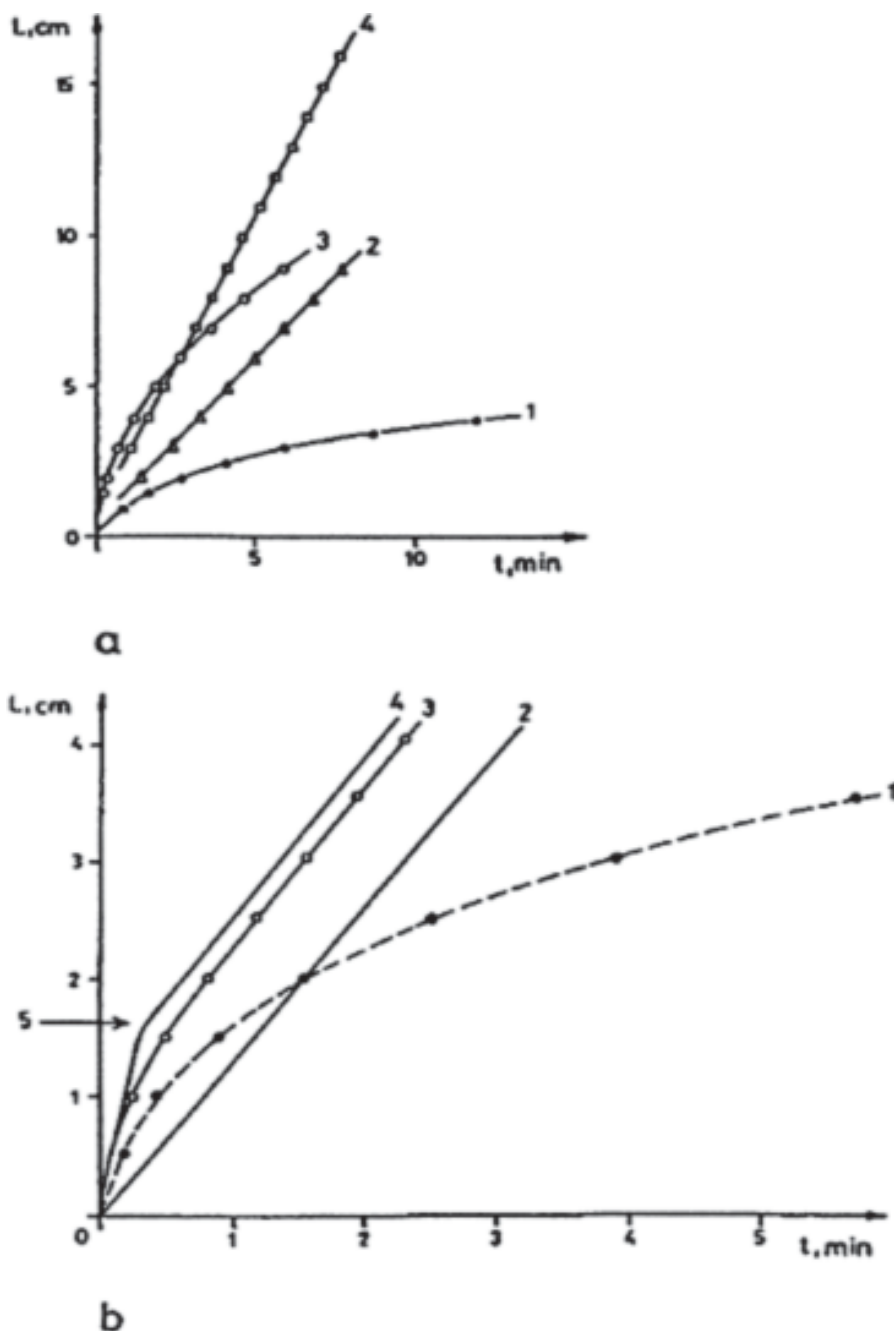


Figure 2 Migration of α front using conventional TLC and various off-line transfusion OPLC developments.

HPTLC silica gel 60 (Merck) is compressed for 10 min at 2.5 MPa prior to development, (a) Whole development. Eluent, carbon tetrachloride; flow rate (OPLC), $0.50 \text{ cm}^3/\text{min}$; temperature, 19.5°C ; 1, conventional development (normal unsaturated chamber); 2, two-directional linear OPLC; 3, circular OPLC; 4, one-directional linear OPLC. (b) Initial period. Eluent, chloroform; flow rate (OPLC), $0.325 \text{ cm}^3/\text{min}$; 1, conventional development (normal unsaturated chamber); 2, theoretical line of linear OPLC; 3, linear OPLC, using rapid eluent admission; 4, linear OPLC, using automated OPLC 50; 5, proposed place of sample application.

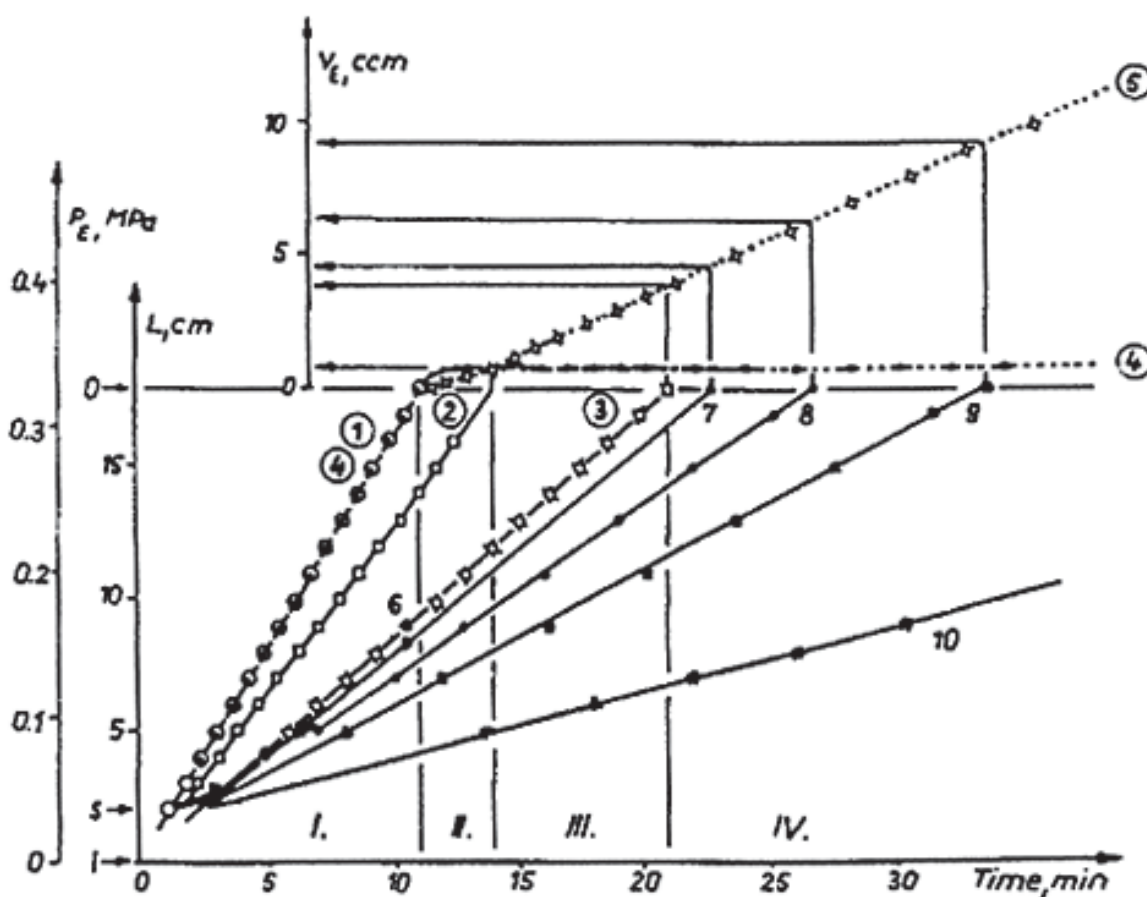


Figure 3 Migration of the solvent fronts and substances during continuous development using transfusion OPLC technique (26). Chromatographic conditions: Chrompres 25 (Labor MIM, Budapest, Hungary); silica gel 60 (Merck); isooctane–THF (100:7.5); external pressure on the membrane, 2.0 MPa. L , migration distance; S , start point; I , eluent inlet point; O , eluent outlet point. 1, α front (F_α); 2, front of total wetness (F_{tw}); 3, β front (F_β); 4, inlet pressure (P_E); 5, curve of eluent volume at outlet (V_E); 6–10, substances separated (6, blue dye, eluting in F_β ; 7, perylene; 8, yellow dye; 9, pink dye; 10, red dye). Stages of continuous development: I, classical, fully off-line OPLC; II, leaving of partially wetted zone; III, leaving of secondary fronts; IV, equilibration.

Using diagonal sample application and a single eluent, R_f values were practically independent of spotting location, and their values were higher on HPTLC layers than on TLC layers (21). Roeraade and Flodberg (38) compressed the sorbent layer prior to OPLC development, and because of the increased packing density, R_f decreased slightly up to 10 MPa and dramatically above this value.

3. Secondary Fronts in Fully Off-Line Systems

It is a well-known fact in classical TLC that the eluent components sorbed strongly by the sorbent sites can cause secondary fronts (F_β , F_γ , ...) (39) that are independent of F_{tw} . This effect can be found during adsorption as well as in reversed-phase development when the eluent consists of solvents of different strengths. The effect of this chromatographic solvent demixing is stronger in fully off-line OPLC systems (26), owing to the total elimination of vapor space, than in chambers with small vapor spaces, e.g., sandwich chambers.

These fronts divide the sorbent layer into zones of different eluting strengths, within which the solvent strength and polarity are practically the same, whereas at the fronts themselves there is a sudden increase in eluent strength that gives rise to “polarity steps.” This phenomenon takes place in HPLC and in fully on-line OPLC as well, but only after

eluent changes during equilibration, when the apparatus is not used for separation (26, 28, 40) (see Fig. 3). The eluent strength (ε) of a given eluent mixture can be calculated according to Snyder and Glajch (41). Eluent strength was correlated with $R_{f,\beta}$ using fully off-line OPLC, silica gel 60, and different apolar and polar eluent mixtures. The mixtures of hexane and ethyl acetate or tetrahydrofuran or acetone show linear relationships between $R_{f,\beta}$ and ε . The mixtures of ethyl acetate and carbon tetrachloride, benzene, or methylene chloride fail to show this type of correlation (28).

The eluent strength of the β zone is regarded as similar to the calculated value. If the secondary front collects analyzable components from the preceding zone (α zone), shorter development or a higher sample origin is needed. When sample components are not sensitive and Z_α can elute the component collected by the secondary front, double development with the same eluent can be used. If the phenomenon cannot be overcome, new eluent should be used. If the polar constituent of the eluent is replaced with a weaker one of the same volumetric ratio, then a higher $R_{f,\beta}$ and lower ε value of the β zone arise. Replacing the apolar constituent of this new eluent with a stronger one results in a higher $R_{f,\beta}$ and a higher ε value of the β zone. At a given eluent composition and sorbent, the $R_{f,\beta}$ value is constant, independent of migration distance and velocity of eluent (25, 26, 37).

The R_f of a secondary front depends on the eluent composition. It was found that a plot of R_m versus the logarithm of the mole fraction of polar constituent used in the mixture did not show a linear relationship, unlike the compounds' migration in the β zone (R_m is equal with the logarithm of $1/R_f - 1$). $R_{f,\beta}$ increases with increasing concentration of polar modifier as well as with decreasing specific surface areas of the sorbent (42). Similar results were found by Wawrzynowicz and Soczewinski (43) in the case of a sandwich chamber and binary eluents. Markowski et al. (44) applied sandwich TLC for the evaluation of adsorption isotherms, comparing this method to the breakthrough and static methods. All three methods gave similar results.

4. Retention Transfer Among TLC, Off-Line and On-Line OPLC, and HPLC

The elimination of the vapor phase above the sorbent layer in OPLC may cause disturbances in the retention transfer from TLC to OPLC. (Recall the previous point.) Retention data obtained in fully off-line OPLC can be converted to on-line separation/detection conditions according to the equation

$$k = \frac{1}{R_f} - 1$$

where k is the capacity factor of a given component in the on-line system (45).

A strong correlation was found on silica gel layers among fully off-line, partially off-line (off-line sample application, on-line separation/detection), and fully on-line OPLC even when eluents were used with more components (28). The slope of the line is not 1, due to the difference in sorbent bed conditions. If the β front collects some components, then the concept of R_m additivity can be used to convert these data into those of the fully on-line system:

$$R_{m,i}^w = R_{m,\beta} + R_{m,i}$$

where $R_{m,i}^w$ is the R_m value in the wet system, $R_{m,\beta}$ is the R_m value of the β front, and $R_{m,i}$ is the R_m value of the given collected components in the α zone.

If the number of silanols in silica gel is reduced by a polar silane reagent such as 3-glycidyloxypropyltrimethoxysilane, then the resulting diol-modified layer is less sensitive to relative humidity, and R_f values are generally higher on it than on bare silica layers (46). Thus the modified layer is suitable for the separation of nonpolar and polar compounds with simple, less polar eluents. The correlation between the retention data of fully off-line and fully on-line OPLC is stronger than it is on silica layers (42) (Fig. 4).

Reversed-phase ion-pair chromatography can be optimized by fully off-line OPLC (47). Good agreement was found in the selectivity of HPLC and OPLC ion-pair systems using the same eluent composition, and this made possible the modeling of HPLC ion-pair systems by fully offline OPLC (48).

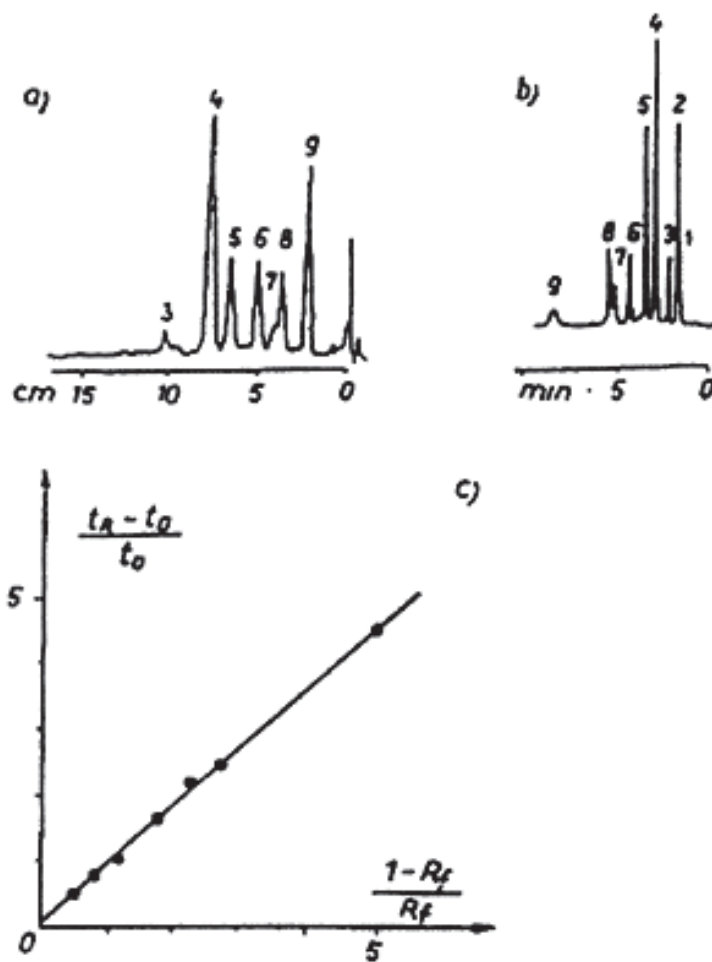


Figure 4 Rapid (a) fully off-line and (b) fully on-line OPLC separation, and (c) comparison of retention data.

Operating parameters: Chrompres 25, external pressure on membrane, 2.8 MPa; temperature, 23°C; layer, diol-

modified HPTLC silica gel 60; eluent, *n*-hexane; flow rate, 2.5 cm³/min; detection: absorbance at 254 nm. Sample volume injected and streaked 3 μL, dissolved in carbon tetrachloride; 1, carbon tetrachloride; 2, toluene; 3, acenaphthene; 4, phenanthrene; 5, pyrene; 6, chrysene; 7, benzopyrene; 8, butter yellow; 9, fat red. (Reproduced by permission of Dr. Alfred Huethig Verlag GmbH, from Ref. 42.)

Selectivity of the mobile phase for coumarins was similar in TLC, off-line OPLC, and HPLC (48a). The change of eluent strength had the same effect on retention using TLC and OPLC as in nonequilibrated systems. In the case of HPLC, the effect of different eluent strengths highly modified the retention behavior.

B. Efficiency Characteristics

1. Theoretical Plate Height and Factors That Influence It in Off-Line and On-Line OPLC

In conventional layer chromatography, the theoretical plate height (HETP) can be calculated according to Guiochon and Siouffi (49), and it is also applicable to off-line OPLC systems (21). HETP (*H*) is

$$H = \frac{\sigma^2}{(L_f - s_0)R_f}$$

where σ is the spot variance, L_f is the front distance, s_0 is the distance between the spotting location and the eluent inlet trough, and R_f is the retention factor.

Owing to the effect of focusing, an initial (starting) spot width may be defined that is different from the spot width deposited. The initial spot variance (σ_{0i}^*) of a given compound (i) is

$$\sigma_{0i}^* = \sigma_0^s(1 - R_{fi}^s)R_{fi}^s$$

where σ_0^s is the spot variance of solvent deposited, and R_{fi}^s and R_{fi}^e are retention factors in the solvent and eluent, respectively (42).

If the bandwidth of the spot or band deposited is very narrow in off-line OPLC, then HETP is practically constant along the sorbent layer and independent of the front distance (Fig. 5) (21, 49a). Figure 5 illustrates clearly the basic difference between TLC, HPTLC, and off-line OPLC with respect to efficiency. Because of the significant reduction of waviness of F_{tw} and also the

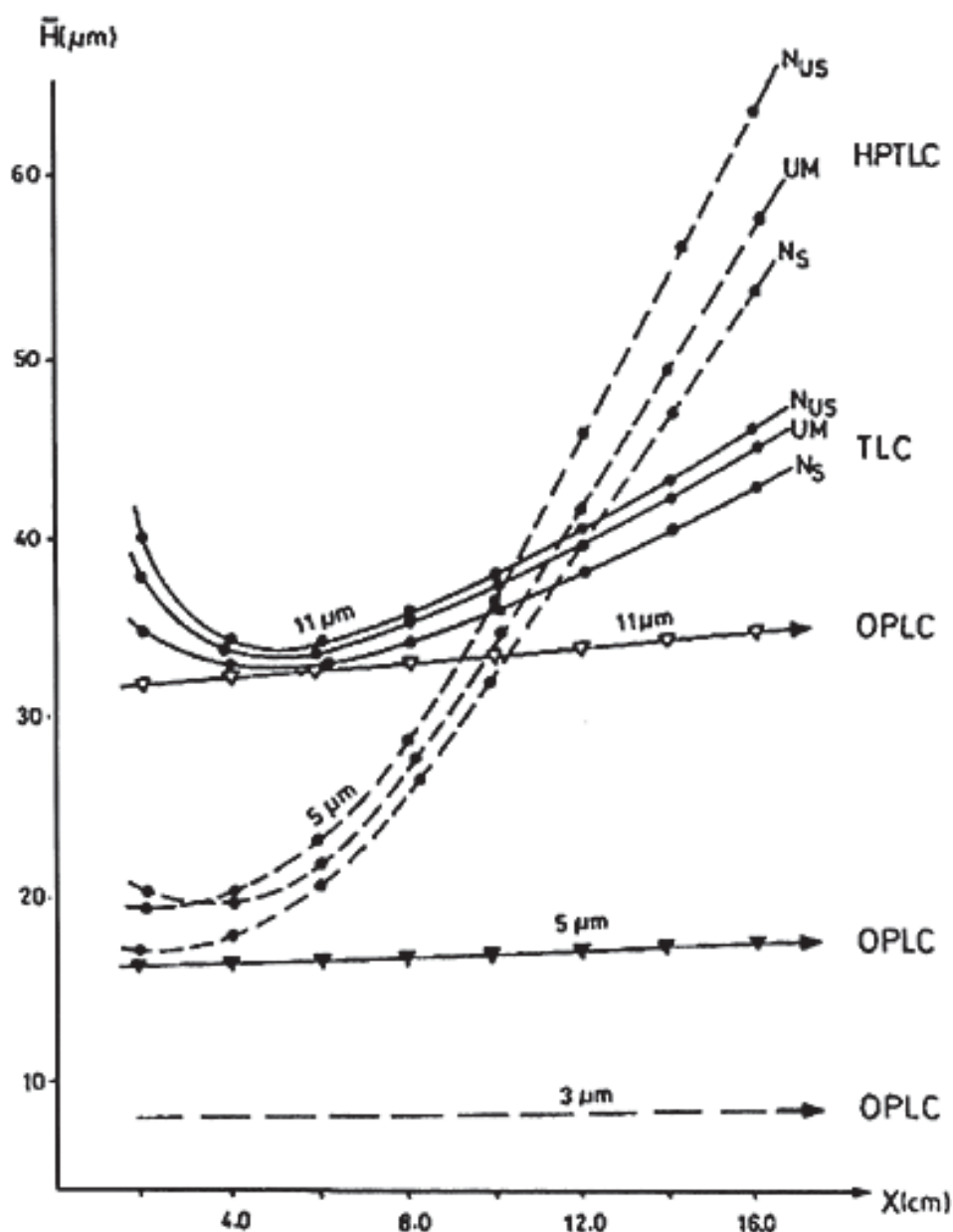


Figure 5 Correlation between the average theoretical plate height (H) and the distance (x) traveled by the eluent on silica gel layers with various particle sizes and in different chamber systems. N_{us} , normal unsaturated chamber; N_s , normal saturated chamber; UM , ultramicro chamber (totally closed layer, without overpressure).

component band sizes of that area, the efficiency is increased during infusion off-line OPLC development (22b).

Hauck and Jost (50) also found that HETP depends on the R_f and front distances. In plotting data for HETP versus the migration distance of various compounds (L_i), a gradually decreasing curve was obtained. This curve was linearized by plotting HETP versus the inverse migration distance (26, 42). The slope of the line depends on the size of the deposited spot.

The linear relationship between the HETP and the inverse migration distance of a component (L_i) can assume the following HETP calculation (42):

$$H_i = \frac{(\sigma_{i0}^*)^2}{L_i} + H_\infty$$

where σ_{i0}^* is the initial spot variance and H_∞ is the HETP at the point of intersection. It was assumed that $H_\infty = h d_p$, where h is the reduced plate height and d_p is the particle diameter according to Knox (51).

The thickness of sorbent layer influences HETP slightly (50). In off-line OPLC, HETP may vary with the linear velocity (50, 52), similarly to HPLC. HETP depends on the characteristics of the plate used and decreases in the following order: preparative layer >TLC, >HPTLC, >Raman plate (26, 28, 49a).

In OPLC, HETP depends on the combination of the off-line and on-line operating steps applied (28, 42). Figure 6 shows HETP (H) versus linear velocity (u) using different operating modes of transfusion OPLC. The curves are very similar, but the values of HETP are different. Fully off-line OPLC produces the lowest, and the fully on-line OPLC the highest, HETP values. Between them are the two curves of partially off-line (or partially on-line) OPLC. The differences among these systems originate from "extracolumn" band broadening, which does not occur in the fully off-line system.

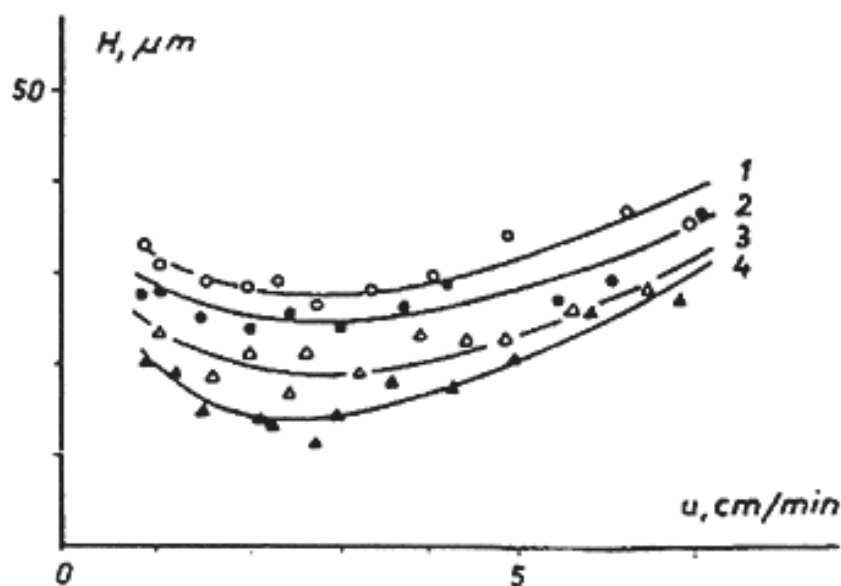


Figure 6 Relationship between theoretical plate height H and eluent

front velocity u for different operating modes of OPLC. Operating parameters: Chrompres 25; external pressure on membrane, 2.8 MPa; temperature, 23°C; layer, HPTLC silica gel 60; eluent, methylene chloride–ethyl acetate (9:1); sample. PTH–methionine; bandwidth deposited and trough width, 0.68 mm; migration distance, 175–180 mm for off-line detection and 180 mm for on-line detection. 1, Fully on-line OPLC; 2, on-line sample application/separation and off-line detection; 3, off-line sample application and on-line separation/detection; 4, fully off-line OPLC. (Reproduced by permission of Dr. Alfred Huethig Verlag GmbH, from Ref. 42.)

The quality of the packing can also influence HETP values, and values can be decreased by compression of the sorbent layer up to a limited external pressure (38). The elevation of external pressure decreases the HETP value in off-line OPLC. But it does not occur during on-line development (52a). The results were similar when a 3 μm spherical sorbent layer was used (52b). Figure 7 shows the effect of linear velocity on HETP at three different external pressures using transfusion off-line operation and fine-particle silica. High external pressure significantly increases the efficiency. Furthermore, the optimum range of linear velocity becomes broader (49a).

2. Theoretical Plate Number

The theoretical plate number (N) can be calculated with the equation

$$N = L_f / H$$

where L_f is the distance between start and front and H is the average theoretical plate height.

If the bandwidth of the deposited spot is very narrow in OPLC, then HETP is practically constant along the plate (21). This means that the theoretical plate number increases linearly with development distance, contrary to TLC/HPTLC. The theoretical plate number as well as the spot and/or peak capacity can be increased by multilayer OPLC using a layer connection in series called “long-distance” OPLC (52c). A

theoretical plate number of 7×10^4 was achieved using butter yellow dye and a 70 cm development distance.

3. Spot or Peak Capacity in OPLC

The spot capacity of conventional layer chromatography is limited (53), and about 20 spots can be resolved with a resolution of unity. In the case of thin-column and fully off-line OPLC, the maximum value of spot capacity (n_M) is

$$n_M = \frac{1}{2} \sqrt{\frac{L}{H}}$$

where L is the distance of development and H is the average HETP value of compounds at given conditions (53, 54).

Peak capacity (n) of a column in HPLC as well as that of the on-line separation/detection OPLC systems is given by (53–55)

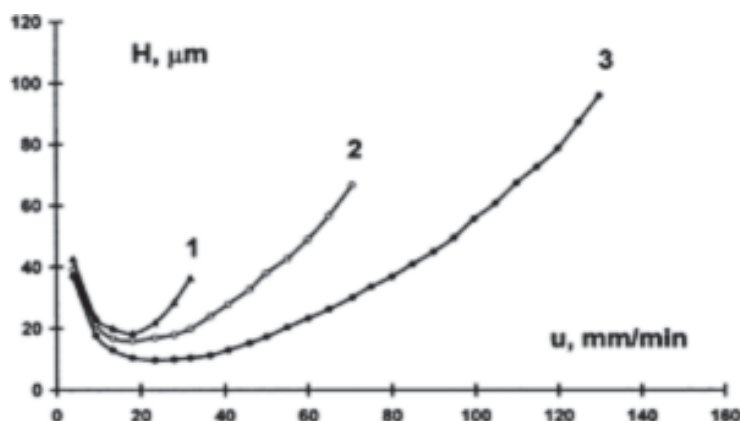


Figure 7 Effect of linear velocity (u) on theoretical plate height (H) using different external pressures. Fully off-line transfusion OPLC; eluent, dichloromethane-ethyl acetate (92:8); HPTLC silica gel; PTH-valine, 0.4 mg/mL; $5 \mu\text{L}/10 \text{ mm}$ band; 1, 1 MPa; 2, 2.5 MPa; 3, 5 MPa. (Reproduced from Ref. 49a, with permission.)

$$n = 1 + \frac{\sqrt{N}}{4} \ln(1 + k)$$

where N is the theoretical plate number of the sorbent bed at given conditions and k is the capacity factor of the most retained compound eluted.

With an openable sorbent bed, compounds separated but remaining on the layer after an online separation/detection can also be detected in situ by densitometry (23a). In this

combined online and off-line OPLC system, higher spot and/or peak capacity can be observed at a given bed length than by single on-line or single off-line OPLC separation, because the peak and spot capacities are additive according to separate measurements (23a).

4. Factors Influencing Resolution

The resolution of a neighboring pair of spots can be described by the equation

$$R_s = \left[\frac{1}{4} \left(\frac{K_1}{K_2} \right) - 1 \right] \sqrt{R_f N (1 - R_f)}$$

where K_1 , K_2 are distribution coefficients of two substances, R_f is the average retention factor of pairs, and the N is the theoretical plate height.

A plot of resolution vs. R_f (Fig. 8) shows the differences between TLC/HPTLC and OPLC. This figure illustrates that in TLC the optimum range of resolution is R_f 0.3–0.4 as opposed to OPLC, where the R_s maximum is R_f 0.5–0.6 (56).

Three factors were studied regarding the resolution in fully off-line OPLC (50). Use of the optimal linear flow velocity in relation to HETP produces the highest resolution. The relationship between resolution and distance of development is approximately linear, and the resolution increases with increasing front distance. The layer thickness shows an optimum value (80–160 μm) in terms of resolution.

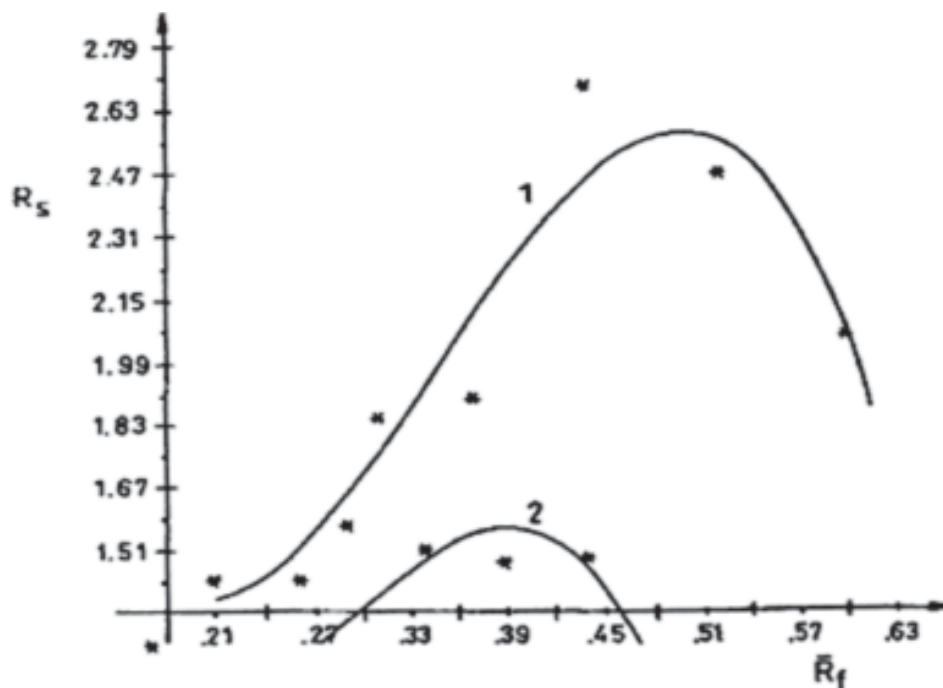


Figure 8 Relationship between $R_s(Y)$ and $\bar{R}_f$ (average R_f value; X) for reversed-phase chromatography of aldehyde DNPH derivatives. 1, Off-

line OPLC; 2, TLC. (Reproduced by permission from Ref. 56.)

III. INSTRUMENTATION AND OPERATION OF OPLC

A. Description of OPLC Instruments

The essential feature of the pressurized ultramicro chamber system is that the sorbent layer is completely covered with a flexible membrane under an external pressure so that the vapor phase above the layer is virtually eliminated (20–22). In this chamber system, it is possible to optimize the flow velocity of the eluent by means of a pump. The principle of a pressurized ultramicro chamber made of polymethyl methacrylate and used mainly for circular separation is illustrated in Fig. 9.

Based on experience gained with experimental pressurized chambers, the LABOR Instrument Works (Budapest, Hungary) developed the Chrompres 10 and Chrompres 25, the first commercial pressurized ultramicro chambers. In the Chrompres 10 the maximum cushion pressure permitted is 1.0 MPa. It can be used with plastic, aluminum, or glass chromatoplates up to 20×40 cm in size, coated with fine-particle (5–6 μm) or superfine-particle (2–3 μm) sorbent (23–25).

In the Chrompres 25 chamber, the maximum cushion pressure permitted is 2.5 MPa, and the maximum size of the layer is 20×20 cm. The optimum eluent front velocity is higher in a superfine-particle (2–3 μm) sorbent layer than in a fine-particle (5–6 μm) sorbent layer, and the increase in the eluent front velocity means a higher solvent inlet pressure (23–25).

Kaiser and Rieder (57, 58) established that high pressure in planar layer chromatography can be applied most easily in the circular and anticircular separation modes. They developed high-pressure planar liquid chromatography (HPPLC), which is theoretically and practically a circular version of OPLC at higher operating pressure with special solutions. This technique exploits all the advantages of the circular technique and uses the experience gained in the field of circular HPTLC.

An instrument was developed for OPLC by Witkiewicz et al. (58a). In this instrument the eluent is fed to the chromatoplate from below by a syringe pump. Gas is used to apply external pressure to the chromatoplate. The instrument can be set up extremely quickly and is very easy to operate.

The latest generation of OPLC is an automated OPLC 50 system (developed by OPLC-NIT Ltd., Budapest, Hungary, and distributed by Bionisis-OPLC SA, Le Plessis Robinson, France) that includes a separation chamber and a liquid delivery system. The separation chamber has four main units: holding unit, hydraulic unit, traylike layer cassette, and attached drain valve (Fig. 10). A microprocessor-controlled system is the heart of the liquid delivery. The pump heads, one for

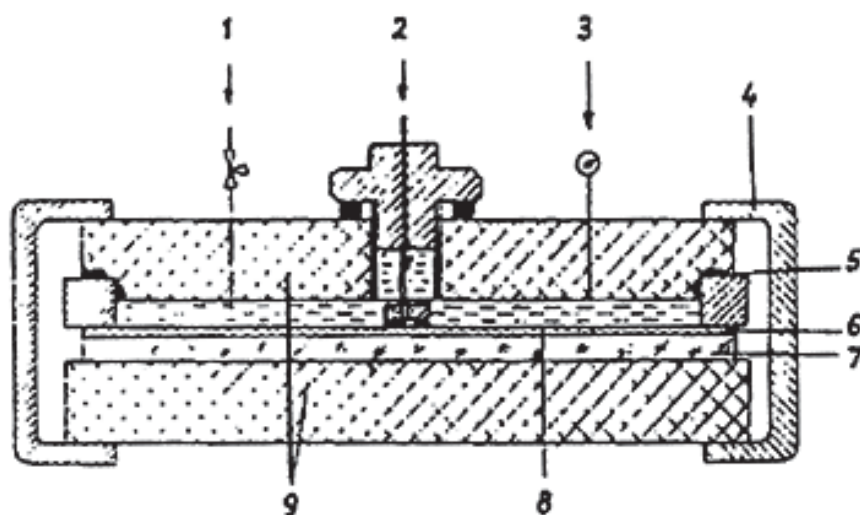


Figure 9 Schematic drawing of the circular-type pressurized ultramicro chamber (PUM chamber). 1, Water inlet; 2, developing solvent inlet; 3, pressure gauge; 4, screw fastener; 5, rubber O-ring; 6, sorbent layer; 7, support plate; 8, plastic foil cushion system; 9, polymethacrylate support blocks.

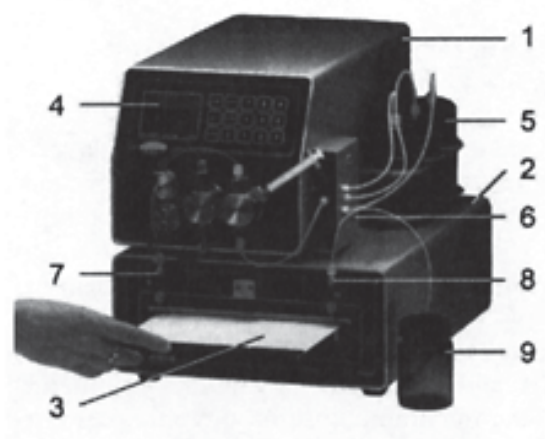


Figure 10 Automated OPLC 50 instrument. 1, Liquid delivery system; 2, separation chamber; 3, cassette; 4, LCD display; 5, eluent reservoirs; 6, eluent-switching valve; 7, eluent inlet; 8, eluent outlet; 9, waste reservoir.

the eluent delivery and the other for the hydraulic liquid delivery, work by means of a common drive. All parameters for single isocratic or step wise gradient (a maximum of three steps) development can be given and stored in the software of the delivery system. The automatic developments are absolutely repeatable, and parameters can be stored during the working period by using a simple fill-in-the-blanks procedure. External pressure (max. 5 MPa), eluent volume of the rapid period and of development (see Fig. 2, line 4), and eluent flow rate can be entered, and developing time is automatically calculated. Linear, one- and two-directional, two-dimensional circular cassettes can be used for infusion off-line, for transfusion off-line, and for on-line development using an analytical or preparative sorbent layer and the appropriate cassette (23d).

The present state of OPLC includes the use of sample applicators of various types, scrapers for the removal of sorbent layers for isolation of the substances separated, eluent connections for the on-line method, staining systems for derivatization, densitometers for off-line quantitative evaluation, and detectors for on-line quantitative evaluation.

B. Preparation of Chromatoplates for OPLC Separation

The OPLC technique of linear development requires a special chromatoplate that is sealed at the edges. This prevents the eluent from flowing off the plate in an unwanted direction. Linear migration of the eluent front in the OPLC chamber in the linear developing mode can be achieved by placing a narrow channel before the eluent inlet. The function of this eluent trough is to direct the eluent and to form a linear eluent front. In practice, a polyethylene or Teflon insert with an eluent through is placed between the sorbent layer and the water cushion to protect the cushion and to direct the eluent along a linear front (23).

Figure 11 shows possible modifications of the chromatoplate. It is obvious that for one-directional development, a plate sealed on three (Fig. 11a) or four (Fig. 11e) sides should be used. If two opposite sides of the chromatoplate are sealed and the eluent inlet is in the middle of the sorbent layer in a channel, this system is suitable for a two-directional separation (Fig. 11b) with a large number of samples. For circular OPLC separation (Fig. 11c), it is not necessary to impregnate the edges of the chromatoplate, and the eluent inlet is placed in the middle of the sorbent layer. For two-dimensional separation (Fig. 11d) in off-line systems, the four sides of the chromatoplate must be sealed beforehand and the seal opposite the actual inlet must be covered with a strip of filter paper, or an eluent outlet should be used in the case of transfusion. For infusion separation (Fig. 11e), the layer is sealed on four sides and the outlet is absent (no overrun possibility).

Chromatoplates for on-line OPLC separation generate two eluent-directing troughs in the sorbent layer or in the Teflon insert cover plate. The eluent inlet trough directs the eluent along a linear front, and an eluent outlet trough collects the eluent at the end of the chromatoplate

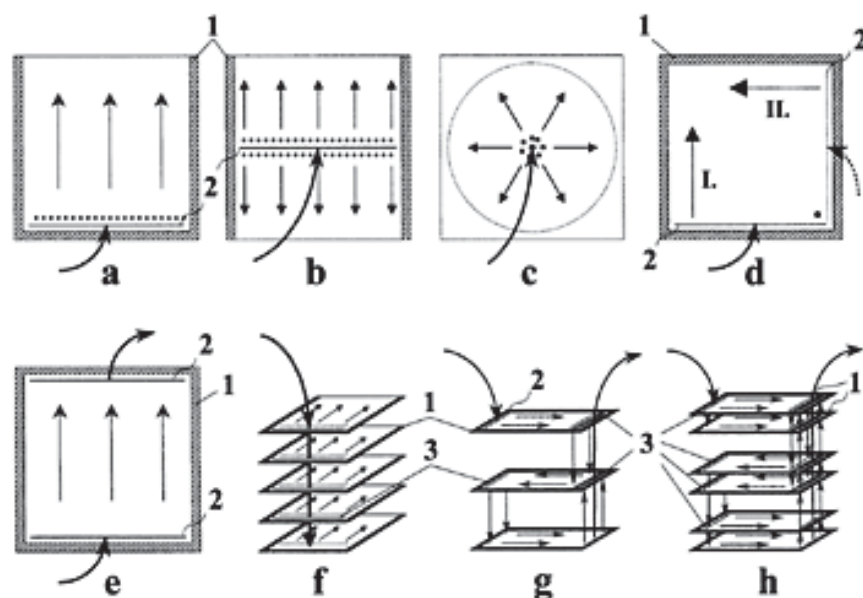


Figure 11 Schematic drawing of chromatoplates used in OPLC separations, (a) One-directional; (b) two-directional; (c) circular; (d) two-dimensional; (e) on-line (f) parallel coupled multilayer; (g) serial coupled multilayer; (h) parallel/serial coupled multilayer. 1, Sealed edge; 2, eluent-directing trough; 3, eluent-directing slit.

connecting to the detector system. The combination of several chromatoplates (Figs. 11f–h) during a single OPLC separation generates special advantages (e.g., a large number of samples). The introduction of the eluent to the multilayer system is a critical matter and is performed by making a perforation of a suitable size and shape in the chromatoplates at the eluent inlet and/or outlet. Ready-to-use OPLC layers sealed on sides are available commercially (Bionisis-OPLC SA., Le Plessis Robinson, France).

C. Main Separation Modes

1. Off-Line Separation

In fully off-line OPLC systems, all the principal steps in the chromatographic process, such as sample application, separation, quantitative evaluation, and isolation, are performed as separate operations. Fully off-line OPLC has two operations, infusion and transfusion. In infusion mode, the eluent is introduced into the totally closed layer (the layer surface is closed by external pressure, the layer edges are sealed on four sides, and

the chamber outlet is closed). The air originally contained in the sorbent layer is continuously compressed during the process. The eluent introduction is finished when the inlet pressure reaches the pressure limit. The transfusion operation corresponds to the classical (original) OPLC technique permitting pass-through of both air and eluent (49a).

In analytical off-line OPLC, several samples can be processed in parallel. This technique offers further advantages, such as that only the spots or bands of analytical interest need to be assessed, quantitative evaluation can be repeated with various detection parameters, and chromatogram spots or bands can be evaluated visually.

In preparative off-line OPLC, the procedures of drying, scraping of the sorbent layer, elution, and crystallization after layer development are similar to those in conventional preparative TLC methods. However, in preparative off-line OPLC, the resolution is considerably increased, and thick, fine-particle sorbent layers can also be used. It is possible to isolate the components of interest from the sorbent layer.

2. On-Line Separation

If the eluent outlet of the chamber is connected to a flow cell detector, eluting solutes can be detected on-line, and fractions can also be collected. The entire chromatographic process can be performed on-line by connecting a loop injector to the eluent inlet and a UV detector to the eluent outlet, in much the same way as in HPLC (Fig. 12).

3. Combined Off- and On-Line OPLC

An OPLC system that is equipped with an injector and a detector provides high flexibility. In addition to the fully off- and on-line process, combinations of the various operating steps are feasible (23a):

Off-line sample application with on-line separation and detection

On-line sample application and separation, with off-line detection.

When using a combined system, some sample components can be measured on-line (as in HPLC detection) and others that remain on the sorbent layer after the separation can be evaluated off-line by means of a densitometer. Figure 12 illustrates the basic elements of the combination of off-line and on-line OPLC. Combining on- and off-line OPLC increases the efficiency of the OPLC system, providing a spot capacity approximately twice that obtained by single systems because the spot and peak capacity are additive (23a).

4. Parallel Coupled Multilayer Separation

Combination of a multilayer system with a forced eluent flow complicates to a certain extent the original simple and flexible TLC technique and also, to some degree, conventional OPLC. However, the result is overpressured multiple layer chromatography (OPMLC), an efficient and prom-

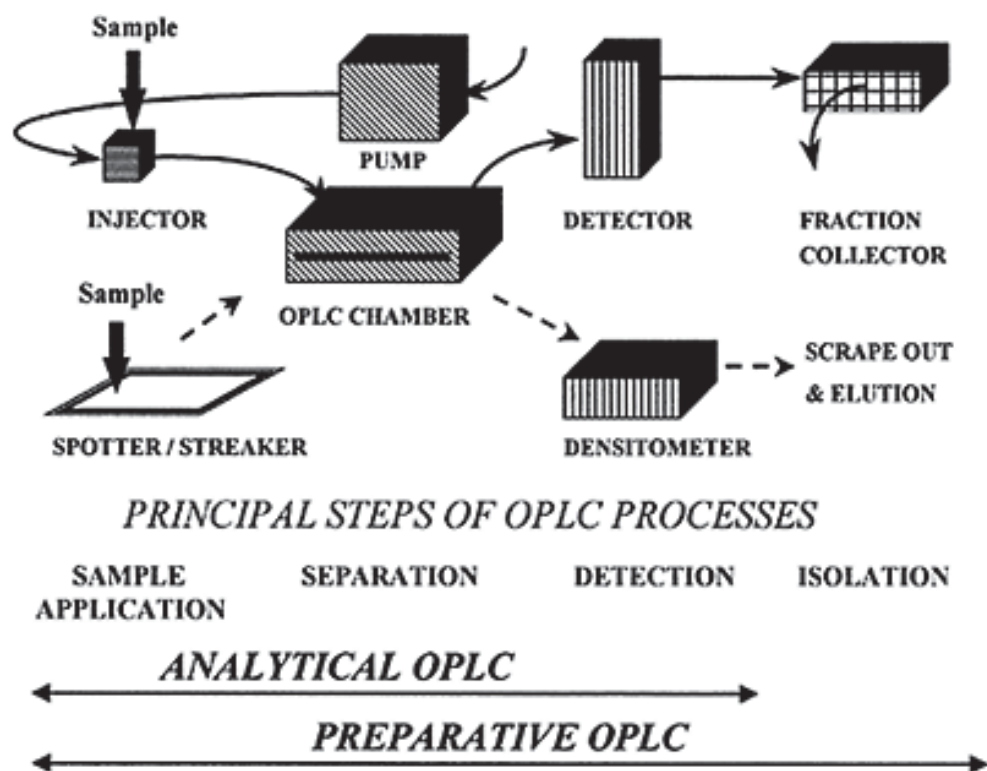


Figure 12 Schematic drawing of OPLC processes. (···) Off-line step; (—) on-line step.

ising technique in the field of layer liquid chromatography that is applicable to analytical and preparative separations in various types of laboratories (23b). The development of OPMLC exploits unique possibilities of the layer liquid chromatography system that are absent from column LC systems.

5. Serial Coupled Multilayer Separation, Long-Distance OPLC

Long-distance OPLC is a multilayer development technique employing specially prepared chromatoplates (23c). In a manner similar to the preparation of layers for linear OPLC development, all four edges of the chromatoplates must be impregnated with a polymer suspension, and movement of the eluent with a linear solvent front can be ensured by placing a thin plastic sheet on the layer or by scraping a narrow channel in the sorbent for the solvent inlet. Several plates are placed on top of each other to extend the development distance (long-distance OPLC). The end of the first (uppermost) chromatoplate has a slitlike perforation to enable the mobile phase to flow to a second layer where migration continues to the opposite end of the chromatoplate. Here the chromatography can be continued on to an adjacent chromatoplate, or the eluent can be led away (Fig. 11g).

Owing to the special arrangement of the prepared layers and the use of forced eluent flow, the mobile phase can travel through the stationary phases at optimum flow velocity.

In this arrangement, the development distance of chromatoplates can easily be increased to the extent desired. Another advantage of the method is that different sorbents can be used so that each part of a complex mixture can be separated on a suitable stationary phase (23c).

6. Parallel or Serial Coupled Multilayer Separation

Layers can also coupled in a parallel or serial mode (see Fig. 11h). This version is well suited for efficient micropreparative isolation.

D. Elimination of Fronts in Fully Off-Line OPLC and Selection of Eluents

Two types of fronts may be formed in fully off-line OPLC: the front of total wetness (F_{tw}) and secondary fronts (F_β , F_γ , ...). The location of F_{tw} can be modified by changing the flow rate; the R_f value of F_{tw} can be increased or decreased by increasing or decreasing the flow rate (36, 37). The total elimination of F_{tw} can be carried out by applying a prerun prior to the separation, in which the components to be analyzed do not migrate and the air is removed from the layer (36). Infusion operation dramatically reduces the effect of component waviness caused by F_{tw} (22b).

Another way to solve this problem in practice is to prewet the sorbent layer with eluent from the outlet direction until the eluent front reaches the point of sample application, then follow conventional development using the eluent inlet (26, 28). A vapor pretreatment of the sorbent layer was applied prior to OPLC development for the elimination of secondary front effects (58b).

Nyiredy et al. (59) developed a model called PRISMA for optimization of the mobile phase for OPLC. PRISMA is a three-dimensional model that correlates the solvent strength and the proportion of eluent constituents, which determine the selectivity of mobile phases by applying Snyder's solvent classification (60). The classes cover 29 solvents commonly used in TLC that are grouped by three criteria: the ability to donate protons, the ability to accept protons, and the ability to undergo dipole interaction. Solvent strength, influencing primarily the R_f value, is represented by the height of the prism. Because the solvent strength of the three solvents selected to define the prism are different, the resulting cover plate will be neither parallel to nor coincidental with the base.

Solvent strength as well as incidental tailing may be influenced by small amounts of additives, which can be symbolized by the base of the prism. If the prism is cut parallel to the basic triangle at the height of the shortest edges (corresponding to the lowest solvent strength of the selected solvents), an upper frustum and a regular prism result. Points in the complete PRISMA model represent the composition of three to five selected solvents for mobile phases of various selectivities and eluent strengths.

The appropriate solvent strength has to be determined experimentally ($0.2 < R_f < 0.8$). If the R_f values of compounds are too low (< 0.2), the solvent strength can be increased by adding modifiers (e.g., water or acetic acid) for normal-phase OPLC. If the R_f values are too high (> 0.8), the solvent may be diluted with hexane, provided it is miscible.

A statistical method for quantifying mobile-phase selectivity was developed for one- and two-dimensional OPLC separations, and it was applied for the separation of steroids (60a).

IV. APPLICATIONS

A. Possibility of Analytical Applications

1. Improvement of Resolution

Resolution in HPTLC is limited by the development distance, because it cannot be increased beyond 8–9 cm. Using OPLC as a forced-flow technique permits longer development distances, and the resolution can be significantly increased. The effect of longer development distance on the resolution can be seen in Fig. 13, where doping agents were separated from a mixture (61). Botz et al. (23c) had the developing distance further increased. By using a serial multilayer, the “long-distance” OPLC technique, they separated materials over a developing distance of more than 50 cm.

The main protein amino acids were separated in a double-layer system by Tyihák et al. (61a). The separation was performed on HPTLC silica plates that were serially coupled so the bed length was 340 mm. The mobile phase was *n*-butanol–acetonitrile–0.005 M KH_2PO_4 –acetic acid (10:5:30:10). The chromatogram was evaluated by densitometry at 490 nm after detection by ninhydrin reagent.

Empore™ silica sheets, because of their physical characteristics, cannot be used in a conventional chamber system over a 5 cm development distance (62). Due to the forced flow, OPLC makes a longer development distance and rapid separation possible on this sorbent. This is promoted also by the higher density of the sheet caused by overpressure (63).

The effectiveness of separation can also be improved with the OPLC technique by using different modified sorbent materials such as diol (64) and amino phases (65). Bis-indol alkaloids extracted from *Catharanthus roseus* were separated (Fig. 14) and determined on a laboratory-modified amino-bonded HPTLC silica gel sorbent (66). Optimization of the mobile phase was performed by the PRISMA model followed by factorial experimental design. Silica gel impregnated with tricaprylmethylammonium chloride (TCMA) was applied for separation of different groups of compounds using eluents containing methanol and water (67). The retention mechanism was not ion-pairing but hydrophobic interaction between the analytes and the caprylic groups of the TCMA.

2. Faster Development with Viscous Solvent Mixtures

Because of the forced flow, OPLC ensures a constant and high flow velocity, even in the case of viscous solvent mixtures with poor sorbent-wetting characteristics. For this reason, development time is significantly shorter than in TLC/HPTLC.

The classes of phospholipids were separated by using an *n*-hexane–2-propanol–water (40:53:7) eluent mixture. The time of development was only 20 min on 17 cm running distance (68).

Polar quaternary alkaloids in a plant extract were separated on a silica gel sheet in a distance of 14 cm; ethyl acetate–tetrahydrofuran–acetic acid (60:20:20) was used as the eluent (69). The development time was 10 min.

The development time was compared in the case of different eluents by using TLC and OPLC techniques for the separation of dinitrophenylhydrazones of saturated aldehydes and ketones (56). It was found that developing by OPLC was about 10 times faster in normal-phase systems and 5 times faster in reversed-phase systems than that in TLC.

The result was similar for the separation of organophosphorus warfare agents using diisopropyl ether–enzyme–tetrahydrofuran–*n*-hexane (10:7:5:11) as eluent (58a). When the development distance was 1.25 cm, the developing time was 59 min by TLC and 9.5 min by OPLC.

Synthetic peptides were separated on a silica gel layer by an optimized eluent system (69a). The eluent was *n*-butanol–pyridine–acetic acid–water (12:4:1:4). The time of separation was approximately 10 min. With this OPLC procedure, the separation of the investigated peptides was better than with an optimized HPLC and CZE system.

Two OPLC systems were developed for the screening of toxicologically relevant drugs in forensic and clinical contexts (58b, 69b). The eluent systems were trichloroethylene–methyl ethyl ketone–*n*-butanol–acetic acid–water (17:8:6:4) and methyl acetate–ethanol–tripropylamine–water (85:9.25:5:0.75). Both of the eluents were used on HPTLC silica plates, and for the last one a presaturation was applied (Fig. 15). The combination of the systems makes possible a fast screening for drugs in urine samples.

3.

OPLC as a Pilot Technique for HPLC

Because of the low solvent consumption and short development time of OPLC, this technique is very useful for preliminary experiments for eluent selection in HPLC. This is possible because of the linear correlation between OPLC relative retention values and logarithms of the capacity ratios obtained by HPLC (47, 70).

4. Sample Cleanup

Analysis is rather difficult when the sample contains impurities in high concentration together with the components to be measured in off-line and on-line OPLC. This situation is typical in the case of biological samples. The sample has to be purified in one or more steps before chromatographic analysis can be carried out. OPLC itself can also be used as a sample cleanup step of multidimensional systems for the separation and identification of components of complex mixtures. Interfering components migrate with the eluent front or remain at the origin on the OPLC

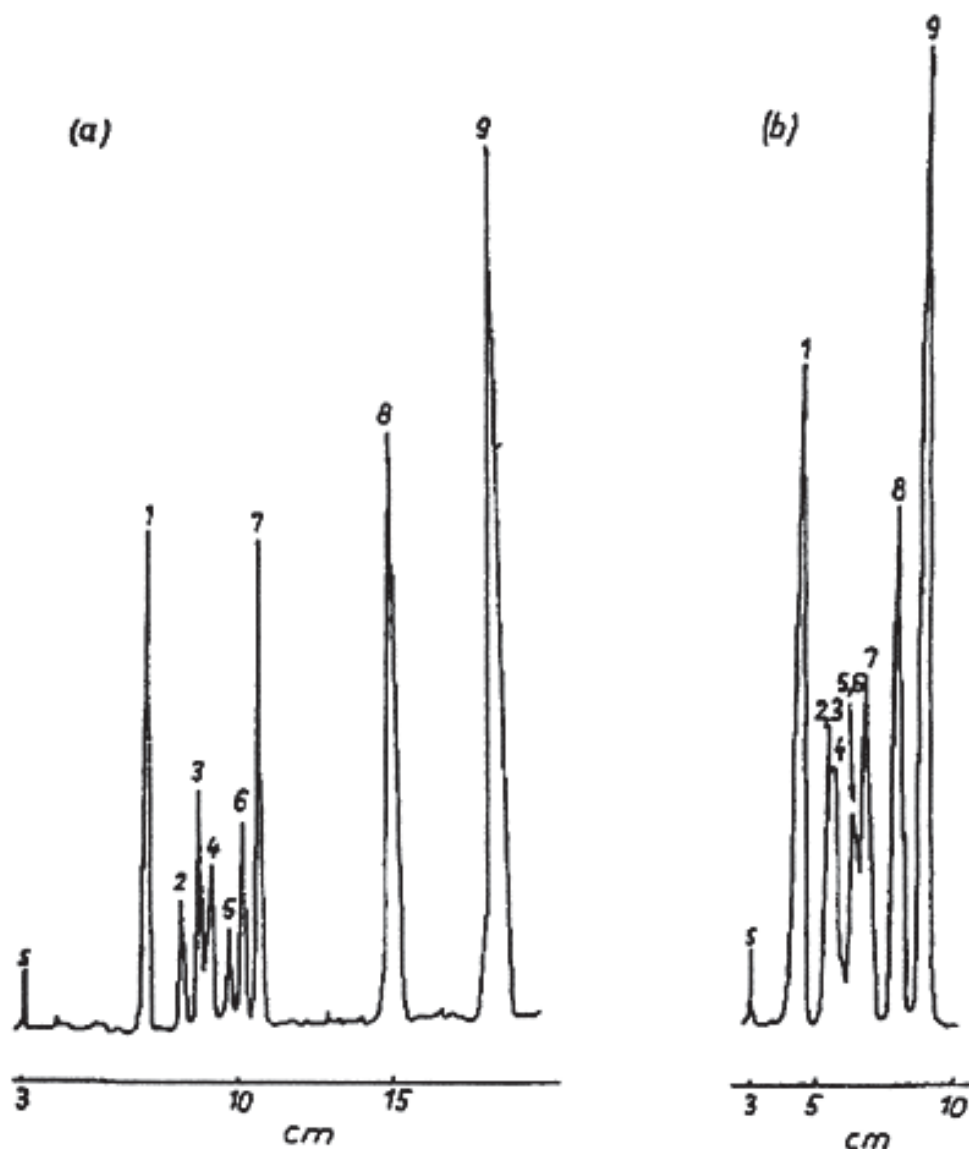


Figure 13 Separation of a mixture of doping agents. Sorbent, HPTLC silica gel 60 F₂₅₄; eluent, *n*-butanol–chloroform–methyl ethyl ketone–water–acetic acid (25:17:8:4:6). (a) OPLC method: Separation distance, about 20% continuous development; development time, 25 min. (b) Conventional TLC: Separation distance, 140 mm; development time, 95 min. Compounds: 1, strychnine; 2,

ephedrine; 3, methamphetamine; 4, phenmetrazine; 5, methylphenidate; 6, amphetamine; 7, Desopimon; 8, Coramin; 9, caffeine. (From Ref. 61.)

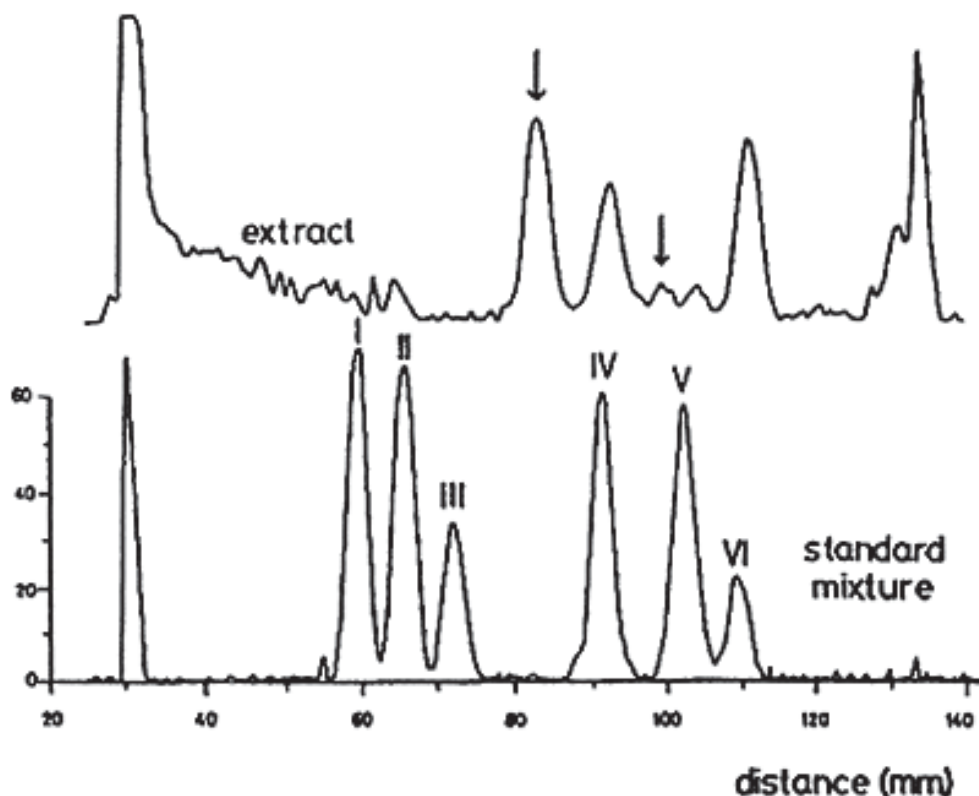


Figure 14 Separation of compounds using the optimum eluent composition achieved by factorial experimental design. Eluent: Hexane–dichloromethane–acetone–2-propanol (65:13:21:0.9); Compounds: I, Deacetyl-vinblastine; II, vincristine; III, *N*-demethyl-vinblastine; IV, vinblastine; V, de-acetoxyvinblastine; VI, leurosine. The arrows show the unidentified peaks adjacent to vinblastine in the plant extract. (From Ref. 66.)

chromatoplate, and compounds investigated can be transferred to the other chromatographic systems.

Sample cleanup for OPLC separation of complex plant extracts can be carried out with a simple gradient technique, where the first step of evaluation serves for purification of the samples. This was the technique of Kátay et al. (70a) for testing fumonisins from inoculated rice culture. The separation was performed on a reversed-phase (RP-18) sorbent layer. The first eluent for sample cleanup was acetonitrile–1% KCl (1:9), and the second eluent for the analytical separation of the components was acetonitrile–4% KCl (2.5:1). The whole process took about 1.5 h.

Using a normal-phase silica gel layer, aflatoxins in wheat were separated by OPLC (70b, 70c). The prepared samples were prewashed in situ on the sorbent layer by predevelopment in the reverse direction, from the outlet side of the OPLC equipment, with diethyl ether–hexane (1:1). The aflatoxins were then separated with chloroform–toluene–tetrahydrofuran (15:15:1).

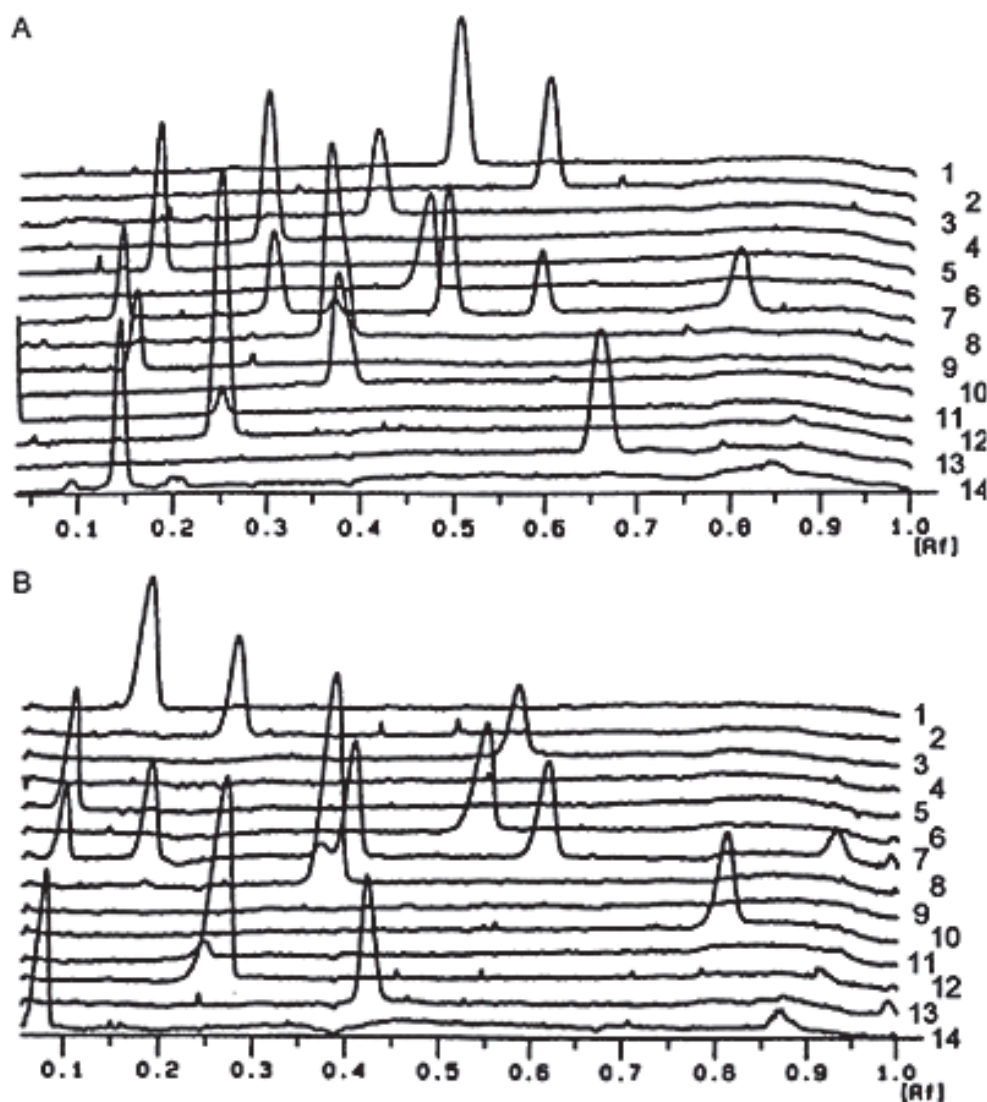


Figure 15 Fully off-line OPLC separation of selected basic drugs using two different solvent systems.

P_{ext} , 5 MPa; flow rate, 450 $\mu\text{L}/\text{min}$; volume of rapid period, 300 μL . 1, flecainide; 2, norverapamil; 3, verapamil; 4, acebutolol; 5, ethylmorphine; 6, aminophenazone; 7, correction standards [codeine ($hR_f=9$), promazine ($hR_f=19$), amitriptyline ($hR_f=40$), levomepromazine ($hR_f=60$), and dextro-propoxyphene ($hR_f=94$)]; 8, chlorpromazine; 9, atenolol; 10, trimipramine; 11, chloroquine; 12, clozapine; 13, caffeine; 14, metoclopramide. (A) Trichloroethylene–methyl ethyl ketone–*n*-butanol–acetic acid–water (17:8:25:6:4); eluent volume, 5500 μL . (B) Butyl acetate–ethanol (96.1%)–tripropylaminewater (85:9.25:5:0.75); eluent volume, 5000 μL ; 0.5 h presaturation with the eluent prior to development. (Reproduced from Ref. 58b with permission.)

The efficiency of HPLC separations can be improved by direct coupling with OPLC to transfer selected, pre-separated components to the column (71).

A coupled OPLC-GC-MS system was used for the investigation of acetylenic thiophene derivatives in extracts of *Tagetes patula* (72). The eluate from the layer, pre-separated by the on-line OPLC method, was collected and injected into a GC-MS system.

5. Simultaneous Analysis of Numerous Samples

The planar layer liquid chromatographic system has an advantage over HPLC in that more than one sample can be analyzed simultaneously. This is an important factor for routine tests on numerous samples. A further advantage of the OPLC technique is the short analysis time. Therefore, OPLC is well suited to fast screening tests. Biogenic amine content was determined in some vegetables by Kovács et al. (72a). The dansylated derivatives were separated on HPTLC silica plates with a step wise gradient elution system. The first eluent was *n*-hexane–*n*-butanol–triethylamine (90:10:9.1) and the second was *n*-hexane–*n*-butanol (8:2). Approximately 26 min was needed for simultaneous separation of sample components.

The number of samples can be doubled on a single chromatoplate by using two-directional development when the eluent is applied to the center of the plate, as can be seen in Fig. 11b. This technique was used for cleaning validation in a steroid plant (72b). Thirty swab samples taken from the hardest-to-clean areas of production apparatus were tested simultaneously by a two-directional separation. The eluent was diethyl ether–*n*-hexane (6:4). This method was suitable for cost-effective control of five steroidal hormone compounds produced in the same equipment at different times. A two-directional separation was also performed for a rapid, fully off-line separation of xanthines in plant extracts using HPTLC silica plates and chloroform–acetic acid (6:4) mobile phase. Seventy samples were processed in parallel, and the separation was complete within 5 min (49a).

A further possibility for the simultaneous analysis of numerous samples is the use of a parallel multilayer OPLC system (23b). In this case, two, three, or more chromatoplates can be used during a separation, so that 50, 100, or more samples can be developed in one run.

6. Increase of Spot and Peak Capacity

a. Combination of Off-Line and On-Line OPLC. In consequence of their additivity, spot and peak capacities can be increased by using a combination of off-line and on-line operation modes. In this case, we could first measure one part of the sample components in on-line systems, as in HPLC detection, while another part of the sample components remained on the sorbent layer after the separation. These compounds can be evaluated by means of a densitometer system (offline detection). The application of this system is illustrated by Fig. 16 (23a).

b. Two-Dimensional OPLC. The spot capacity in conventional two-dimensional TLC is increased considerably in comparison to one-dimensional TLC (31). Using overpressure to introduce a mobile phase into a pressurized chamber, the peak capacity is significantly improved in the case of two-dimensional separation (73).

Guiochon et al. (54, 74) and Beaver and Guiochon (75) integrated the advantages of two-dimensional separation in a planar system with overpressured layer chromatographic development. In two-dimensional thin-column chromatography (TCC), the flow velocities of two eluents in a closed pressurized chamber (practically a column) are controlled during the entire process, permitting the choice of an optimum eluent velocity, irrespective of other parameters of the chromatographic system.

Two-dimensional off-line OPLC was used for the perfect separation of five organophosphorus warfare agents in the presence of 17 pesticides (76) and in case of 16 closely related coumarins (77). In the latter paper, the optimization process of the eluent system and its transfer from TLC to 2-D TLC and 2-D OPLC were also discussed.

Another, more attractive, version of two-dimensional thin-column chromatography is elution of the solutes out of the bed of stationary phase and on-line detection (in real time) as in HPLC. Guiochon et al. (54) called this procedure two-dimensional column chromatography (CC).

c. Multiple Development OPLC. By multiple development a virtual lengthening of the chromatographic running distance can be achieved. Although multiple development is known in classical TLC too, it is more efficient in OPLC because of the decreased band-

broadening effect. Multiple development can be performed by consecutive development using the same or different eluents and running distances. The polar related substances of the drug levonorgestrel were tested by this separation technique (77a). Toluene–ethyl acetate–chloroform (50:10:40) was used as the eluent, with increasing consecutive running distances.

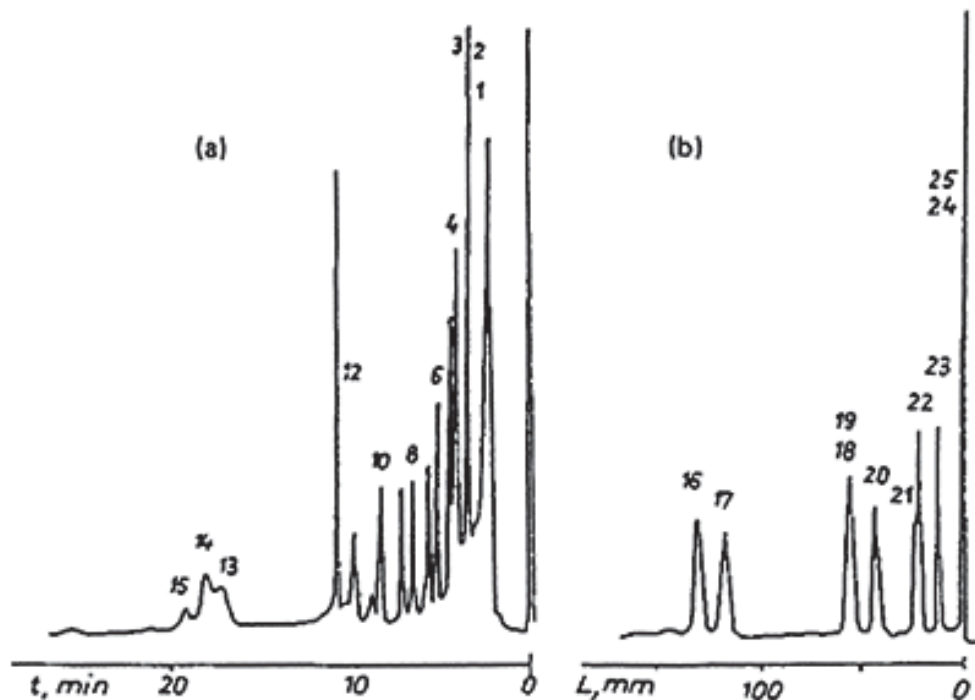


Figure 16 OPLC separation of PTH amino acids using longer elution ($k=10.5$) and combined (a) on-line and (b) off-line detection at 270 nm. Off-line sample application and prewetting from the outlet direction were used prior to separation. Chromatoplate, HPTLC silica gel 60 (Merck), preconditioned at 76% humidity; eluent, dichloromethane—ethyl acetate—acetic acid (95:5:0.5). Samples: 1, Pro; 2, Leu; 3, Ile; 4, Nle; 5, Val; 6, Phe; 7, Met; 8, Cys(Me); 9, Ala; 10, Trp; 11, Gly; 12, Tyr; 13, Lys; 14, HyPro; 15, MetO₂; 16, Thr, 17, Ser; 18, Glu; 19, Asp; 20, Asn; 21, S-CM-Cys; 22, Gln; 23, His; 24,

CysO₃K; 25, Arg. (Reproduced by
permission from Ref. 23a.)

In some cases, depending of the solubility of the sample in the eluent, disturbing “sattellite spots” can remain on the plate in the consecutive developments (77b). In those cases, overrunning can be advised to improve resolution, instead of multiple development.

7. Application of Special Detection

a. A Complex Bioautographic System. In biological systems, factors that promote and retard (inhibit) cell proliferation play a determining role. Among these factors, antibiotics are the most important molecules that originate from the antibiosis processes (77c). Recently, the resistance of microbial strains to antibiotics has become a big problem in the field of antimicrobial animal and human therapy (77d, 77e). Significant increases in the prevalence of resistance to antibiotics have been observed in common pathogens of humans worldwide (77d). Bacteria have developed mechanisms of resistance to all classes of antibiotics available for systemic use in humans (77e). Therefore, the research on these resistance mechanisms and the search of new antibiotics are important areas of pharmaceutical research (77f).

The sorbent bed in a layer arrangement is suitable for the direct bioautographic detection of biologically active substances using biological detection systems. Column techniques are not usable for such investigations. The maximum exploitation of this special, unique advantage of layer liquid systems is a logical step.

BioArena is a complex bioautographic system that integrates the up-to-date methodological and biological results of conventional bioautography with the potential of OPLC (77g). This new separation and detection system attractively exploits the possibilities of interaction between the microbes and the dye substance as well as other small molecules and macromolecules as cofactors in the sorbent bed after OPLC separation. This technical solution transfers the known advantages of OPLC to TLC/HPTLC. The advantages of the BioArena system are the optimum, changeable incubation time and the parallel or consecutive application of necrotrophic and biotrophic microbes. There is an unlimited possibility for interactions between microbes and biologically active compounds in the sorbent bed of the chromatoplate in OPLC. The cell proliferation—promoting and/or -retarding factors, as well as trace elements and other cofactors, can be used in the sorbent bed and/or inoculation (culture) media. There is a possibility, furthermore, for the application of abiotic stressors such as radiation and temperature. It seems that the BioArena system will be an indispensable methodological part of the modern OPLC technique. It has already been illustrated in the case of *trans*-resveratrol, *trans-trans*-farnesol, and capsaicin (77g).

b. Combination of OPLC with Digital Autoradiography. Discovering the metabolic pathways of a drug in animals and humans is a long process. Investigation of drug metabolites in different matrices requires combined analytical and preparative methods. To maintain the detectability of a drug and its metabolites in a biological matrix at low concentration, efficient separation techniques must be combined with various radioactivity detection methods.

Combination of off-line OPLC separation with digital autoradiography (DAR) is a powerful hyphenated technique in metabolite research (77h, 77i). After off-line OPLC separation, the ^3H -and/or ^{14}C -labeled drug and its metabolites were sensitively detected and quantified in situ on the layer by DAR. The separated components localized by DAR can be transferred for structure elucidation by various spectroscopic methods, e.g., FAB-MS (77j). OPLC-DAR was investigated to find glucuronide conjugates of ^{14}C -labeled compounds in human urine (77k).

8. Validation in Quantitative OPLC

In OPLC, development, detection, and quantitative evaluation of chromatograms may be performed in off-line and on-line modes. Off-line detection and densitometric determination of the analyte are performed in the same manner in OPLC as in classical TLC/HPTLC.

In the case of continuous development, similarly to HPLC, the OPLC chamber is connected on-line with a flow-cell detector. Ultraviolet detectors are most frequently used, but refractive index detectors (78), radioactivity detectors for radiolabeled substances (79), and mass spectrometers (80) have also been mentioned in published works as on-line connected OPLC detectors. On-line OPLC chromatograms are quantitatively evaluated just as in the case of HPLC.

In combined off-line/on-line OPLC separation, the substances remaining on the plate can be determined densitometrically, and those eluted from the layer are analyzed by an on-line flow cell detector (23a).

When developing a new method for purity testing or an assay procedure, it is necessary to prove its long-term suitability for its intended purpose i.e., it should be validated. Standardization of validation procedures can be based on the guidelines (81, 81a) of the International Conference on Harmonization (ICH), in which the different validation characteristics are clearly defined. When devising a validation plan, one should take into account these definitions and the special features and error sources of the method tested.

In consequence of the same type of sample application and detection, the validation steps of a fully off-line quantitative OPLC purity test or assay method are the same as those in classical TLC, HPTLC (82, 82a, 82b, 82c), and fully on-line OPLC as in HPLC (83–85). When the different steps are combined (e.g., off-line sample application and on-line detection), the validation procedure is specially combined, and it should be worked out.

In what follows, some experience in comparison of validation characteristics of quantitative TLC and fully off-line OPLC is discussed with reference to definitions of ICH draft guidelines (81).

“*Specificity* is the ability to access unequivocally the analyte in the presence of components that may be expected to be present” (81). For verification of specificity of a planar chromatographic method, the R_f and R_s values of the substances and their expected impurities, degradation products, or placebo ingredients may be determined.

One of the main advantages of OPLC over TLC is its better specificity and the higher available R_s , partly due to the reduced band-broadening effect. Furthermore, due to the forced flow, HPTLC plates can be used for a 15–18 cm or longer development distance in

OPLC without decreasing the efficiency of separation (Fig. 13). To obtain optimal specificity (maximal resolution) in OPLC, in addition to optimizing the mobile phase the linear velocity of eluent should also be optimized.

“*Accuracy* of an analytical procedure expresses the closeness of agreement between the value that is the accepted reference value and the value found” (81). The accuracy of a method may be characterized by the recovery rate of the analyte added to samples in known quantity. There are no data for comparing the accuracy of TLC and OPLC methods. The quality of sample preparation has an influence on accuracy; therefore, significant differences should not be expected between OPLC and TLC.

“The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be performed at three levels: repeatability, intermediate precision, and reproducibility” (81). The measure of *repeatability* is the standard deviation calculated from the results of identical samples determined not less than six times on the same chromatoplate. If determination is performed by different analysts at different times on different chromatoplates but in the same laboratory, the standard deviation of the results shows the *intermediate precision*. The variance among the results of different laboratories is called *reproducibility*.

Repeatability and intermediate precision were compared in a purity test of a drug substance determined by TLC and OPLC techniques (86, 86a). Using the optimal eluent flow rate, the relative standard deviations were lower in the case of OPLC than in conventional TLC, so the repeatability and intermediate precision were better.

“The *detection limit* (DL) of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected” (81). DL may be calculated according to the equation $Y = x \pm 3 \text{ SD}$, where x is the average, SD is the standard deviation of not less than 15 blank peak heights, and Y is the peak height for calculating the DL by use of the calibration line. Using the optimal eluent front velocity, DL was found to be lower in OPLC than in TLC because of less band broadening (86), even if the running distance was longer than that in the TLC method (87).

“The *quantitation limit* (QL) of an individual procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy” (81). QL may be calculated according to the equation $Y = x \pm 10 \text{ SD}$ (terms defined above) or based on the repeatability of peak areas of decreasing amounts of substance applied. QL is the amount at which the RSD of repeatability is equal to the repeatability of the method. Similarly to DL, QL was found to be lower (i.e., better) in OPLC at optimal linear velocity of eluent than in TLC (86).

“The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample” (81).

“The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity” (81). The regression line is constructed from peak areas plotted versus the amount of analyte applied, by using the least squares method. The linearity of the range may be tested by plotting the residuals. If residuals exist

uniformly around the regression zero line and there is no trend or one-directional variation, the calibration graph is considered to be linear.

In Ref. 86, the linearity of some calibration curves was proved by using the *F*-test. The linear range was found to be wider toward a higher quantity of analyte in TLC than that in OPLC. Exploiting the better sensitivity, the lower limit of detection and quantitation, the linear range of the calibration graph in OPLC is transferred to the smaller quantities applied.

Fluorescent derivatives of prostaglandins were separated and determined by OPLC on HPTLC plates using ethyl acetate–diethyl ether–benzene–dioxane–hexane (45:12:5:8:30) as eluent (88). The linearity of the calibration graphs was good in the range of 1–100 ng. This range was satisfactory for the determination because the detection limits of the analytes were 10–40 pg.

“The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage” (81). The robustness test of a quantitative off-line OPLC assay procedure was reported (89). The test was performed by fractional factorial design and evaluated by a half-normal probability plot. The effects of seven factors were investigated on two levels, and the method was found to be robust.

Comparison of OPLC with TLC has not yet been performed from the point of view of robustness. The difference between the features of these planar chromatographic techniques has to be taken into consideration by choosing the factors investigated. Because the vapor phase above the layer is completely eliminated, environmental circumstances have smaller effects on results in OPLC methods than in TLC.

B. Preparative Application

As with analytical OPLC, off-line and on-line methods can be distinguished in preparative OPLC applications. In the off-line OPLC method, the steps of operation after development are similar to those of conventional TLC methods: drying, scraping of the sorbent layer, elution, and crystallization. Phorbol diester constituents of croton oil were identified by off-line OPLC separation followed by extraction and chemical ionization mass spectrometry (CI-MS) (90). The on-line method is more effective for preparative applications because time-consuming scraping and elution can be eliminated.

On-line OPLC method was used for the isolation of hemp constituents (91). The cannabinoid acid fractions were analyzed by various spectroscopic methods without further purification. Biologically active compounds of plants were separated by this method using a system optimized with the PRISMA model (27). The quantities of the separated materials were between 50 mg and 0.5 g when a sorbent layer 2 mm thick was used. The development distance was between 17 cm and 36 cm, and the development time was several hours.

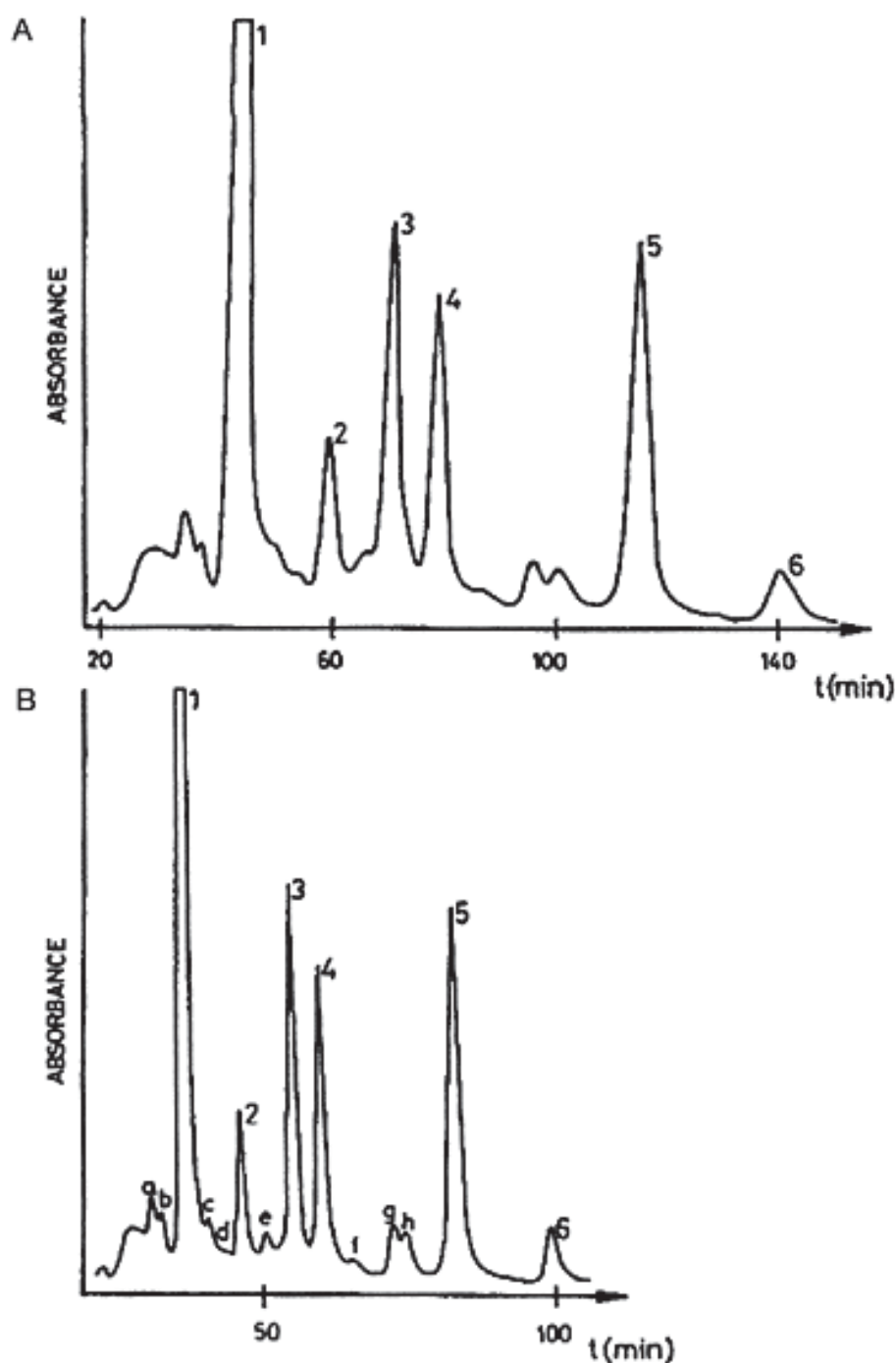


Figure 17 Fully on-line long-distance (51 cm) separation of raw extract from roots of *Pseuedanum palustre*. (A) Flow rate, 0.13 mL/min; (B) flow rate, 0.27 mL/min, on-line injection of 80 $\mu\text{g}/10 \mu\text{L}$; counterpressure, 24 bar; detection, on-line UV at $\lambda=313 \text{ nm}$; 1,

isoimperatorin; 2, columbianadin; 3, (+)-oxypeucedanin; 4, ostruthol; 5, isobyakangelicin angelate; 6, ($\pm$)-oxypeucedanin hydrate. (From Ref. 52c.)

The on-line preparative method was applied for the separation and isolation of synthetic isomers from a crude reaction mixture (92). The pure components were isolated from 70 mg of mixture during 30 min and, after identification, were used for other reactions.

Snini et al. (78) performed preparative isolation of phenolic dialdehydes from a reaction mixture by on-line OPLC. The unknown isolated materials were identified by UV, IR, and H-NMR spectrometry.

Preliminary results are available for a directly coupled on-line OPLC-MS system (80). This method proved to be very useful for detection, quantitation, and structure elucidation of different compounds.

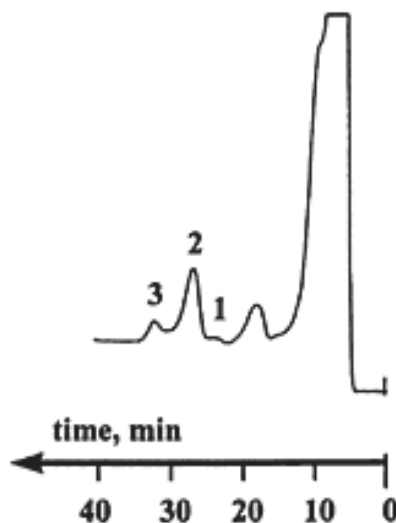


Figure 18 Fully on-line OPLC separation of tea leaf extract on a 0.5 mm thick preparative silica gel plate. P_{ext} , 5 MPa; flow rate, 1.5 mL/min; volume injected, 1750 μL . [Reproduced from Ref. 49a with permission.]

The serial multilayer development method (“long-distance” OPLC) makes a longer running distance possible. Thus, compounds from extremely complex biological matrices can be separated and isolated by this technique. Figure 17 shows the results of a fully on-line long-distance separation of a raw extract from an herb (52b).

Micropreparative OPLC separation and isolation were modeled by fully off-line OPLC and TLC on silica using various sample volumes of a tea leaf extract and 10 mm band sizes. The selected volume of optimum linear loading capacity was converted to a 0.5 thick preparative silica layer. Figure 18 shows a fully on-line OPLC separation of a tea leaf extract using a 1750 μL , (4.55 mg) injection (49a).

A combination of on-line OPLC-radioactivity detection (RD) and HPLC-RD was applied for isolation of urinary metabolites of a ^{14}C -labeled drug candidate (93). The isolated fractions of peaks of normal-phase on-line OPLC-RD (first dimension) were further purified by reversed-phase HPLC (second dimension). DAR measurement was applied to control the sorbent layer after OPLC-RD separation.

Preparative OPLC techniques and other preparative methods are reported in detail in Chapter 11.

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8

Detection, Identification, and Documentation

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I. INTRODUCTION

For detection and identification of chromatogram zones, in situ techniques are generally employed. As in an analytical disk (1), the information stored in the chromatogram can be used for various detection and identification methods, even successively, because the processes of chromatographic development and detection or identification are independent in both time and space. Detection in HPTLC takes place in the absence of the mobile phase and therefore offers much greater choices than any other chromatographic technique. This means that

1. Multiple subsequent detection of the same chromatogram is possible. In addition to recording, e.g., an absorbance or fluorescence scan using visible or ultraviolet (UV) light, a Fourier transform infrared (FTIR) or Raman spectrum can be recorded, and these methods can be followed by a suitable microchemical reaction or mass spectrometry (MS) to provide additional information.
2. Detection can be repeated with different parameters, e.g., portions of the chromatogram can be selectively evaluated.
3. Postchromatographic derivatization can easily be performed on the plate. A great variety of selective or specific reagents can be used to ease detection and identification.

Absorbance or fluorescence spectrometry and microchemical detection are commonly employed in TLC (Fig. 1). Bioactivity-based reactions, i.e., microbiological and biochemical detection methods, have gained interest for toxicologically relevant substances. In situ FTIR spectroscopy has become a practical method for detection and identification, and Raman spectroscopy has gained importance with the introduction of lasers as the light source. Furthermore, the combination of TLC with in situ MS (see

Chap. 9) can be employed. Detection and identification of radio-actively labeled substances by autoradiographic, fluorographic, spark chamber, or scanning techniques are discussed in a special chapter of this Handbook (see Chap. 12). The combination of TLC with flame ionization detection (Chap. 13) is used only for special purposes (2).

With all of these methods, accurate documentation is necessary to provide reliable and reproducible results. Factors of influence must be documented in detail. Nowadays, protocols and image documentation of the plate involve the use of computers, but drawing, sketching, tracing, photocopying, or photographing can also be used to obtain images of the plate.

II. DETECTION

For detection, physical, microchemical, microbiological, and biochemical methods are available in HPTLC. Physical detection methods mainly include either absorbance or fluorescence mea-

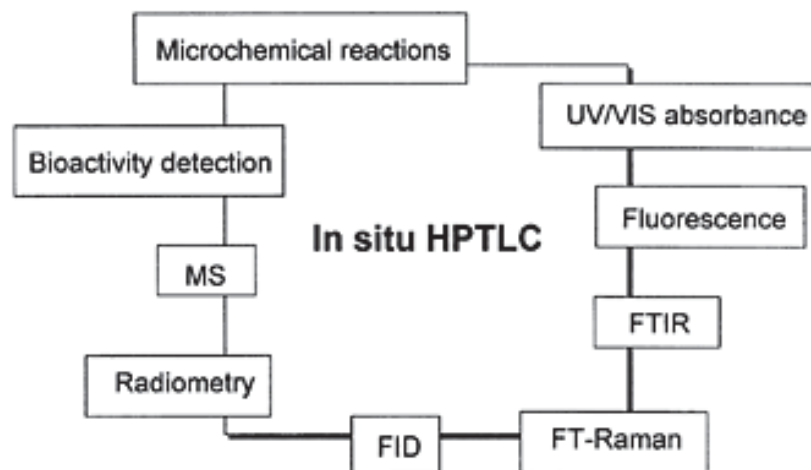


Figure 1 In situ detection and identification methods.

surement. Other physical methods are based on the difference in solubility, iodine vaporization, the addition of pH indicators, or the detection of radioactively labeled substances. Microchemical detection methods can be carried out either before chromatography (prechromatographic derivatization) or afterward (postchromatographic derivatization). Therefore, a variety of universal reagents or group characterizing reagents are available (3). Microbiological or biochemical detection methods take account of the biological and/or physiological activity of the separated components independently of their physical or chemical properties.

Detection is performed on a dry plate. After development, the plate is dried with either air or nitrogen gas, e.g., by means of a plate heater, an oven, or a hair drier, to remove the residual mobile phase. The zones are now ready for various detection methods or detection sequences, if more than one detection method is successively used.

A. Physical Detection Methods

Physical detection methods are nearly nondestructive. They mainly include the photometric measurement of either absorbance or fluorescence, i.e., the emission of electromagnetic radiation. Suitable detectors may be the eye (visual detection) or more sensitive sensors, such as a photo-multiplier for TLC scanners or a charge-coupled device for image processing systems (photometric detection). Other physical methods are based on the difference of solubility, iodine vaporization, the addition of pH indicators, or the detection of radioactively labeled substances.

1. Visual Detection

Colored substances may be viewed in daylight. Owing to the fact that such compounds absorb a particular portion of polychromatic light in the visible range, the remaining reflected radiation can be detected by the eye as the visible color of the substance zone.

Colorless substances that can be excited to produce fluorescence or phosphorescence by mostly longwave (366 nm) UV radiation can be irradiated under a UV lamp. The emitted longer wavelength visible radiation (above 400 nm) can be viewed as red, yellow, orange, green, blue, or violet zones against the dark layer background.

Colorless, nonluminescent substances that show self-absorption in the shortwave UV region can be visualized under a UV lamp (254 nm) by using HPTLC plates with a fluorescent indicator. On layers containing a fluorescent indicator, the emission is reduced in regions where UV-active substances absorb the UV light with which they are irradiated. Such substances appear as dark zones on a fluorescent background. This effect is wrongly referred to as fluorescence quenching; it should be described as *phosphorescence inhibition* because the decay of emission of radiation lasts longer than 10^{-8} s after exciting radiation is cut off. As fluorescence indicators (correctly, phosphorescence indicators), inorganic substances are mainly used, for instance, acid-resistant alkaline earth metal tungstates (4) that emit blue light, e.g., for reversed phases, or manganese-activated zinc silicates (5) that emit yellow-green light, e.g., for silica gel plates.

Various UV lamps are commercially available. The plates are best viewed in a darkened room or corner. UV lamps can be equipped with a stand that shields off extraneous light on three sides (Fig. 2). Objects up to 2 mm thick can be pushed through under the back screen. For inspection without a dark room, UV viewing cabinets (Fig. 3) are recommended. UV lamps incorporate longwave (366 nm) or shortwave (254 nm) UV light, or both. Usually the supply voltage is converted to a high-frequency current (25–30 kHz) on which the tubes operate. This ensures instantaneous illumination at the selected wavelength as well as the absence of the flickering that is observed with 50/60 Hz systems.

2. Photometric Detection by TLC Scanners

Photodetectors are more sensitive sensors than the human eye. Generally, photomultipliers are employed; these have replaced photocells in TLC scanners. Photomultipliers depend on the external photoeffect and are evacuated photocells that incorporate an amplifier. The photocurrent is



Figure 2 CAMAG dual wavelength (254/366 nm) UV lamp with stand. (Photograph courtesy of CAMAG.)



Figure 3 CAMAG UV cabinet for inspection without a dark room.
(Photograph courtesy of CAMAG.)

amplified by a factor of 10^6 – 10^8 by using secondary electrodes (dynodes). Various types of photomultipliers, e.g., “side on” or “head on,” can be employed. Photodetectors that depend on the internal photoeffect, such as photoelements and photodiodes, are also used in TLC. Photodiodes are used, e.g., for gel electrophoresis as an additional detector for transmission measurements. Photoelements such as the charge-coupled device (CCD element) are used for detection with video technology.

A diffraction grating is usually employed as the monochromator. Grating monochromators have an approximately linear wavelength scale, which can easily be automated, a constant and non-wavelength-dependent dispersion, and a higher light transmission (above 270 nm) than prism monochromators.

As light sources, continuous and spectral line sources are installed. In the UV region, hydrogen, deuterium, or high-pressure xenon lamps, and in the visible range incandescent tungsten lamps or halogen lamps are employed as continuous sources to record absorbance scans or spectra. Fluorescent substances are commonly excited with a mercury vapor lamp, a spectral line source that radiates more powerful major bands than a xenon lamp. Furthermore, lasers are being discussed for use. However, they should be tunable to afford a wider choice of usable wavelengths.

Densitometric measurements of planar chromatograms are made by reflectance, in either absorbance or fluorescence modes. Transmission measurement was used at the

very beginning of densitometry in planar chromatography, and today it is still used for the evaluation of gel electrophoresis.

a. Transmission. Densitometry of TLC plates started with the measurement of transmission in the 1960s analogously to the photometry of solutions using the Lambert—Beer law. However, an important prerequisite of that law is that the measured TLC zone does not scatter the measured light. This is not fulfilled, because the sorbent layer scatters the light to a great extent, and that is why there is often not a linear relationship between extinction and amount of substance per zone. Moreover, the adsorbent and its support (glass plate) absorb UV light, which means that transmission measurement beyond 325 nm is not possible. There are still more reasons, such as the bad signal-to-noise ratio, why transmission measurement is not reasonable in TLC. Nowadays, transmission measurement is used only for gel electrophoresis. A photodiode mounted below the object is used as detector, and a higher step resolution, e.g., 25 μm , is necessary.

b. Reflectance. The diffuse reflection of the measured light on the sorbent layer is used for reflectance measurements. Therefore, the measuring photomultiplier is aligned at an angle of 30° to the normal. The physical facts of diffuse reflection, i.e., what happens in the sorbent layer concerning reflection, refraction, and diffraction, are best described by the Kubelka—Munk equation. Reflectance can be measured in either absorption or fluorescence modes.

Absorbance measurement. Substances that absorb light in the UV or visible range can be measured by absorption. Generally, they are determined at the maximal absorption wavelength. The principle of direct absorbance measurement functions as follows. The reflected light energy is detected by the photomultiplier, i.e., photons strike the photomultiplier cathode and are intensified by the dynodes. As the chromatogram is scanned, the voltage differences produced at the detector are plotted as a function of position of measurement to yield an absorption scan. If the plate background is scanned, the full light intensity is reflected and generates the 100% signal because there are no substances that absorb the light. Thus the signal has to be inverted to get the familiar representation of the baseline. If a chromatographic zone is scanned, it absorbs a proportion of the light irradiating it and emits a lower light intensity than the plate background. This negative peak has to be inverted to generate the usual analytical peak.

The same principle is used by indirect absorbance measurement, i.e., substances that absorb between 250 and 300 nm are detected on HPTLC plates with fluorescence indicator, also called UV indicator. HPTLC plates with fluorescence indicator enable visual evaluation and ease of positioning the plate in the TLC scanner. Because fluorescence indicators absorb in the same wavelength range (between 250 and 300 nm), the stimulation of luminescence is diminished by the substance because it absorbs energy in the excitation range of the indicator. When a deuterium lamp is used, the radiation energy is low. Consequently, unlike the total radiation, the fluorescence radiation makes up only a small signal. Thus, results of indirect absorbance measurement between 250 and 300 nm on HPTLC plates with fluorescence indicator are changed by only a small extent when the deuterium lamp is used.

A further principle of indirect absorbance measurement is fluorescence quenching (correctly, phosphorescence inhibition). When the deuterium lamp is replaced with a mercury vapor lamp, the radiation intensity is much greater. The short-wavelength

emission line of 254 nm excites the fluorescence indicator much more intensely. Before the detector, a cutoff filter is inserted in the light beam to absorb the excitation wavelength of 254 nm. The plate background, i.e., the fluorescence of the indicator, is set to 100% emission. Substances that absorb in the wavelength range around 254 nm reduce the emission of the fluorescence indicator and generate negative peaks that have to be inverted. By scanning the chromatogram, the inverted voltage differences produced at the detector are illustrated as a function of measurement position, thus producing the fluorescence quenching scan. Indirect absorbance measurement is generally not as sensitive as direct measurement. The detection limits of absorbance measurement are 0.01–0.2 μg of substance per chromatogram zone in the most favorable cases.

Fluorescence measurement. In fluorescence measurement, substances are irradiated at a definite wavelength to generate the fluorescent light that is measured. For irradiation, a high-pressure mercury vapor lamp is used. It provides wavelengths of high energy that are listed in Table 1. Fluorescent substances emit the absorbed light energy instantaneously as radiation, usually of a longer wavelength than the incident light. To measure just that fluorescent light of longer wavelength, a special filter is positioned before the detector to block out the excitation wavelength.

Table 1 Emission Lines and Their Relative Intensities for the High-Pressure Mercury Vapor Lamp (St 48)

Wavelength (nm)	Relative intensity
238; 240	3
248	8
254	55
265	25
270	5
275	4
280	10
289	7
297	18
302	3
313	69
334	7
366	100
405; 408	43
436	81
546	108

Either a cutoff or monochromatic filter can be used. A cutoff filter blocks out the light beyond a definite wavelength, e.g., a K 400 filter blocks wavelengths beyond 400 nm. A monochromatic filter passes the light at a definite wavelength, e.g., an M 460 passes light at wavelengths of 460 nm.

Fluorescence measurement functions as follows: In a scan of the substance-free background, no signal is measured because the excitation wavelength is blocked out by the filter. If a fluorescent zone is scanned, it emits light of longer wavelength that passes through the filter and thus generates a signal at the detector, i.e., a peak. The most luminescent substance is set to 100% emission.

Fluorescence measurements have the following advantages compared to fluorescence quenching or absorption measurements:

Increased selectivity. An increased selectivity is caused by two factors: (a) matrix that is not fluorescent is not measured and (b) both excitation and emission wavelengths can be selected. The ideal combination of these wavelengths eases detection and quantitation. For example, with a 600 nm filter, an orange fluorescent substance can be detected and easily quantified even when a blue fluorescent substance is overlapping that zone. In this case, bad resolution is compensated for by good detection selectivity.

Increased sensitivity. Compared to absorption measurements, fluorescence measurements are more sensitive by a factor of 10–1000. Normally, substances on the plate can be detected in the picogram and lower nanogram range.

Signal is independent from zone shape. The distribution of the substance within the zone has no influence on the signal if the scanning slit passes over the whole zone. Thus, for fluorescence measurements a slit length longer than the zone diameter or length is selected.

Increased linearity. Between fluorescence intensity and substance concentration there exists a linear relationship over a wide concentration range due to the applicability of the Lambert—Beer law (Chap. 10).

For these reasons, for inherently fluorescent substances, fluorescence measurement should be preferred, and for nonfluorescent substances, derivatization reactions to render them fluorescent should always be considered for optimal detection and quantification.

3. Photometric Detection by Video Technology

Photodetectors depending on the internal photoeffect, such as the charge-coupled device (CCD element), are used for detection by video technology and are incorporated in video cameras or digital cameras. As a video camera, a 3-CCD color camera, a 1-CCD color camera, or a 1-CCD monochrome (black-and-white) camera can be employed. All cameras are equipped with the long-time integration feature because the standard exposure time of 20 ms is often not sufficient to detect weakly fluorescent substances.

Images recorded with a video camera are digitized, creating a color or gray scale image of the chromatogram. This is performed by a video documentation system (Fig. 4), which is composed of

A high resolution, highly sensitive CCD camera, capable of long-time integration

Special hardware, normally called a frame grabber, that digitizes the analog video signal (supplied by the CCD camera) into the digital PC memory

A lighting module with direct UV light of 254 or 366 nm wavelength, direct or transmitted white light, or all combined, and a cabinet cover to shield off extraneous light, with a camera stand



Figure 4 CAMAG Reprostar 3 with cabinet cover, camera bellows, camera support, and 3-CCD camera with zoom objective. (Photograph courtesy of CAMAG.)

A computer with monitor, printer (with good color and graphics capabilities), and special software for chromatogram documentation

Using professional software for chromatogram evaluation, tracks are marked on the chromatogram image and converted to analog curves by considering the average gray scale level of the pixels in each line of the selected track. Integration of the analog curves and their quantitative evaluation are easy to handle and very fast because all tracks are simultaneously integrated and evaluated in response to a mouse click. As an additional new feature, tracks of different plates can be compared with each other by superimposing the analog curves. This kind of profile comparison of several tracks on different chromatograms is used, e.g., for pattern recognition in drug analysis.

Strong points of chromatogram evaluation with video technology are speed of evaluation, low cost (only additional software for evaluation is needed), and time-independent evaluation, i.e., electronically saved chromatograms can be evaluated at any time. However, only the visible part of the spectrum is used (similarly to the human eye). Thus, only visible substances, fluorescent substances excited at a wavelength of 254 or 366 nm (offered by the lighting module), or substances that absorb around 254 nm on fluorescent plates (fluorescence quenching) can be detected. Therefore, a spectral range comparable to that offered by TLC scanners is not available, and spectral selectivity and recording of spectra are not possible. Sensitivity, accuracy, and precision may become comparable to those of TLC scanners, but only in certain cases, e.g., when the absorbance of the substance is at or near the excitation maximum of the fluorescence indicator (254 nm). In general, sensitivity, accuracy, and precision are not quite as good as those achieved by densitometry with TLC scanners, but in most cases they are sufficient for the analytical task. That is why, especially with its rapidity and ease of handling, video technology is used for detection and evaluation of the chromatograms. However, the strong points of densitometry with TLC scanners are spectral selectivity, use of the entire UV range down to 190 nm, recording of spectra, and high accuracy and precision.

4. Other Physical Detection Methods

Another physical method is based on lipophilicity. Lipophilic substances on hydrophilic adsorbents such as silica gel or aluminum oxide can be viewed and marked by spraying or dipping the plate in water (using special plates that can be wetted with water). Transparent layers result that show the lipophilic substances as dry white zones that can be recognized best by holding the TLC plate against the light. With this kind of solubility detection, compounds such as herbicides, hydrocarbons, sapogenins, phosphoinositides, and triterpene derivatives can be detected.

In the same way, aqueous dye solutions such as methylene blue or patent fast blue are employed instead of water. Lipophilic substances such as anion-active detergents appear pale on a transparent blue background. This phenomenon is the reverse on reversed phases, in which case the lipophilic part of the detergent is aligned with the RP chains whereas the hydrophilic part is colored by the dye, and therefore deeply colored blue zones appear on a pale background. Using lipophilic dye solutions for the detection of lipophilic substances on a hydrophilic phase yields dark zones on a pale background.

The fact that substance zones dipped in or sprayed with fluorescent solutions lead to increased fluorescence can be exploited as well. For example, spraying lipophilic substances with a dichlorofluorescein solution produces yellow-green fluorescent zones on a purple background.

Moreover, the reversible reaction of iodine vaporization can be employed as a universal detection method for lipophilic substances such as indoles, amino acids, steroids, or lipids. The solvent-free chromatogram can be treated with iodine vapor or dipped in or sprayed with an iodine solution. Iodine dissolves in or forms weak charge transfer complexes with most organic substances, leading to first slightly yellow then dark brown zones on a pale yellow or tan background. To stabilize the iodine on the chromatogram plate, the plate can be immersed in or sprayed with a dilute starch solution to produce blue iodine inclusion compounds that are stable for a long time.

For detection of acidic or basic substances, pH indicators can be employed. Dipping or spraying the chromatogram with an indicator solution whose pH is adjusted to be close to the endpoint of the basic or acidic substances results in a change in their color.

B. Microchemical Detection Methods

In addition to physical methods of detection, chemical derivatization methods can be employed to yield or complement results. Derivatization to colored, fluorescent, or UV-absorbing compounds can be carried out pre- or postchromatographically. Prechromatographic derivatizations, during sample preparation or on the starting zone of the HPTLC layer, are employed primarily to improve selectivity of the separation by changing of substance properties and to stabilize labile substances that would degenerate during chromatography. The main purpose of postchromatographic derivatizations, however, is to visualize substances, i.e., to render them detectable, and to improve detection limits and the linearity of the calibration function.

1. Prechromatographic Derivatization

In contrast to derivatization during sample preparation, in which case samples and standard solutions have to be treated individually one after the other, derivatizations performed as in situ reactions at the starting zone or in the concentration (preadsorbent) zone of the TLC plate offer the following advantages:

1. Derivatization reagents can be automatically applied on the layer, and derivatization is performed simultaneously on all tracks. In contrast to derivatization of each single sample and standard solution, prechromatographic derivatization on HPTLC plates is rapid and easy to handle.
2. If substances are not stable, prechromatographic derivatization can produce stable zones for analysis.
3. Reactivity of substances, e.g., toward the stationary phase, can be reduced.
4. Linearity of calibration curves can be improved.
5. Sensitivity of detection can be increased by adding a chromophore or fluorophore to the analyte molecule.
6. Chromatographic selectivity can be improved by specific chemical derivatization.

In practice, the derivatization reagent is applied as a band first. Then the sample or standard solution is applied onto the same starting zone. This method of application is called overspraying. The solvent in which the sample or standard solution is dissolved should not cause the reagent to spread outward. If necessary, the reagent solution can be

applied once more so that it is present in excess or a second reagent solution, necessary for the proper reaction, can be applied. Finally, the starting zone should be covered with a glass strip before being placed on a hotplate or in an oven if heat is necessary to accelerate the reaction. The derivatization reagent can also be applied as a vapor. The layer, except for the applied substance zones, is covered with a glass plate and placed in a chamber over the vapor of the reagent that produces the derivatization reaction. Examples of prechromatographic in situ reactions are compiled in Table 2. For quantification, derivatization products have to be proportional to the quantity present on the layer, and derivatization reagents in excess should not interfere with the subsequent chromatographic separation. If necessary, a prechromatographic run can be used to separate the excess derivatization reagents.

2. Postchromatographic Derivatization

Colorless, nonluminescent substances that cannot be detected by UV absorbance, fluorescence quenching, or prechromatographic derivatization have to be derivatized after chromatography to render them detectable. The primary purposes of postchromatographic derivatizations are to

- Visualize substances
- Increase selectivity of detection

Table 2 Examples for Prechromatographic Derivatizations In Situ

Reaction	Compound class	Derivatization reagent	Ref.
Acid hydrolysis	Cardenolide glycosides	37% Hydrochloric acid	6
Alkaline hydrolysis	<i>n</i> -Hexadecyl esters	Methanolic sodium hydroxide solution	7
Enzymatic hydrolysis	Digitalis glycosides	Luizyme solution	8
Oxidation	Geraniol	20% Chromic acid in glacial acetic acid	9
Reduction	Alkaloids	Sodium borohydride solution	10
Chlorination	Acetanilides	Chlorine vapor	11
Bromination	Capsaicinoids	Bromine vapor	12
Iodination	Phenolic steroids	Iodine vapor	13
Nitration	Phenols	Nitrous vapor	14
Diazotization	Estriol	Saturated ethanolic Fast Dark Blue R salt solution	15
Hydrazone formation	Aldehydes and ketones	2 N 2,4-Dinitrophenylhydrazine in acetic acid	16
Esterifications	Aflatoxins	Trifluoroacetic acid	17

Etherifications	Carboxylic acids and organophosphoric acids	Ethereal diazomethane solution	18
Dansylation	Fatty acids	Dansyl semicadaveride solution; <i>N,N'</i> -dicyclohexylcarbodiimide solution	19

Improve sensitivity of detection

Improve the linearity of the calibration function

The substance concentration needed for derivatization is reagent-dependent because not every derivatization reagent can detect the substance at the same sensitivity level. Therefore, a separation can seem to be a good one when the outer part of the substance zones is not detected because substance concentration is too low for reaction with the derivatization reagent. This effect can confuse the results. With increasing R_f values diffusion increases, thus leading to a lower zone concentration and less detection sensitivity. That is why visualization of substances in the chromatogram is reduced by increasing R_f values.

Postchromatographic reactions can be performed as universal reactions or with functional group selectivity. Universal reagents react with a wide variety of compound types:

Hydrochloric acid vapor reacts with organic substances to form colored products and finally dark brown carbon (20).

Phosphomolybdic acid causes blue-black zones to appear against a yellow background (21).

Anisaldehyde-sulfuric acid reacts with natural products and leads to differently colored zones, whereby zone identification is possible (22).

Antimony(III) or (V) chloride produces zones of different characteristic colors on a white background (23).

Ammonium hydrogen carbonate vapor reacts with many organic compounds and leads to fluorescent products when heated (15).

Zirconium salts form mainly yellow green to blue fluorescent zones (24).

Sequences of microchemical detection (3), i.e., the consecutive application of different derivatization reagents onto the plate, can be used for complex mixtures. An intermediate drying or heating step is employed, and the chromatogram documented and/or evaluated after each derivatization step.

Examples for functional group-specific reagents are listed in Table 3.

Table 3 Functional Group-Specific Postchromatographic Derivatizations

Functional group	Derivatization reagent	Reference
Acetylene compounds	Dicobalt octacarbonyl	25

Aldehydes	2,4-Dinitrophenylhydrazine	26
Alcohols	Lead(IV) acetate dichlorofluorescein	26
Amines	Ninhydrin	26
Carboxylic groups	2,6-Dichlorophenyl indophenyl (Tillmans' reagent)	26
Halogen derivatives	Ammoniacal silver nitrate (Dedonder's/Tollens' or Zaffaroni's reagent)	27
Ketones	2,4-Dinitrophenylhydrazine	26
Nitro derivatives	Benzocyanide benzyltrimethylammonium hydroxide	28
Peroxides	1-Naphthol/ <i>N</i> ⁴ -ethyl- <i>N</i> ⁴ -(2-methyl-sulfonamidoethyl)-2-methyl-1,4-phenylenediamine	26
Phenols	7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride)	26
Thiols	7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride)	26

In practice, the derivatization reagent is added by exposure to vapor, by immersion, or by spraying after evaporation of the mobile phase. The procedures for chromatogram immersion or exposure to vapor are preferable because of their precision and repeatability. The derivatization reagent may also be added to the mobile phase if the reagent is evenly spread over the layer and if it elutes with the solvent front. For example, acids have been added to the mobile phase for detection of quinine alkaloids (29), and fluorescamine has been added for detection of biogenic amines (30).

a. Immersion. The procedure of dipping or immersing the chromatogram plate into a derivatization reagent solution offers three advantages:

1. Contamination with toxic derivatization reagents is reduced in comparison with spraying or evaporation. When the immersion device is not in use, it is always covered with a stainless steel lid.
2. Consumption of derivatization reagents is low if reagents are used repeatedly.
3. The layer is homogeneously coated with the reagent. This results in a better baseline structure, consequently a lower detection limit, and better reproducibility compared with spraying methods.

A chromatogram immersion device is available from CAMAG (see Chap. 5). The derivatization reagent is poured into a dip tank that holds either 20×20 cm or 20×10 cm plates. Then the dried chromatogram is automatically immersed and withdrawn at a uniform speed. The advantage of using an immersion device instead of manual dipping is the uniform speed of immersion and withdrawal of the plate. Thus, irregularities of manual dipping, such as tide marks that can interfere with densitometric evaluation, are avoided. The vertical speed (selectable between 30 and 50 mm/s) as well as the immersion time (selectable between 1 and 8 s) can be set as required. After immersion, the chromatogram is removed slowly to allow excess reagent to drain back into the dip tank and the back of the plate is cleaned off. Then the plate is dried with either air or nitrogen gas, or it is heated with a plate heater to start the derivatization reaction.

Generally, dipping solutions are about 80% less concentrated than corresponding spray solutions, and if necessary they can be modified during preparation. For instance, water is often replaced with an alcohol or another lipophilic solvent because on the one hand water can dissolve the silica gel layer and on the other hand it cannot penetrate reversed phases. But, of course, dipping solutions must not dissolve the substances or their reaction products out of the stationary phase. If the dipping solution is too polar, it can penetrate the layer, thus leading to more intense zones at the back of the layer than at its surface. In this case, the dipping solution has to be rendered less polar. A chromatogram immersion device can also be employed to impregnate adsorbent layers with a detection reagent prior to sample application. This preimpregnation method has been used successfully with silver nitrate and with phosphomolybdic acid.

b. Exposure to Vapor. The most homogeneous way to cover the chromatogram with a derivatization reagent is by exposing it to vapor. For instance, iodine can be sprayed onto the chromatogram as a 1% alcoholic solution, but it is simpler to place the plate in a closed standard developing chamber that contains a few iodine crystals at the bottom and is saturated with iodine vapor. Twin-trough chambers or special conditioning chambers can be used for this purpose as well. Surprisingly good quantitative results can be obtained using another HPTLC plate, which can be exposed to iodine vapor for several days and then used as a source of vapor for the investigated plate for a time ranging from a few minutes to a couple of hours (31). To stabilize the iodine on the chromatogram plate, the plate can be immersed into a dilute starch solution to produce blue iodine inclusion compounds that are stable for a long time. Iodine vapor allows nonspecific, and in most cases nondestructive, detection of many lipophilic substances such as indoles, amino acids, steroids, and lipids. Besides iodine, bromine, chlorine, formaldehyde, ammonia, diethylamine, ammonium hydrogen carbonate, acids, or sulfur dioxide can be applied as vapors.

c. Spraying. For spraying the chromatogram plate with derivatization reagents, electropneumatic sprayers (Fig. 5) or simple glass sprayers with a rubber bulb pump are mainly used. Alternatively, a computer-controlled instrument, the Chromajet (Desaga), can be employed to spray on defined amounts of derivatization reagent. Derivatization reagents are atomized into a fine aerosol spray with particles in the range of 0.3–10 μm . Glass sprayers can also be operated using a compressed air or an inert gas. Usually the derivatization reagent solution is sprayed onto the layer at a pressure of 0.6–0.8 bar. Spraying should be performed in a spray cabinet, which ensures the complete removal of excess spray from the sprayer and of spray particles that have rebounded from the chromatogram plate. The spray jet is not deflected before it reaches the chromatogram, an effect that often occurs in a normal laboratory fume hood. Spraying is carried out manually from a distance of 20–30 cm. It is performed two-dimensionally (first horizontal then vertical lines) in a meandering pattern, returning outside the chromatogram. The very first spray should be directed beside the TLC plate until a very fine aerosol spray is supplied. However, sprayers are operated manually and can never be used very uniformly. That means that the resulting chromatogram visualization differs from individual to individual and from spraying to spraying. Reproducibility is not as good when a chromatogram immersion device or evaporation application is used. However, spraying cannot be circumvented when two derivatization reagents



Figure 5 CAMAG TLC sprayer.
(Photograph courtesy of CAMAG.)

have to be applied successively without intermediate drying. Aerosol sprayers using fluorogenated hydrocarbons, etc. as the propellant gas, quite common in former years, should be avoided for environmental considerations. After each use, the sprayer should be cleaned by spraying with a suitable solvent to prevent clogging. Some derivatization reagents, like manganese heptaoxide and perchloric acid—containing reagents, sodium azides, and iodine azide solutions can cause explosions in the exhaust ducts of the spray cabinet and should never be sprayed. Plates should preferably be immersed into such reagents.

d. Heating. After immersion into, spraying with, or vapor application of the derivatization reagent, heating is often necessary to produce the required color or fluorescence of the substance zones, i.e., to start and complete the derivatization reaction. Instead of a plate heater, which is commonly used, an IR source, a microwave apparatus, or an oven can be employed. A plate heater (Fig. 6) can usually be regulated over a temperature range of 25–200°C. The temperature is uniformly maintained over the entire surface of the heating plate. However, chromatogram plates should be positioned in the center of the heating plate. Programmed and actual temperatures are digitally displayed. Temperature and heating time depend on the derivatization reagent and sorbent layer used.

3. Stabilization or Intensification

Generally, chromatograms should be protected from light and oxygen during storage. After de-derivatization, it should be determined that the chromatogram will be sufficiently stable until it is evaluated. Various stabilization treatments can be employed if a reduction in the fluorescence or color intensity is observed (3). For colored substances, for example, cadmium or copper salts can be added if a ninhydrin reagent has been used, a sodium nitrite solution can be sprayed to stabilize

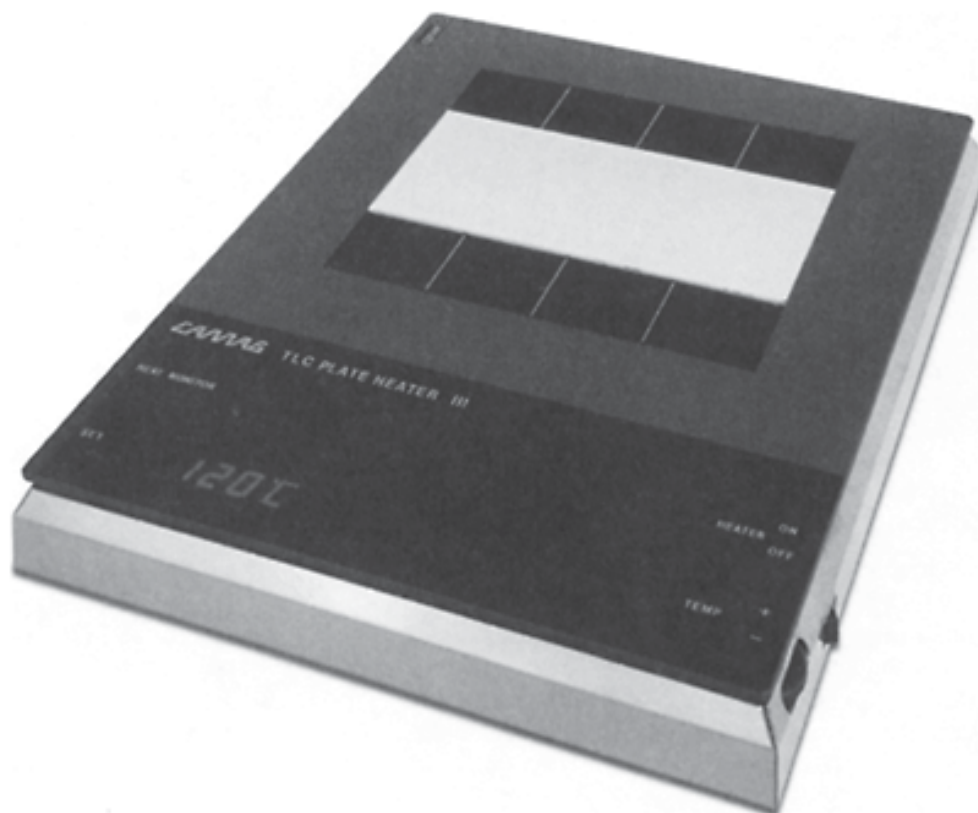


Figure 6 CAMAG TLC plate heater.
(Photograph courtesy of CAMAG.)

the van Urk reaction with lysergic acid derivatives, or an ammonia solution or ammonia vapor can be employed to stabilize the reaction of tryptamine with 2,6-dibromoquinone-4-chloroimide. For fluorescent zones, the chromatogram plate is treated with a viscous lipophilic or hydrophilic agent. These agents evidently influence the rotation of the molecules and keep out the ambient air to help eliminate quenching. As lipophilic stabilization agents, particularly liquid paraffin, but also silicone, kerosene, isooctane, or dodecane are used at low concentrations. Often, more concentrated solutions additionally yield an intensification of the fluorescence. As hydrophobic stabilization agents, for example, polyethylene, triethylamine, triethanolamine, or Triton X-100 are used.

C. Bioactivity-Based Detection Methods

Microbiological and biochemical methods of detection do not exploit chemical or physical properties but the biochemical or biological-physiological activity of substances. Bioactivity-based reactions are employed mostly for the detection and determination of environmental or toxic compounds such as pesticides (insecticides, fungicides, herbicides), antibiotics, alkaloids, myco-toxins, cytotoxins, hot or bitter substances, and saponins. Such compounds have in common that they stimulate or inhibit an appropriate enzyme or test organism during incubation. Either enzymes or test organisms can be applied directly onto the sorbent layer (bioautographic detections) or the sorbent layer

itself is placed on the test organism medium (reprint methods). For biochemical detection, enzymes are used. Appropriate test organisms for microbiological detection can be mold spores, yeast cells, cell organelles, or bacteria in a nutrient medium.

For instance, saponins are detected by blood cells. After chromatography, a blood—gelatin suspension is directly applied onto the layer. Then active agents diffuse from the layer to the blood—gelatin suspension and stimulate or inhibit the test organism during incubation. Saponins cause hemolysis of blood cells, so they are visible as transparent, nearly colorless zones on a turbid red blood—gelatin background.

Antibiotics in environmental samples can be detected by the bacterium *Bacillus subtilis*. The plate is dipped in the bacterial solution. After incubation, the plate is sprayed with MTT—tetra-zolium salt reagent, which, after incubation, gives a blue-violet background. Antibiotics inhibit the growth of the bacteria and cause bright zones of inhibition on the colored background (Merck Chrom Biodip[®], bioautography test kit).

The principle of enzymatic reactions is the formation of an enzyme—substrate reaction (32). The developed chromatogram is dipped in an enzymatic solution, e.g., a solution of cholinesterase, and incubated for a short period. Then it is dipped into a substrate solution, e.g., 1-naphthyl acetate/Fast blue salt B. In presence of the active enzyme, 1-naphthyl acetate is hydrolyzed to 1-naphthol and acetic acid. Further, 1-naphthol is coupled with Fast blue salt B to form a violet-blue azo dye. This enzyme-substrate reaction is inhibited by pesticides, such as organophosphates, organochlorines, carbamates, or pentachlorophenol, which inhibit the enzyme cholinesterase. Consequently, such substances cause bright zones of inhibition on a violet-blue background (33, 34). Also, other enzyme test systems, such as (chymo)trypsin, elastase, urease, amylase, aminolevulinic acid dehydratase, vegetable peroxidase, or catalase can be applied.

Advantages of bioactivity-based detection are

1. High specificity and reduced interference of the matrix, leading to a reduced need for sample cleanup.
2. More sensitive detection limits, i.e., typical detection limits are found to be in the sub-nanogram and even in the lower picogram range.
3. Coupling of chromatography with bioactivity, allowing identification of toxic compounds, degradation products, or metabolites, not just the summation of damaging effects in a specified test system as in biomonitoring tests. Separated fractions are stored in the chromatogram and can easily be used for bioactivity—based reactions.

Coupling of bioactivity detection with TLC enables the assignment of physically detected substances to a specific activity, which means that toxic active substances can be identified, not just detected. Thus, in a sample, further unknown toxic compounds that affect the test system can be detected, leading to a complete toxicity profile of the sample related to the test system.

III. IDENTIFICATION

In planar chromatography, a substance is identified by comparison with an authentic standard cochromatographed on the same plate. Parameters that are compared are the hR_f

value, the analog curve (absorption at a definite wavelength), the color of the zone if the zone is visible or inherently fluorescent, the spectrum, and/or the reaction with a derivatization reagent.

Unknown substances can be detected and identified by a special feature, called a multiwave-length scan (CAMAG TLC ScannerS), with which the plate is automatically scanned at up to 30 different wavelengths. The analog curves at the different wavelengths are overlaid in one graphic (Fig. 7), and the spectral and chromatographic properties are compared to a series of identification standards. Thus, unknown constituents of a sample can be detected and identified over a wide wavelength range.

For fingerprint identification, all samples on one plate are compared to one another at the same time. If necessary, analog curves of samples on different plates are overlaid by a special feature of the CAMAG VideoScan. Thus, for example, in plant analysis the chemical constituent profile is linked to the botanical identity of a plant.

Generally, UV/vis spectra are recorded because they can easily be measured with a conventional TLC scanner. The spectrum can then be compared with a standard cochromatographed on the same plate or with a spectral library. However, if possible, more information is given by recording an FTIR, Raman, or mass spectrum. In former times, zones of interest were recovered by extraction from the adsorbent and were then characterized by FTIR-, Raman, or mass spectrometry. Nowadays, there seems little need to go through time-consuming recovery procedures. FTIR or Raman spectra can be directly recorded on the plate using appropriate instrumentation. Recording of in situ mass spectra is described in detail in Chapter 9, and the detection and identification of radioactively labeled substances in Chapter 12. For identification of very complex mixtures, coupling of separation methods (especially HPTLC with HPLC) is used to cope with difficult separations and to get rid of interfering matrix.

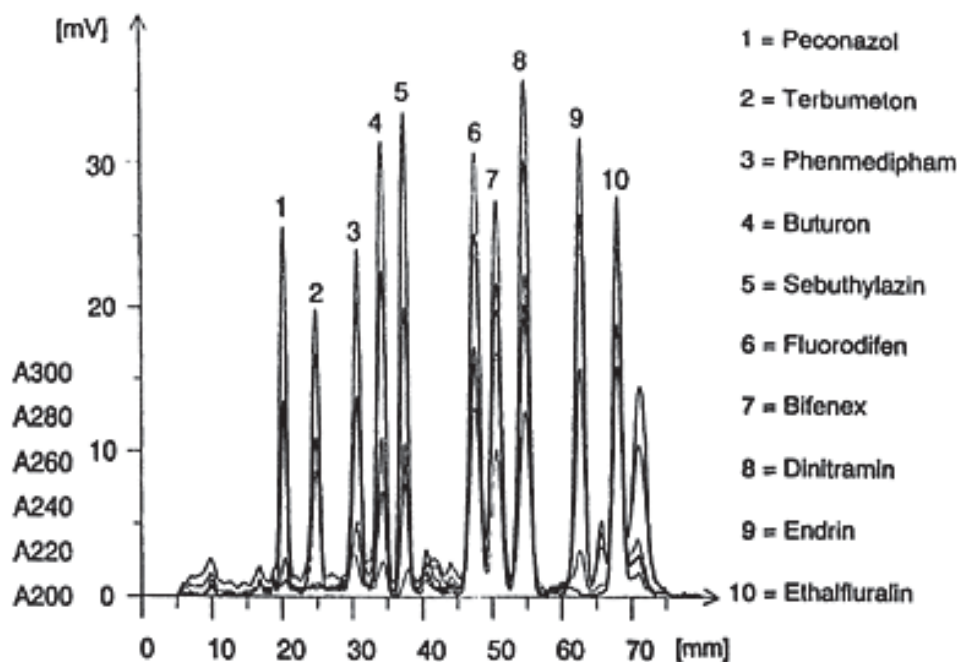


Figure 7 Multiwavelength scan of pesticides recorded at six different

wavelengths that are super-imposed to get a spectral scan of the track.

A. Ultraviolet/Visible Spectra

Spectral data can be processed after the chromatographic run to identify individual fractions by comparison with spectra of authentic standards cochromatographed on the same plate or stored in a spectral library (Fig. 8). Spectra can also be recorded to check identity by superimposing the spectra of all fractions within the same R_f window. Moreover, the spectral data allow the determination of the optimum wavelength(s) for quantitative scanning and the checking of the purity of fractions by superimposing the spectra from different positions within a spot.

If substances are well separated and possess different chromophores, their UV/vis spectra can be used for recognition and identification, as shown in Fig. 9. However, in the case of unknown mixtures, it is necessary to employ other identification methods such as direct in situ FTIR measurement, because for example, phenazone scarcely differs from other pyrazolone derivatives such as propylphenazone, and caffeine does not differ from purine derivatives such as theophylline or theobromine. The situation is similar for designer drugs of the 3,4-methylenedioxybenzene series. They can be well separated by chromatography, but they cannot be distinguished at all by means of their UV spectra (Fig. 10a). However, this is possible after derivatization with a definite reagent solution (Fig. 10b).

Ultraviolet/visible (UV/vis) spectra can easily be recorded by a conventional TLC scanner that is described in detail in Chapter 5. The spectrum is automatically corrected by measuring the spectra of the lamp or light, the plate background, and any solvent traces thereon, i.e., a substance-free area of the layer:

$$\lambda_{\text{corrected}} = \lambda_{\text{substance on HPTLC plate}} - \lambda_{\text{lamp}} - \lambda_{\text{plate background}}$$

HPTLC spectra usually correspond to the spectra of the same substances in solution. However, either bathochromic or hypsochromic shifts can be caused by interaction of the substance molecules with adsorbents (e.g., silanol, amino, or poly amide groups) and with any solvent traces still on the plate (e.g., if acids or bases have been used in the solvent mixture). Thus, HPTLC spectra are compared to authentic standards chromatographed on the same plate or are searched for in a self-made spectral library.

HPTLC spectra are dependent upon the amount of substance, especially in the range of the detection limit. At low amounts, the bondings between adsorbent and substance influence the reflection, whereas at high amounts only the substance itself contributes to the reflection. This means that spectra have to be compared at similar concentration levels.

For spectral comparison, the correlation or difference of spectra can be displayed as well as the overlaying of the spectral shape. Spectra of unknown substances can be searched for in libraries. As search criteria, the following are used:

The characterization number (wavelength maxima, the number of wavelength maxima, and similar features of the spectra)

The position [migration distance, R_f value, hR_f value (corrected R_f value)]

The correlation (statistical comparison of two spectra)

A list shows the best matching spectra. Up to 1200 self-recorded spectra can be saved in one library file. Besides comparing spectra of unknown substances with those in a library file, spectra of two different library files can be compared. In this way, application-specific library files can be compiled.

B. FTIR Spectra

Direct in situ HPTLC-FTIR measurement is carried out by diffuse reflectance using a diffuse reflection infrared Fourier transform (DRIFT) spectroscopic unit (Fig. 11) (35). It is necessary to take into account the fact that at wavelengths at which the absorption is great and the refractive index is high, the incident radiation is almost 100% normally reflected at the surface so there is scarcely any diffuse reflection. However, only this part of the reflection contains the spectral information concerning the sample, in contrast to the normal (Fresnel) reflection. This means that reflectance minima, and not the expected reflectance maxima, are obtained at wavelengths of

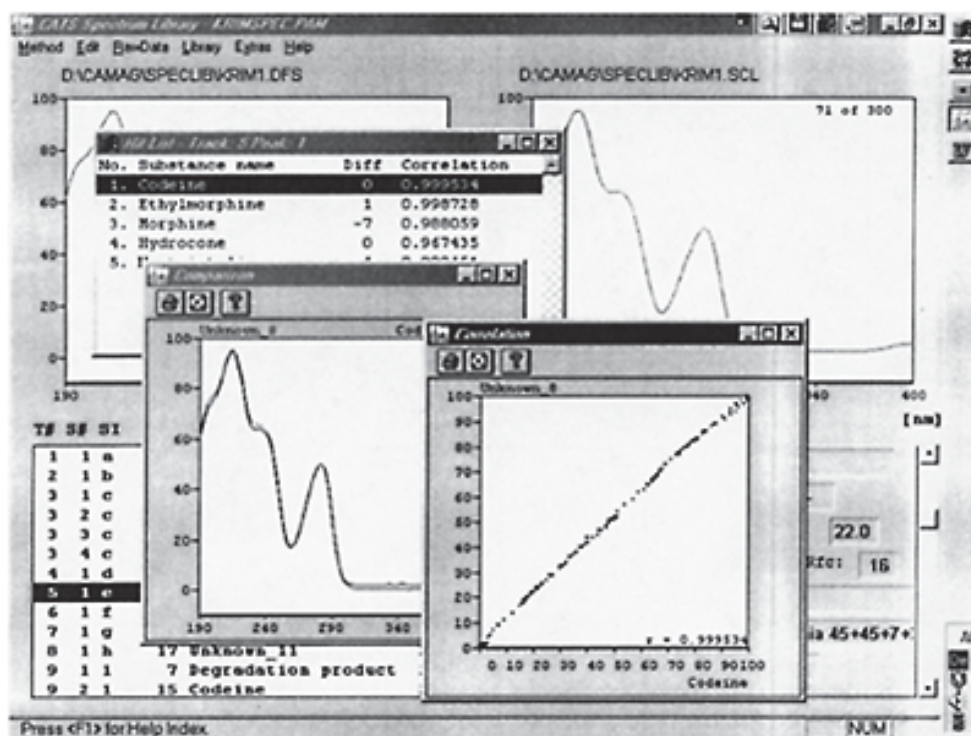


Figure 8 Spectrum library search; codeine was found to be the best hit for the unknown. (Photograph courtesy of CAMAG.)

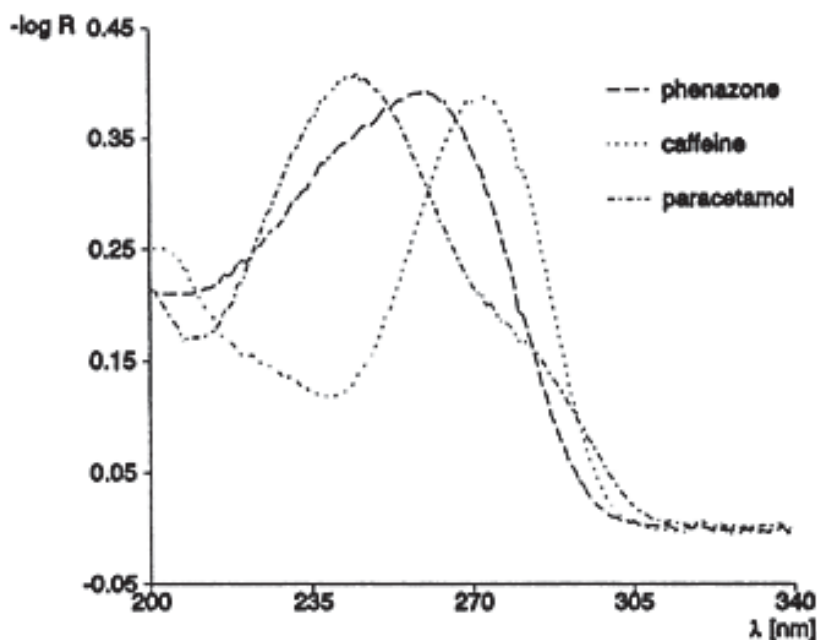


Figure 9 HPTLC UV spectra of phenazone (---), caffeine (···), and paracetamol (—·—)

strong absorption. With silica gel, the absorption maxima, also known as residual radiation bands, dominate considerably in the 1300 to 1000 cm^{-1} region, so the diffuse reflectance of interest is negligibly small. Therefore, measurements in this region are not possible on this sorbent. In contrast, it is possible to make measurements up to a wave number of 1000 cm^{-1} on cellulose stationary phase. In spite of the limited wavelength range, it is still possible to carry out in situ measurements on silica gel to characterize and identify substances that have been separated by HPTLC if use is made of an HPTLC-FTIR reference library with automatic comparison of band position, width, and intensity and if this is supplemented by comparison of the sample spectrum with the best matching spectra.

The Fourier transformed interferograms provide IR spectra that can be recorded and converted at will of the library search into normalized reflectance spectra (reflectance units R) (Figs. 12A and 12B), into quasi-absorbance units that are not proportional to concentration ($-\log R$) (Fig. 12C), or into Kubelka-Munk units that are proportional to concentration (Fig. 12D). The sub-(Fig. 12E) or the Gram-Schmidt technique (Fig. 12F). The first of these two methods can be used to increase selectivity (e.g., the spectral window can be chosen so that it detects only compounds stances can be localized on the TLC plate by using either spectral windows chosen at will with carbonyl groups), whereas the second is universally applicable and independent of wave number.

The large quantity of data generated by HPTLC-FTIR coupling can be printed out as a three-dimensional plot of a spectral series, with the wave numbers on the x-axis, the distances on the z-axis, and the absorptions on the y-axis. However, because the whole

picture can then become very complex, a two-dimensional contour plot is better suited for the recognition of band overlaps and small quantities of impurities.

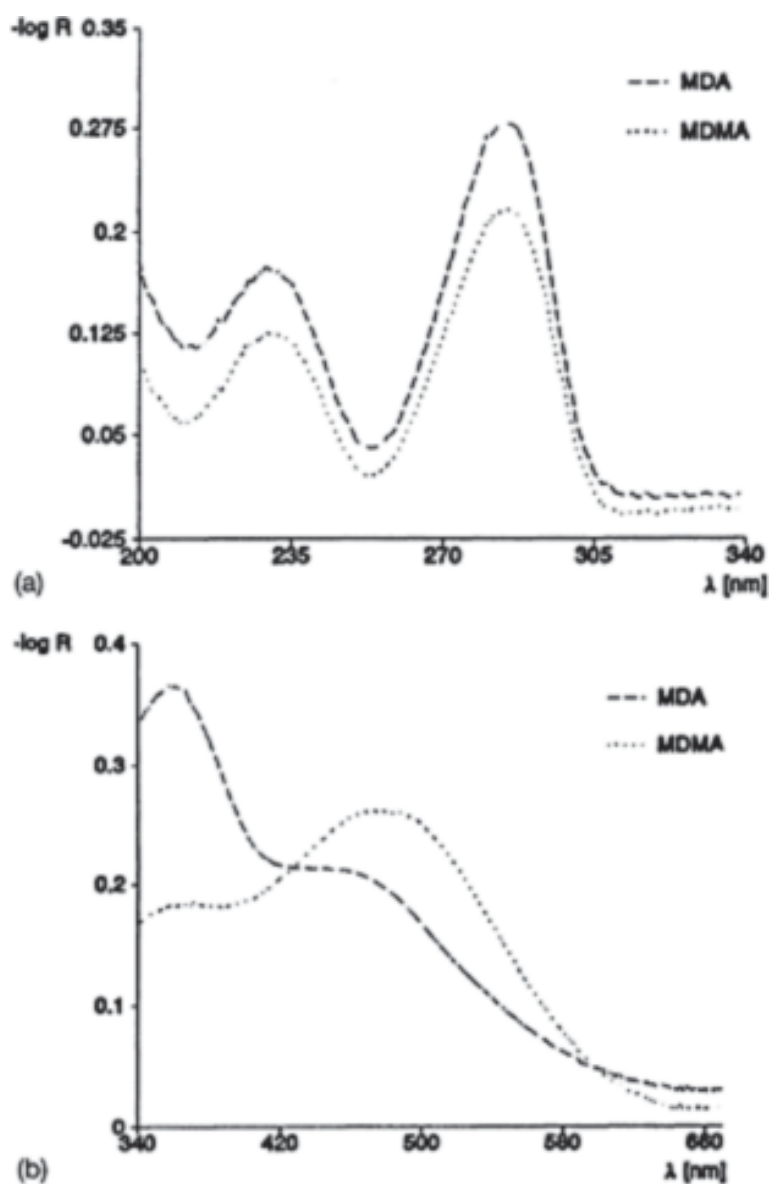


Figure 10 HPTLC *UV* spectra of MDMA (---) and MDA (···) (a) before and (b) after dipping in an *o*-(benzenesulfonamido)-*p*-benzoquinone solution.

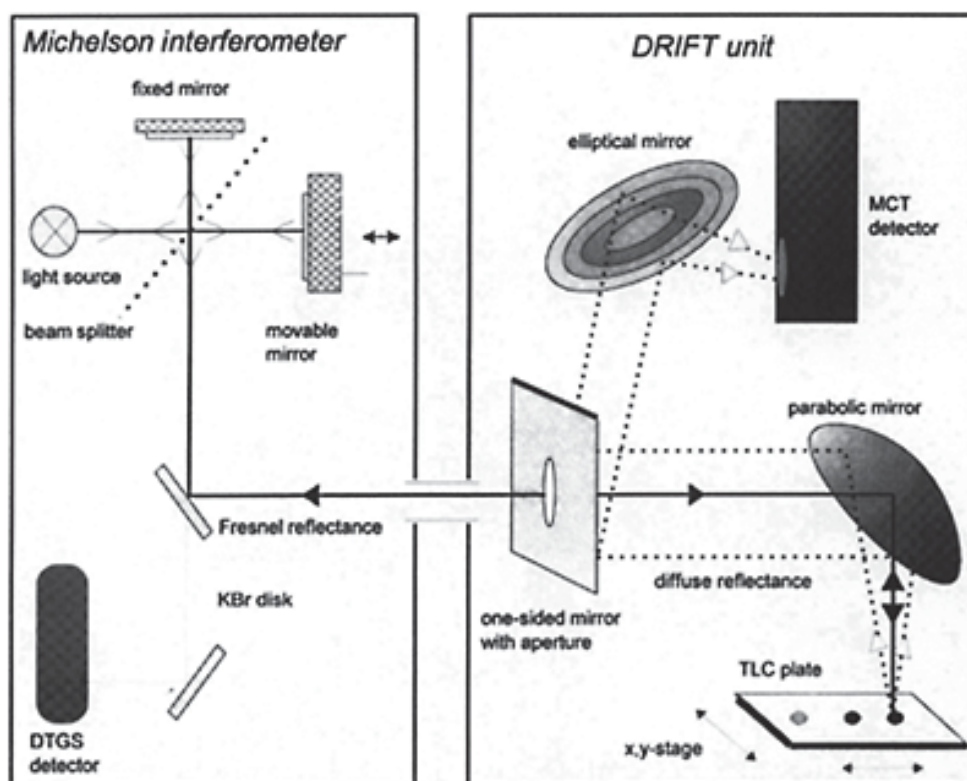


Figure 11 Schematic overview of the Bruker HPTLC-DRIFT unit for on-line measurement.

The HPTLC-FTIR method is particularly suitable for identification and quantification of substance mixtures. Depending on the specific IR absorbance of the substance and the distance run in the chromatogram, the limits of identification, the validated detection limits, and the limits of quantification lie between 15 ng and 2.5 μg .

The power of this coupling method is confirmed by examples from various fields of analysis such as drug identification (36), forensic chemistry (37), environmental analysis (38), and quality control of essential oils (39). The most recent developments include the design of a silica gel sorbent containing 50% magnesium tungstate, which considerably enhances the interpretable wavelength range (40). This allowed an efficient HPTLC-UV/FTIR coupling procedure for the separation and rapid identification of flurazepam hydrochloride and its related substances in bulk

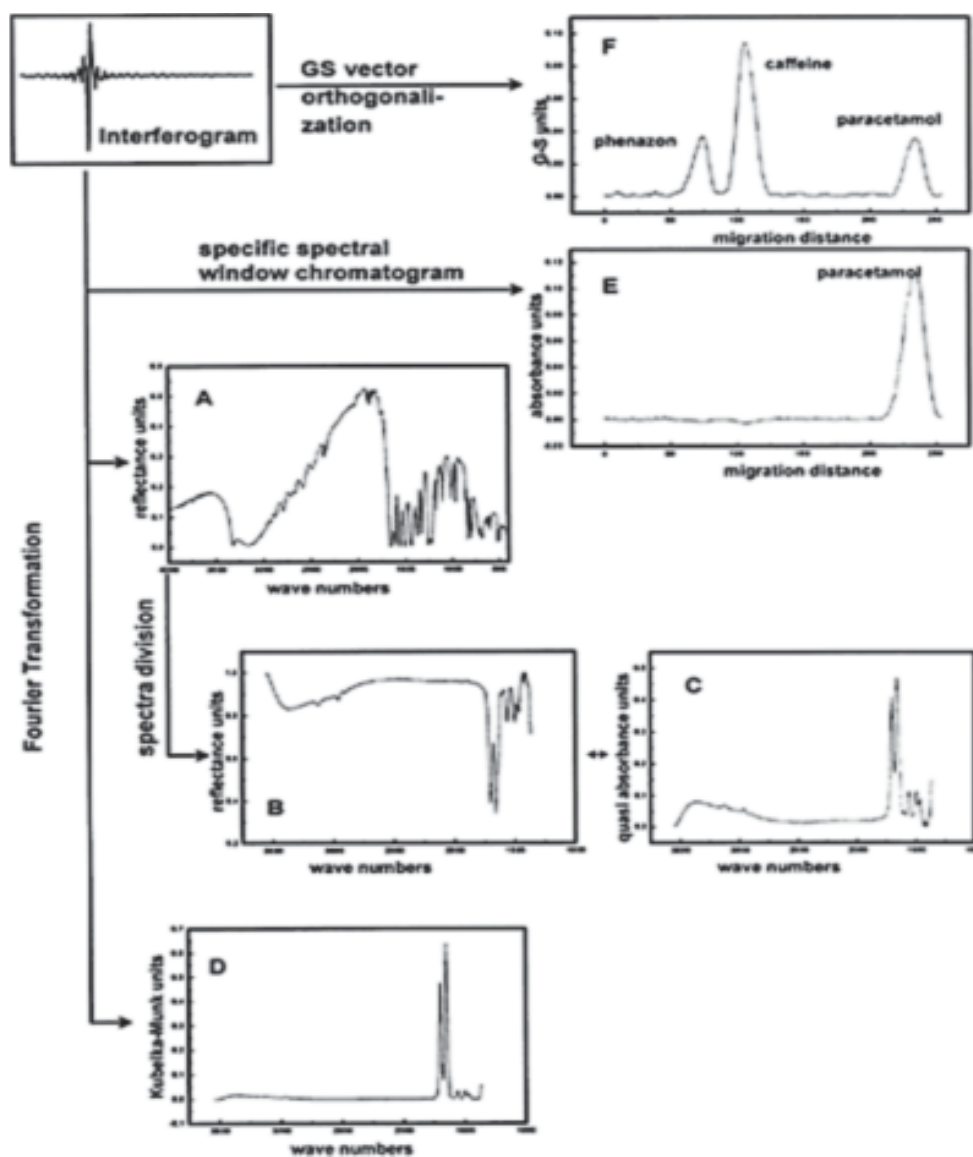


Figure 12 Schematic overview of data presentation possibilities.

powder and capsules (41). Compared to the related compound test of the Pharmacopoeia, this procedure shows several advantages, e.g., baseline separation of the known impurities and detection of the substances as peaks in the UV region (Fig. 13) as Gram-Schmidt or window chromatograms (Fig. 14). Furthermore, unambiguous identification is obtained by postchromatographic extraction of the DRIFT spectra and comparison with reference spectra in the library. Quantification of the related compounds was carried out densitometrically.

C. Raman Spectra

With the use of argon ion, HeNe, or YAG lasers as monochromatic light sources and the improvement of detection methods by the employment of more sensitive CCD detectors instead of photomultiplier tubes, Raman spectroscopy has gained in importance. This identification technique serves primarily for the investigation of apolar atomic groups and of symmetrical groups of atoms that are infrared-inactive. It is also possible to assign vibrations from FTIR spectroscopy with the aid of Raman spectra. However, little progress has been made with quantitative evaluation.

For in situ identification in TLC especially, the surface-enhanced Raman scattering (SERS) technique is used in the subnanogram range. After development and drying of the chromatogram, the plate is dipped in or sprayed with a colloidal silver suspension (42). The silver colloids (about 15 nm particle size) are prepared by reduction of silver nitrate with sodium citrate. With the use of this technique, the investigated substances experience an intensity enhancement of about 10^6 due to the metal microstructure on the surface of the chromatogram, thus leading to greater electron—photon coupling at the atomically rough metal surface and simultaneous charge transfer to orbitals of the adsorbates. Consequently, one of the advantages of the SERS technique is the

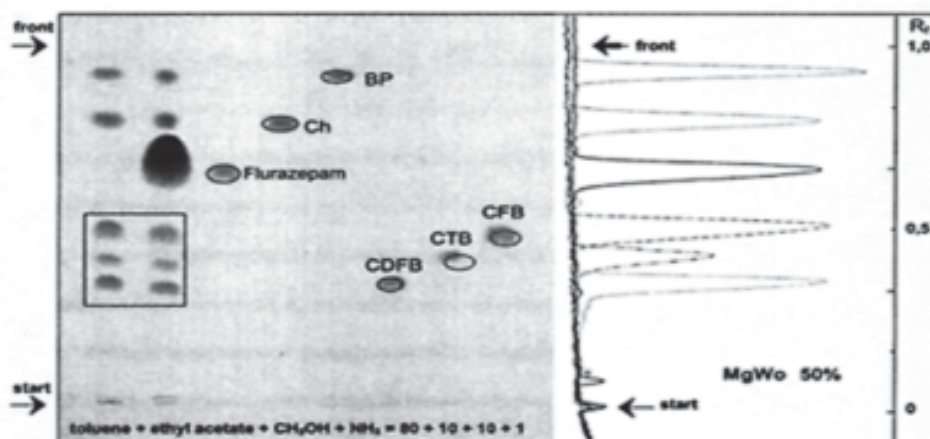


Figure 13 Separation and detection of Flurazepam and its impurities, Ch, 3-Amino-6-chloro-1-[2-diethylamino)-ethyl]-4-(2-fluorophenyl)-chinolin-2-one hydrochloride; BP, 5-chloro-2-[2-(diethyl-amino)ethylamino]-2'-fluorobenzophenone hydrochloride; CDFB, 7-chloro-1,3-dihydro-1-[(2-ethyl-amino)-ethyl]-5-(2-fluorophenyl)-2*H*-1,4-benzodiazepin-2-one hydrochloride; CTB, 7-chloro-1-

[(2-ethylarmno)-ethyl]-5-(2-fluorophenyl)-2*H*-1,4-benzodiazepin-2-one hydrochloride; CFB, 7-chloro-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

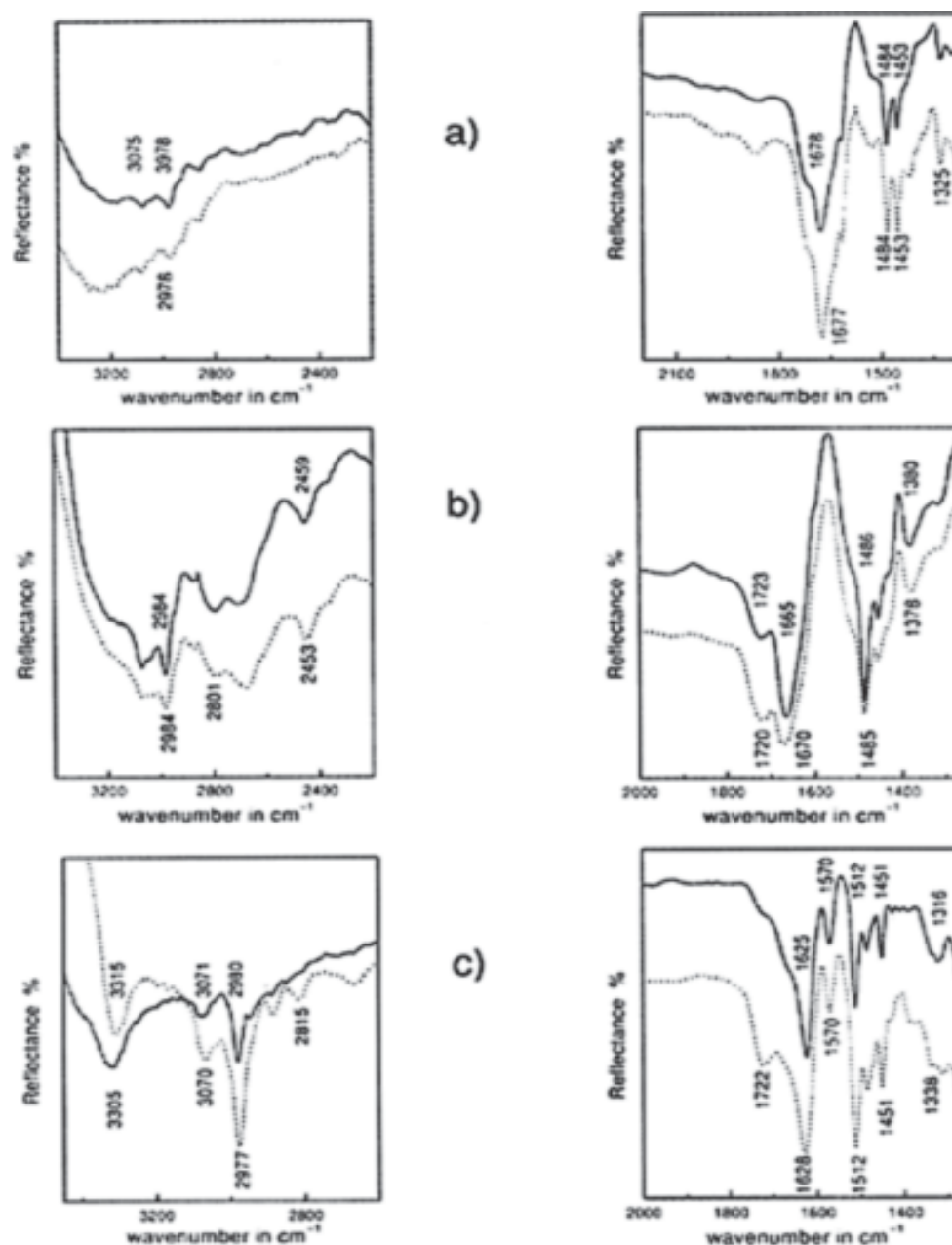


Figure 14 DRIFT spectra of degradation products (solid curves) in capsule after stress treatment [hit qualities (a) CDFB (652), (b) CTB

(711), (c) BP (839)] and reference (dotted curves).

high enhancement factor, permitting in situ analysis of TLC zones even down to picogram amounts.

To avoid diffusion effects at the zones of interest when the plate is dipped in or sprayed with an aqueous colloidal silver suspension, the silver molecules can be evaporated onto the HPTLC plate (43). Plates (10×10 cm) are placed in an evaporation device (Fig. 15) in which silver (about 600 mg) is evaporated at high temperature under high vacuum. Figure 16 shows the intensity enhancement by evaporation with silver molecules very clearly.

Highly Raman-active compounds such as optical brighteners (Fig. 17) (M.Moss, M.Zeller, personal communication, 1995) can also be detected without surface-enhanced scattering on specially modified silica gel plates. The identification limit is about 25 ng for these substances and about 100 ng for dyes.

D. Mass Spectra

For this in situ identification method, FAB (fast atom bombardment), liquid SIMS (secondary ion MS), or laser desorption is generally employed as the ionization technique (44, 45). The analytes are sputtered directly from the TLC foil (Fig. 18) (46), or the TLC plate is placed on a movable table. However, the amount of substance needed for recording reliable mass spectra still lies in the submicrogram range. More details are supplied in Chapter 9.

E. Coupling of Separation Methods

Coupling of TLC with gas chromatography, supercritical fluid extraction, or the thermal separation technique (TAS) has been employed for special analytical tasks. Coupling of HPLC with either rotation planar chromatography (RPC) or overpressured layer chromatography (OPLC) (47) and the coupling of different stationary phases, known as long-distance OPLC (48), have also been demonstrated.

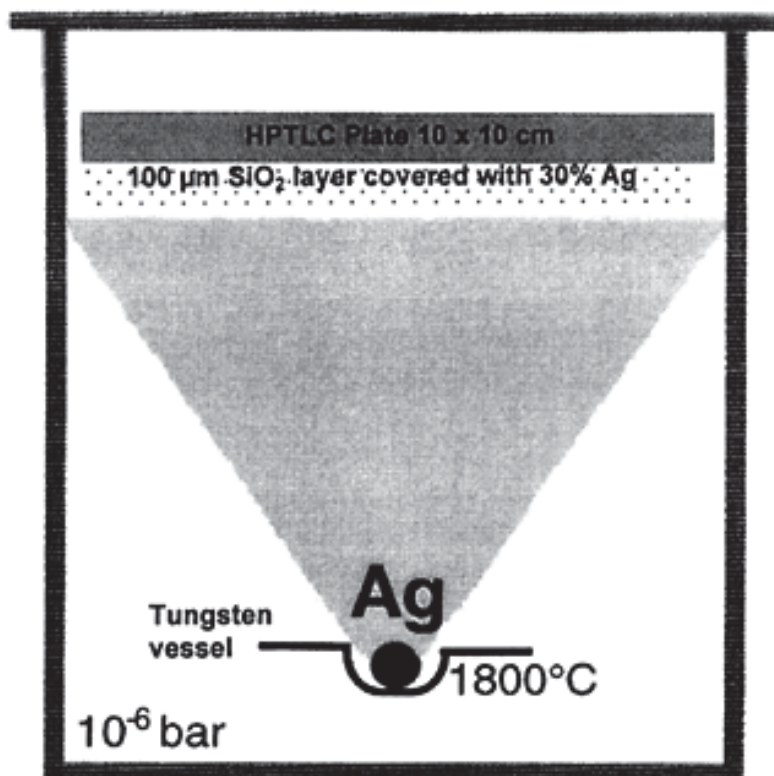


Figure 15 Evaporation device for SERS-Raman spectroscopy.

Of major interest is the coupling of HPLC with automated multiple development (AMD) because of the immense increase in separation power it achieves. It seems to afford a low-price and rapid way to cope with difficult separations and to get rid of interfering matrix components of complex mixtures. HPLC separations are primarily carried out by bonded phase partition chromatography, whereas TLC separations on silica gel take place according to the principles of adsorption chromatography. Coupling of these two highly efficient separation methods greatly increases the information content of analyses (Fig. 19) (K. Burger, personal communication, 1994). In practice, a complex mixture is first separated on a microbore system, thereby providing a low flow rate of about $60 \mu\text{L}/\text{min}$. This low flow rate enables a connection without a splitter. Selected HPLC fractions are automatically transferred onto the HPTLC plate by using a special application device (CAMAG DuoChrom) that can cope with an application flow rate of about $60 \mu\text{L}/\text{min}$ and can be heated if desired. Thereafter, planar chromatography is continued as usual.

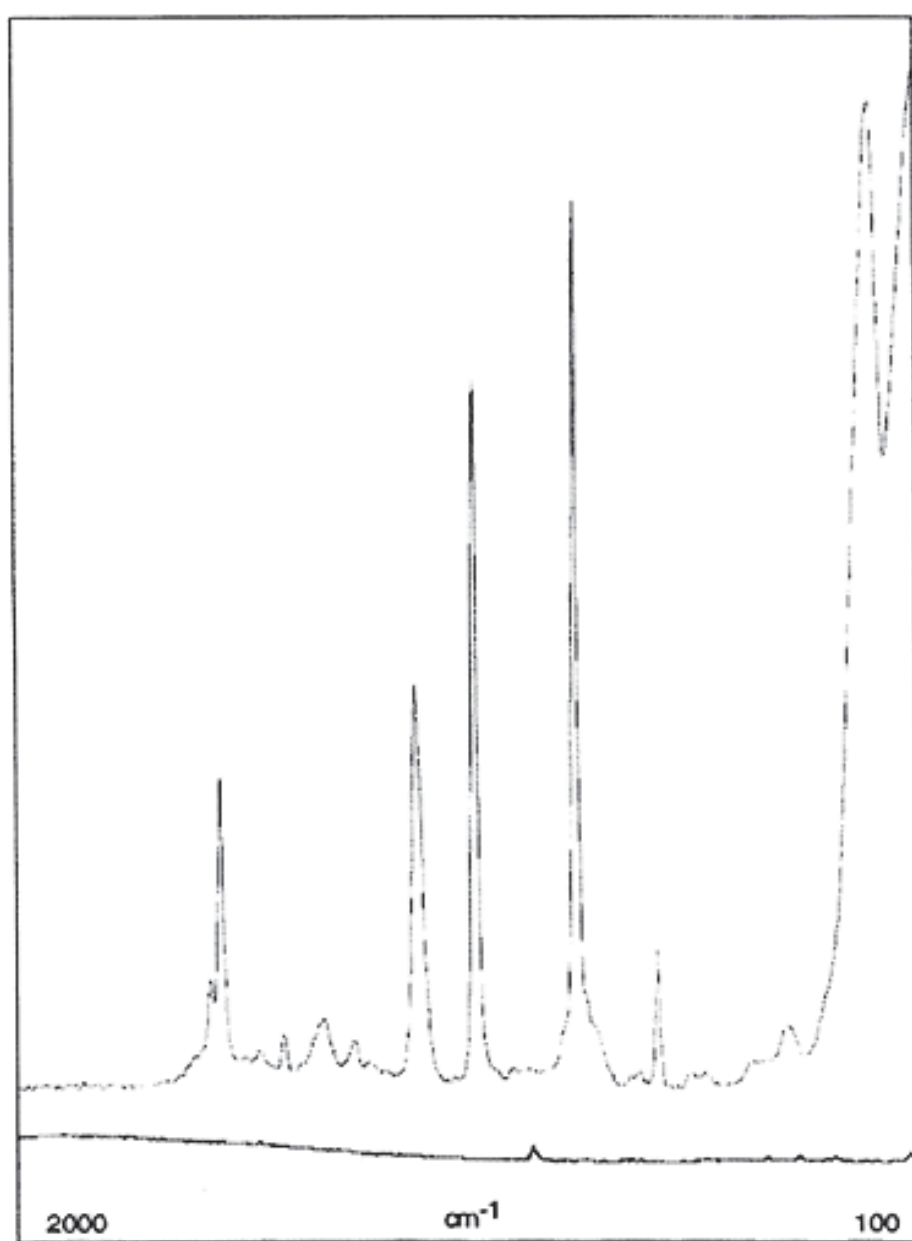


Figure 16 Intensity enhancement by evaporation with silver molecules. Raman spectra of 300 ng phthalic acid before (lower) and after (upper) evaporation.

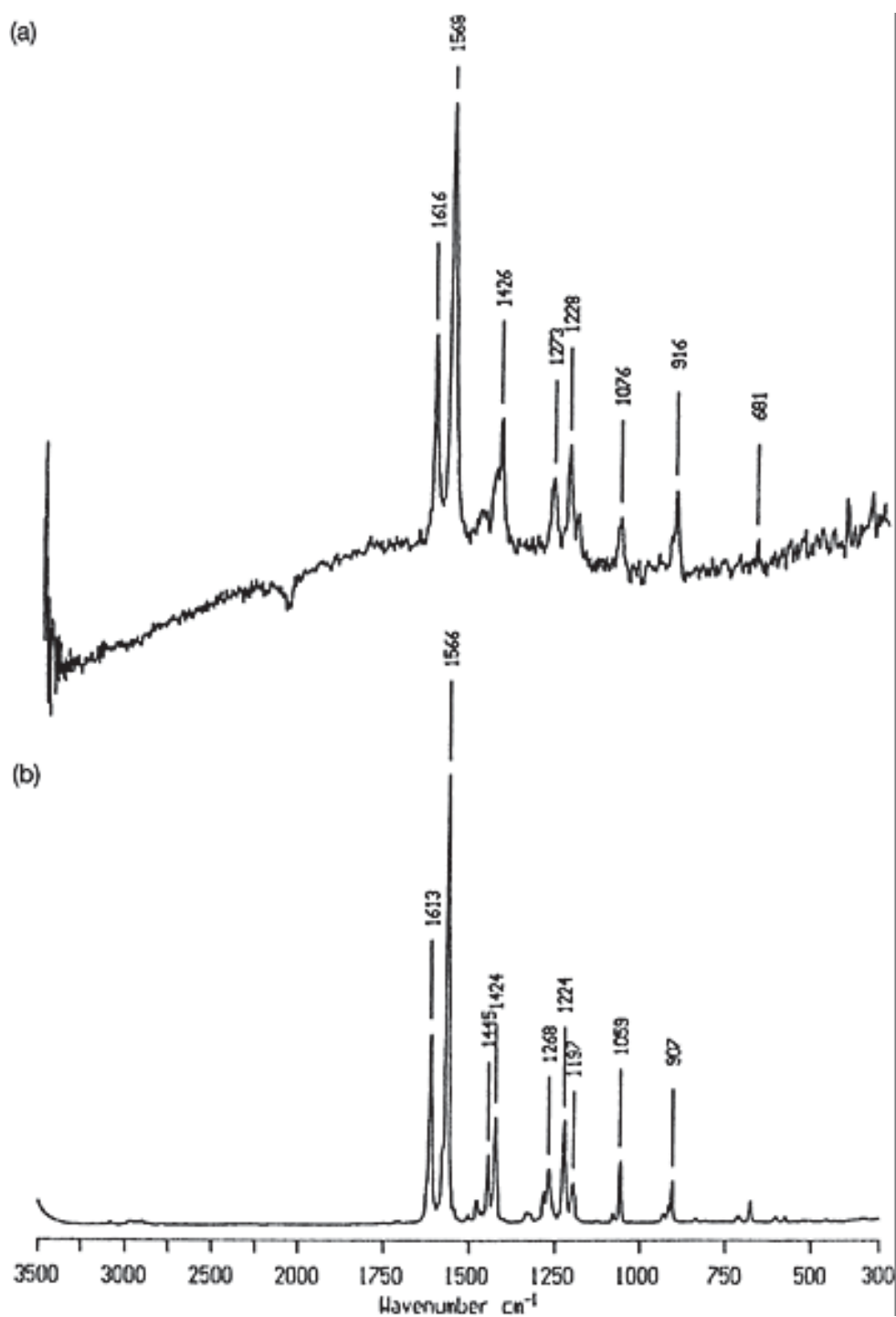


Figure 17 In situ Raman spectra of 100 ng of an optical brightener (a) and its reference substance (b).

IV. DOCUMENTATION

Planar chromatography is an open system, in contrast with high-performance liquid chromatography or gas chromatography. Thus, it can more easily be affected by the environment, and possible factors of influence have to be monitored more consciously and documented in detail (49). Accurate documentation seems to be the basis for reproducible planar chromatographic results.

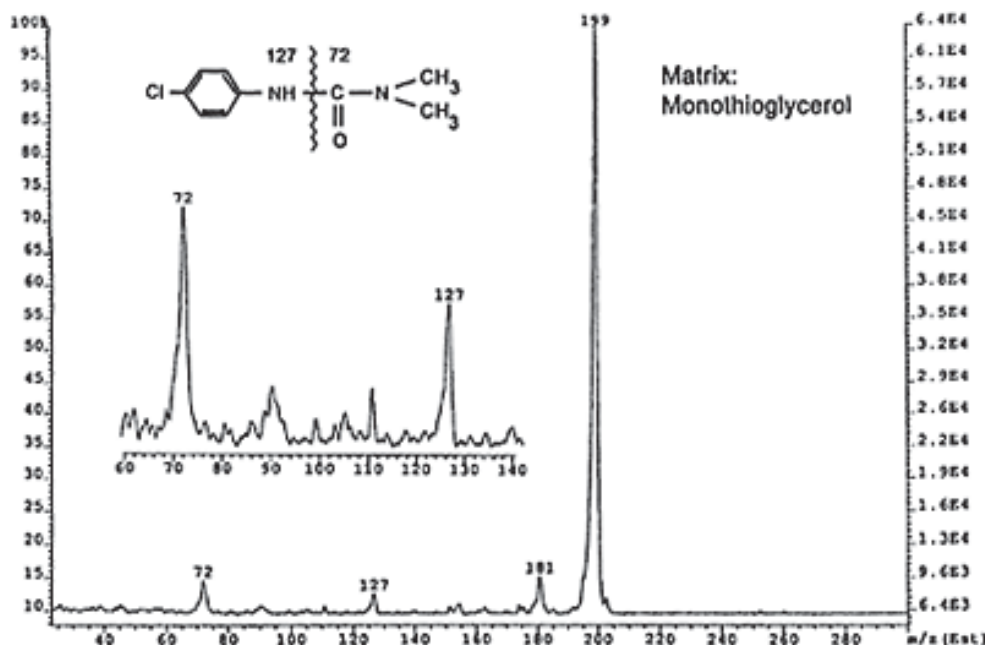


Figure 18 In situ positive-ion FAB-MS/MS analysis of the phenylurea herbicide Monuron.

A. Documentation of the Method

Good laboratory practice (GLP), good manufacturing practice (GMP), and standard operating procedures (SOP) and procedures such as accreditation, auditing, and certification involve nothing more or less than determining a range of parameters and demonstrating their reliability by means of statistical methods. Thus, it is necessary to ensure the quality of the working instructions and to document the chromatographic conditions for reproducible and reliable results. Some important items of documentation are compiled in Table 4.

These items can easily be documented with a computer. Nowadays, software [e.g., winCATS, (CAMAG)] is specially designed to manage, monitor, and control all constituent steps of the planar chromatographic procedure. From sample application to TLC plate development to classical densitometry and image documentation, all necessary parameters are documented in one data file. The software manages and supervises all

software-driven instruments. For nonsoftware-driven equipment, e.g., development in a glass tank or pre- or postchromatographic derivatization, the user can manually enter all related parameters, and the software will archive and report them in compliance with GMP/GLP. That means that one type of software, one data file, and one protocol are sufficient for the entire TLC procedure inclusive of the devices used.

B. Image Documentation

For documentation of the size, shape, and color of the individual zones, the chromatographic result can be reproduced graphically or stored as a whole (manual documentation), or it can be recorded as a photocopy, photograph, or electronic image (electronic documentation).

1. Manual Documentation

In former times, the original chromatograms were stored, i.e., the plate itself was the document. Storage of chromatograms was more convenient if TLC foils had been employed or if the adsorbent layer was fixed and removed from the plate as a whole. The latter was achieved by smoothly pressing cellophane tape or clear contact paper on top of the layer so that the adhesive came into uniform contact with the layer. Then the tape and the attached layer were carefully peeled away and fastened into a notebook. Treatment of the chromatogram with collodium (50) or plastic dispersions based on polyacrylic ester, polyvinyl chloride, or polyvinyl propionate (E.Merck, company literature about Neatan, 1975) was used also. These kinds of storage methods often entail degradation, fading of the zones, as well as changing of the color or blurring of the contours.

Furthermore, TLC separations can be reproduced by drawing, sketching, or tracing. For example, transparent paper can be placed on top of a glass-covered chromatogram, and the zones can be traced directly and colored with crayons or pens or marked in accordance with a color key system to reproduce the impression of color. However, these methods are tedious, time-consuming, and subjective.

2. Electronic Documentation

Direct copying on Ozalid or Ultrarapid blueprint paper (51) and contact printing (52) have been replaced by photocopying, photographing, or electronic image processing. Such phototechniques allow rapid retakes to produce the best possible result. Instant photography, photocopying, and electronic image processing even provide for immediate reproduction and decision making regarding acceptance or retake under different conditions.

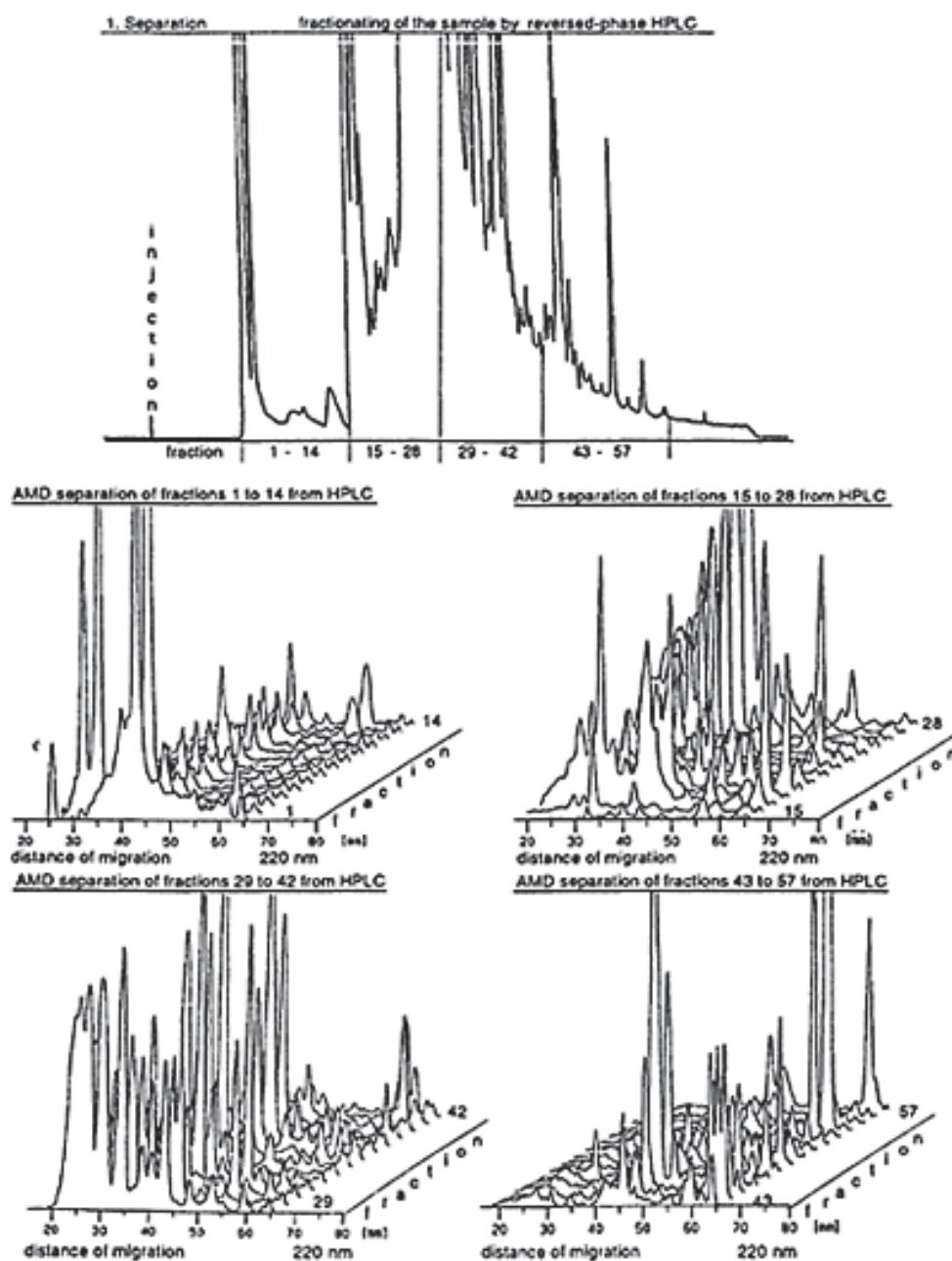


Figure 19 On-line HPLC/HPTLC (AMD) analysis of wastewater.

Table 4 Important Items for Method Documentation

Sample preparation	Reference substance: name, amount, dilution factor, manufacturer, batch, purity. Solvents: manufacturer, batch, purity, stabilizer. Sample preparation or cleanup procedure.
Stationary phase	Type of plate, plate size, indicator, layer thickness, manufacturer, batch, description of pretreatment, impregnation, or conditioning.

Mobile phase	Composition of mobile phase, equilibration of vapor phase. Solvents: manufacturer, batch, purity, stabilizer.
Application	Application device, spot or spray technique, application scheme, application volume and other application parameters, drying mode after application.
Development	Technique of development, developing chamber system, volume of mobile phase, migration distance and time, temperature, humidity, drying mode after development.
Derivatization	Pre- or postchromatographic derivatization, preparation of derivatization reagents (manufacturer, batch, purity, stabilizer, etc.), detailed derivatization technique (spraying, evaporation, immersion). Reagents for stabilization or intensification of zones. Heating mode, temperature, and heating time of the plate.
Evaluation	Detection mode, principle of measurement, software version, scanner type, parameters of measurement, integration, quantification or spectroscopic identification.
Documentation	Date, time, user name, identification number, parameters of image acquisition, comments.

a. Photocopying. Photocopying is the simplest way to record visible chromatogram zones. Relatively good reproductions can be achieved in black and white or even in color. Intense zones can be duplicated better than light ones.

b. Photographing. Chromatograms can be photographed in black and white or true color under visible or UV light with appropriate filters. Aside from electronic image processing, color photography is probably the best method for documenting chromatograms. When a long exposure time is necessary, especially for photographing fluorescent zones or for using filter combinations, handheld lights and cameras are undesirable and do not provide exact documentation. Therefore, commercial camera stands and suitable lighting units that can be combined with a large variety of conventional and instant cameras, for example, a Polaroid multipurpose reflex camera or a standard 35 mm camera, should be used. Lighting units feature direct shortwave UV light (254 nm), direct longwave UV light (366 nm), and direct and/or transmitted white light (400–750 nm). Additionally, transmitted midrange UV light (302 nm) is offered. The tubes operate with high-frequency (25–30) current; this ensures optimum light efficiency and eliminates synchronization problems with electronic cameras. The cabinet cover ensures complete exclusion of ambient light, so image capturing under all kinds of light is feasible in an undarkened room.

Ultraviolet photography. For UV photography, the entire chromatogram has to be illuminated uniformly by the UV light source. This is more difficult than with brighter, more intense white light sources. Illumination strikes the chromatogram at a proper angle from two sides in the reflected mode. In addition to completely excluding ambient light by using a cabinet cover, the excitation wavelength has to be cut off with a filter (barrier filter) placed before the camera lens. Further, UV tubes have to be covered with a special filter (bandpass filter) that permits only UV light to pass through and illuminate the zones, because otherwise a “wash-out” effect due to the excessive contamination of white light emitted from the UV tubes is observed. The effectiveness, in other words the transparency, of the blue bandpass filter can be reduced with increasing duration of irradiation, especially in the shortwave UV range. The resulting slight blue coloration of

photos can be avoided by using a yellow or pale orange filter. In the transmittance mode, the frosted glass that is used as support for the HPTLC plate is replaced by a bandpass filter that allows through the midrange UV light (302 nm) emitted by tubes in the base of the instrument. This mode is used mainly for electrophoresis gels.

The above-mentioned barrier filter is used to absorb or remove unwanted UV radiation to prevent it from being recorded on the film because it is much brighter than fluorescence and causes the film to be overexposed. Thus, the more residual UV radiation is absorbed, the darker the background will become on the photograph. A correctly chosen barrier filter (Table 5) (53) will transmit only the visible wavelength of the fluorescent zone. Generally, a Wratten 2 E filter, which blocks all UV radiation but also cuts into the visible range, is recommended for recording yellow-green fluorescent zones at an excitation wavelength of 365 nm. For blue to indigo fluorescent zones, a Wratten 2 A or 2 B filter can be recommended. If all fluorescent zones should be recorded on the film, a Wratten 2 C filter can be used, but the residual UV irradiation between 385 and 400 nm will pass the filter and cause a grayish appearance on black-and-white film or a brownish background on color film. Wratten 3, 4, and 8 filters produce a very dark black background but cut off almost all of the visible blue spectrum. Consequently, violet and blue fluorescent zones are lost when these filters are used.

After the proper choice of the UV barrier filter, contrast and rendition can be enhanced by controlling the exposure time. The exposure time is primarily dependent on the intensity of the fluorescence and has to be optimized for each chromatogram. Experience has shown that operating with a range of exposure times, i.e., an aperture of f/8 with exposures of 15, 30, 60, 120, and 240 s, always leads to one optimal exposure time. In certain situations, substances can be adversely affected by UV light and fade rapidly under prolonged exposure (photobleaching). The exposure time for photographing zones of fluorescence quenching at a wavelength of 254 nm often applies for several recordings using the same conditions. A special glass filter (GG 435) placed in front of the camera lens often improves rendition (3). Color-correction filters are used in UV photography to lessen the amount of yellowness created by Wratten barrier filters (53). For example, a G (green) color correction filter, which absorbs red and blue, or an R (red) filter, which absorbs blue and green, can be used. Moreover, contrast filters for black rendition (mostly Wratten filters Blue 47 or Red 25) are employed for black-and-white UV photography to darken the zones against a bright fluorescent background. Corresponding contrast filters for white rendition (e.g., Wratten filter Green 58) brighten specific fluorescent colors (e.g., green) and make them appear white against a dark background (53).

CAUTION: All radiation below 350 nm is considered to be dangerous. Therefore, protective gear must be worn to protect the eyes and skin.

White light photography. In white light photography, a frosted glass plate serves to support the HPTLC plate as well as to diffuse the light. In normal cases, the zones are more visible in

Table 5 Wratten Barrier Filters for UV Photography

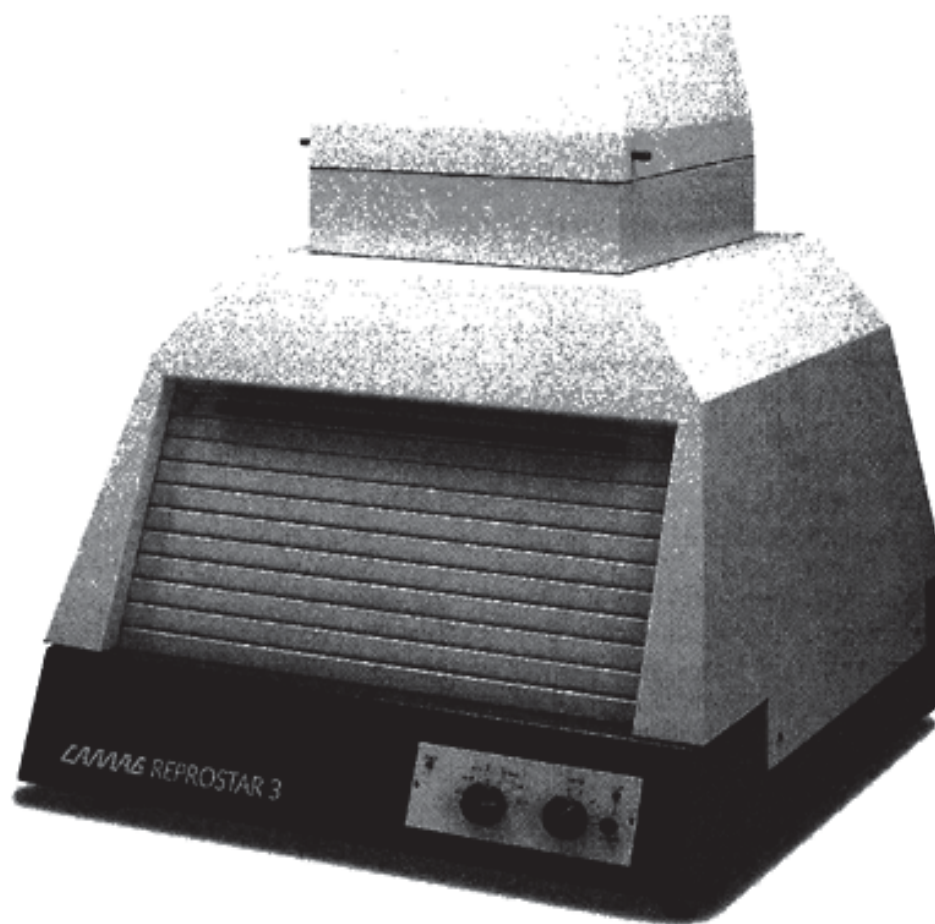
Wratten gelatin filter number	Absorption of UV radiation (at and below)
2 C Pale yellow	385
2 B Pale yellow	390
2 A Pale yellow	405
2 E Pale yellow	415
3 Yellow	440
4 Yellow	450
8 Yellow	465

the transmission mode, with illuminating white light tubes at the instrument base, than in the reflection mode. Color-corrected white light is recommended rather than cool or warm white illumination for obtaining better color rendition. Most color films are designed to perform best at 5500 K. Therefore, when using warm light UV tubes of about 4000 K for illumination, a color temperature filter (Table 6) (53) is usually employed for color correction. Usually a Wratten gelatin filter is positioned between the camera lens and the UV barrier filter. Moreover, color correction filters are used to accentuate the color and control the contrast. Photographing through a filter of a complementary color (e.g., a yellow filter for a blue zone) makes the zone appear darker. The blue zone will appear lighter when photographed through a blue filter.

c. Electronic Image Processing. Video documentation systems for acquiring, printing, and archiving images of planar chromatograms have largely replaced instant photography systems. Their salient advantages are low cost per image, previewing and immediate optimization of the images on the screen, full compatibility with GMP requirements, high user-friendliness, and rapid data storage on the PC, all of these leading to durable results. The chromatograms are photographed in direct and/or transmitted light, depending on their quality. Even multiple detections of the chromatogram, i.e., several images of the same plate (visualization under white light, fluorescence quenching at UV 254, fluorescence at 366 nm), can be easily documented. The appropriate configuration, which includes the electronic settings for the CCD camera and frame grabber for a special illumination mode, has to be chosen. After the optimum contrast, contour, sharpness, illumination, etc., have been determined, images are captured, i.e., a digital “snapshot” is taken to create a colored or gray-scale image of the entire chromatogram. Single tracks or fractions of the chromatogram can be edited very comfortably, and annotations can be made. Raw data and all parameters of their acquisition are stored in a secure file format that cannot be manipulated. The images can be exported in various open image formats. An image database makes it possible to manage many images along with their (computer-generated) ID, date and time of capture, infor-

Table 6 Illumination Filter Correction for Color Film (5500 K)

Illumination source	Blue filter number	Increase in exposure stops
3200 K	80 A	~1
3400 K	80 B	~1 $\frac{2}{3}$
3800 K	80 C	~1
4200 K	80 D	~ $\frac{1}{3}$

**Figure 20** CAMAG Reprostar 3 with cabinet cover and mounted digital camera. (Photograph courtesy of CAMAG.)

mation about the user, and special notes. Display and print formats can be selected. Images from the database can be selected at will for comparison, and all entries are searchable.

Photo Scanners and digital cameras are less expensive electronic image processing systems than CCD cameras. If photo scanners are used for image documentation, only visible wavelength zones (those illuminated in direct white light) can be documented. With a high-resolution digital camera (Fig. 20), the image quality is comparable to that of pictures taken with a conventional or instant camera. However, digital cameras have relatively low data transfer rates and are slower than image documentation systems that use a CCD camera. The software supplied with the digital camera or photo scanner is usually suitable for simple applications but is unfortunately not GMP/ GLP-compliant so far because of the open file format. If this problem is solved in the near future, then high-resolution digital cameras will probably replace the more expensive video cameras.

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9

Thin-Layer Chromatography Coupled with Mass Spectrometry

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I. INTRODUCTION

The overall performance of a separation method is intrinsically linked to the performance of the detector used as part of the system. Other handbook chapters detail principles, operation, and applications of common detectors for thin-layer chromatography (TLC), many of which have been in use since the beginnings of TLC. In contrast, mass spectrometry (MS), especially in an imaging mode, is a relatively new detection method for TLC. Mass spectrometry has been used with gas chromatography and liquid chromatography, and with supercritical fluid chromatography and capillary electrophoresis, to provide a balanced combination of separation and detection capabilities. Benchtop GC/MS systems (available for about \$50,000 USD) are operated directly by the end user. Other low-cost, high-performance chromatography/mass spectrometric combinations will follow with continued development of a new generation of smaller, more automated mass spectrometers. These same technological developments have also led to TLC/MS in several different forms. Moreover, renewed emphasis on the measurement of two-dimensional imaging data from mass spectrometry holds genuine promise for TLC/MS and for planar chromatography coupled with mass spectrometry in general. This chapter summarizes the approaches that have characterized TLC/MS since its first inception through to the more recent one- and two-dimensional imaging systems.

Commercial analytical instruments are developed when manufacturers perceive that a profit can be derived from meeting the demand of the marketplace. Demand in the marketplace develops when consumers are convinced of the practical value of the instrument in the solution of problems at hand and when an instrument is readily available and supported by the manufacturer. Demonstrations of feasibility are the break in this circular conundrum. Over the past 15 years, TLC/MS has been shown to be technically feasible and applicable to a wide variety of problems in both qualitative and quantitative analysis. Commercial interest in TLC/MS, however, is still limited. The same path of development was followed for TLC coupled with infrared spectrometry.

TLC/MS is only part of the more general area of planar chromatography coupled with mass spectrometry (PC/MS). However, applications and research that involve mass spectrometry as a detector for planar chromatography continue to emphasize thin-layer chromatography. TLC, in classical and high-performance formats, is widely used in analytical laboratories around the world, and the advantages of the additional specificity derived from the mass spectrometric detection have been evident for some time, as covered in previous reviews. The most relevant analytical points for PC/MS and TLC/MS are identical.

Although feasibility has been demonstrated and the instrument technology is in place, TLC/MS is still not offered as a stand-alone instrument within the commercial marketplace. The MS market itself has changed significantly over the past five years. Fewer general-purpose mass spectrometers are sold than in the past, and more instruments are sold as specific “problem solvers.” The instruments are (in general) smaller, cheaper, and more sensitive than those of a decade ago. However, at the same time, the new instruments are usually not as flexible, and they cannot be easily reconfigured to meet new analytical needs or reengineered into different formats, such as must still be done to assemble a TLC/MS instrument.

Mass spectrometry has successfully infiltrated many aspects of the analytical market. The combinations of gas chromatography with mass spectrometry (GC/MS), of liquid chromatography with mass spectrometry (LC/MS), and of capillary electrophoresis with mass spectrometry (CE/MS) became sustainable markets when (a) the analytical and regulatory demand for the data that these methods could uniquely provide was in place; (b) the instruments became extraordinarily reliable, easy to operate by nonspecialists, and reasonably priced; and (c) the numbers of instruments in use reached a critical community mass. For regulatory purposes, there is a need for analytical instrumentation that can be put in place in multiple locations, instrumentation that provides the same result for the same samples each time, and instrumentation that is widely available so that the results can be independently verified. A one-of-a-kind, special-purpose instrument such as a combination of a thin-layer chromatograph with a mass spectrometer might well be used to highlight analytical capabilities and potential, but sustained commercial growth can occur only when the number of instruments to be sold can be counted in the hundreds. Analytical and regulatory demands are currently met with other chromatography/mass spectrometry combinations. The speed with which these methods (GC/MS, LC/MS, and CE/MS) have been adapted to pressing analytical needs (higher separations resolution, shorter analysis times, combinatorial analyses) has reduced the opportunities for unique contributions by planar chromatography and by TLC/MS.

Basic principles of instrument interface design, sample transport and ionization, and mass spectral data manipulation developed for TLC/MS are covered in this updated review within the same organization as in previous editions. Current applications are updated at the end of each appropriate section of the review. More general analytical attributes are discussed in a closing section.

A. Capabilities of Mass Spectrometry

A mass spectrum is a compilation of ions of measured mass plotted against the measured intensities of the ion signals. The mass scale (given in units of mass-to-charge ratio,

referenced to the ^{12}C mass of 12.00000 daltons exactly) can be measured to a variable degree of accuracy, ranging from integral mass numbers to exact mass measurements to a few millidaltons (mDa). The intensity scale is most often marked in terms of relative abundance (RA), in which the most intense ion signal within the plotted mass range is arbitrarily assigned a relative abundance of 100%, and the abundances of all other ions are scaled to that value. Mass spectral interpretation provides the molecular mass of the compound that provides the mass spectrum and, in many cases, its molecular structure via rationalizations of the fragmentation patterns of the ions.

Central to any measurements in mass spectrometry are the ionization of the sample molecules and transfer of those ions into the vacuum required for operation of the mass spectrometer. The choice of ionization methods available to analytical and organic mass spectrometrists has expanded greatly in the past 15 years and now includes means for the ionization of nonvolatile as well as volatile molecules. Any of several methods might be chosen to address a particular problem, and each might provide satisfactory results. Because there is no single ionization method used exclusively with TLC/MS, this section contains an overview of the most common methods of sample molecule ionization in mass spectrometry.

The classical ionization methods of electron and chemical ionization have been refined over years of application to the study of volatile organic molecules. The vast majority of samples that are analyzed by mass spectrometry are volatile. In terms of the mass spectrometer, "volatile" means that there is a sample vapor pressure of at least 1×10^{-6} torr at a temperature of 250°C . Electron ionization (EI) remains the most widely applied method of ionization in mass spectrometry, and it is the sole method for which a time-tested base of mass spectral data exists. For samples that are sufficiently volatile, gas chromatography can be used for separation of mixtures. Thin-layer chromatography can, of course, also be used. As later parts of the chapter describe, desorption of volatile molecules from the TLC plate into a gas stream then becomes a straightforward means of interfacing TLC with mass spectrometry.

In a typical electron ionization source, electrons are emitted from a heated filament of metal, often tungsten, and accelerated to an energy of 70 electronvolts (eV). Interaction of the sample molecules with the relatively high energy electrons leads to the formation of a molecular ion of the sample, defined as an ion in which one electron has been lost to form M^+ , or an ion in which one electron has been gained to form M^- . If the odd-electron molecular ion formed in electron ionization is especially unstable, the relative abundance of the molecular ion may be reduced below the noise level. In these cases, determination of the molecular weight of the sample, often the first information sought from a mass spectrometric analysis, is made much more difficult. The inherent instability of the molecular ion formed by electron ionization for certain classes of organic compounds provided the original impetus behind the development of chemical ionization mass spectrometry.

Chemical ionization (CI) also deals with the ionization of volatile gas-phase samples, and it can be applied across-the-board to the same types of samples as electron ionization. Chemical ionization provides abundant molecular ions for those compounds that do not produce a discernible molecular ion by electron ionization. The molecular weight of the sample molecules of interest is reflected in the mass of the protonated molecule $(\text{M}+\text{H})^+$ in positive-ion chemical ionization or the mass of the deprotonated molecule $(\text{M}-\text{H})^-$ in

negative-ion chemical ionization. These positive even-electron ions are formed in an ion/molecule reaction between a gas-phase sample molecule M and a strong acid such as CH_5^+ (formed from methane) in which a proton is transferred to the neutral sample molecule to form $(M+H)^+$. Both electron and chemical ionization can be used in TLC/MS experiments in which the samples within the TLC plate are independently evaporated into the gas phase and swept into the source of the mass spectrometer in a stream of gas.

The need for reliable and sensitive analytical methods for nonvolatile and thermally labile compounds, including those of high molecular weight such as biomolecules, has encouraged development of new ionization methods in mass spectrometry (1, 2). For TLC/MS, the most important of these new methods are fast atom bombardment (FAB) ionization and liquid secondary ion mass spectrometry (SIMS) (3, 4), in which organic molecules are sputtered from surfaces by the impact of an energetic particle beam, and laser desorption (LD), in which sputtering of organic molecules from a surface occurs as a result of the high thermal energy imparted by the laser beam to the surface. The sample ions formed by these methods are usually the same even-electron ions such as $(M+H)^+$ formed in chemical ionization, and spectral interpretation proceeds along the same lines. A difference between these methods and electron and chemical ionization is that the sample is not evaporated in a separate step, and both volatile and nonvolatile materials can be sampled.

A key difference between EI and CI on the one hand and FAB, LSIMS, and LD on the other is the fact that sampling in FAB and LSIMS is from a specified location that corresponds to the impact footprint of the primary particle beam. If the sample is a solution, as it often is for FAB and LSIMS mass spectra of discrete samples, then diffusion within the solution blurs the spatial resolution of the ionization method. If the sample is held in a solid state, in a diffusion-controlled liquid state, or within a substrate such as a thin-layer chromatogram, the spatial resolution inherent to the sampling method is preserved. The natural compatibility of FAB, LSIMS, and LD with the direct mass spectrometric analysis of TLC plates is readily apparent.

Briefly, a beam of energetic atoms (FAB) or energetic ions (LSIMS) is generated in a particle beam source. The particles move with a velocity of about 10^5 m/s and are focused into a spot size of $0.01\text{--}1\text{ mm}^2$. The energy imparted to the sample or sample solution by the impact of the particle beam initiates a collision cascade in which molecules and ions are set into motion. Protons and electrons are also released from within energized areas of the sample, and a number of ionization reactions can result.

A method growing in popularity for the direct analysis of TLC plates and planar electro-phorograms as well is matrix-assisted laser desorption ionization (MALDI). Laser desorption, as we shall see later in this chapter, has been used for direct desorption of sample molecules from TLC plates since the early years of TLC/MS. In direct laser desorption, the photon energy must be absorbed by the components of the chromatogram or by the sample itself. Most early work used infrared lasers for this reason. In MALDI, the sample molecules are cocrystallized with a matrix (often in 1000–10,000-fold excess) that absorbs laser photons at the chosen wavelength. The photon energy is directed into the matrix rather than into the sample molecules. The matrix molecules respond by undergoing a variety of electron transfer, proton transfer, and, most important, phase transfer reactions. As the matrix molecules and ions leave the surface, sample molecules and ions can also be transferred into the gas phase without degradation. In application to

individual samples, a solution of the matrix and the sample molecules is placed on an inert metal support, and crystals coalesce as the volatile solvent evaporates. The thin film sample is then placed into the ionization source of the mass spectrometer. In MALDI analysis of TLC plates or planar electropherograms, other means of adding the matrix to the sample molecules already separated within the chromatographic matrix must be found.

Electrospray ionization (ESI) is a newer ionization method, and it is unique in that it generates ions directly from within a solution that is sprayed from a fine needle at atmospheric pressure. A stainless steel capillary tube carries solvent at a flow rate of 2–5 $\mu\text{L}/\text{min}$. A potential difference of 3000–4000 V is maintained between the needle and a counter electrode, which can be a wall of the source or a skimmer cone with an aperture that passes the ions into the mass spectrometer. A spray is generated at the tip by the solvent flow emerging at atmospheric pressure, and the potential difference ensures that the droplets emerging from the needle are charged, aiding in their dispersal. As the solvent first emerges from the charged capillary, it forms a cone (called a Taylor cone) that results as the droplet shapes itself to minimize electrostatic repulsion. Desolvation involves the loss of neutral solvent molecules from the droplet. As the droplets decrease in size, the charge density increases until an instability limit is reached and the droplet dissociates into still smaller highly charged droplets. Residual solvent finally evaporates to leave only the charged ions themselves to be transferred into the mass spectrometer. Protonation, and in fact multiple protonation, is commonly observed. Positive ions of the general form $(M+nH)^{n+}$ are formed by multiple protonation of larger biomolecules (molecular mass as designated by M) such as peptides and proteins. One effect of multiple charging is to bring multiply charged higher mass molecules within the mass range of commonly used mass spectrometers, because the mass analysis is actually a measurement of the mass-to-charge ratio (m/z). Further, because M is constant between the series of peaks observed as adjacent multiply charged ions, the multiple measurements of mass of these ions constitute a series of simultaneous equations that can be solved to determine M, the molecular mass, to a precision of $\pm 0.005\%$. The applications of electrospray in TLC/MS have been minimal, but the ability to use the solvent for both extraction of the sample from the TLC plate and spraying through the needle to cause ionization can be advantageous. However, currently ESI is used for analysis of higher molecular mass samples than those usually separated by TLC.

B. Interfaces in Chromatography/Mass Spectrometry

The development of GC/MS and LC/MS was possible only with the invention of interfaces that bridged the gap between the working parameters of each independent method. For GC/MS, the GC column operates above atmospheric pressure and, for packed columns, dilutes the sample in a considerable flow of carrier gas. For LC, a continuous stream of solvent exits the column, carrying sample along in dilute solution. The interface of each method to a mass spectrometric detector must be designed to transport sample molecules efficiently through to the ionization source while discarding as much of the carrier gas or solvent as possible, with no degradation in separation resolution. In both GC/MS and LC/MS, the interface must operate in real time, enriching the sample and transporting it to the mass spectrometer even as the actual separation is

carried out on the chromatographic column. Interfaces for the combination of mass Spectrometry with supercritical fluid chromatography and capillary zone electrophoresis must deal with similar disparities in sample pressures and operate efficiently in real-time separations. In many instances, the interface operates independently of the ionization method used and is therefore generally applicable. In other instances, as in the interface with capillary zone electrophoresis, there is a strong connection and a specific design.

The interface between TLC and mass spectrometry can be considerably simplified in terms of the element of time. As with most other detectors used for TLC, the mass spectrometer is operated in an "off-line" configuration. The development of the chromatogram is complete before the detection of the sample spots on the TLC plate begins. This is the same mode of operation as, for example, in a scanning densitometer used to evaluate the chromatogram after the development of the plate. Solvents that are used to develop the plate can be removed before the sample plate is submitted to mass spectrometry for evaluation. The removal of time as a factor in the detection method allows for much greater flexibility in terms of instrument and interface design. Better sensitivity, increased selectivity, and wider dynamic range can accrue as a result. As new instrument designs appear, however, it is worthwhile to note that mass spectrometric detection need not be operated in an off-line manner. With proper consideration given to the need to maintain a vacuum within the mass spectrometer, it may ultimately be possible that TLC/MS can be operated in an on-line mode to monitor the progress of a planar separation.

1. TLC Operation Relevant to Mass Spectrometry

The coupling of TLC with MS involves identification of the sample molecule in a mixture. Consider a pure sample in a spot on a standard silica gel TLC plate, and the components of the mixture in which the identification must be made. In addition to the sample molecules, the silica of the plate is present in excess, as well as binder material, along with the fluorescent indicator(s) that may be incorporated into the plate. Residual solvents and salts from the developing solvent system will be present. Water will be held within the silica, even under the vacuum of the mass spectrometer. If the silica has been modified with an organic adsorbent, this organic compound or mixture of organic compounds will also be present. Any methods used to visually locate the sample spots (derivatization reactions) will leave reaction residues on the silica gel chromatogram. Mass spectrometry may be sufficiently sensitive to detect side products of the derivatization reaction that are often not characterized by other detection techniques. A mixture is invariably present at any sample spot on the layer; logically, the best analytical results can be obtained when the complexity of that mixture is minimized or when the detection experiment is designed to maximize the sample signal relative to the signal from other mixture components.

Normal- or reversed-phase silica TLC is now complemented with separation methods that use novel stationary phases, such as silica gel particles bound into a polymer membrane (available commercially as Empore media). Although the predominant use of these carriers has been in solid-phase extraction, TLC can be accomplished with silica gel in an Empore membrane. Affinity chromatography can also be accomplished in a planar format and, especially in conjunction with MALDI mass spectrometry, will constitute a

growing segment of planar chromatography. In these cases, the background materials expected to contribute to the mass spectrum may vary. However, the basic aspects of planar chromatographic separation remain similar.

In TLC, the sample is present as a spot distributed in the xy -surface of the planar chromatogram, extending into the z -dimension of the chromatogram as well. In TLC, sample concentration is as much of a concern in designing a viable interface as it is in gas or liquid chromatography. Consider a sample spot or band with a total xy -surface area of 2 mm^2 and a penetration into the silica gel to the $200\text{ }\mu\text{m}$ thickness of the silica layer. The amount of silica present in this area is about 0.4 mg (depending, of course, on the type of silica gel used to prepare the TLC plate). Assume that the sample spot contains $1\text{ }\mu\text{g}$ of sample material. The concentration of sample in the spot is about 0.25% w/w, considering only the sample and the silica. An interface must be designed to introduce sample and not silica into the mass spectrometer; the experiment should provide maximum signal for the sample and minimum signal for common components of the chromatogram. Both strategies have been developed in TLC/MS. Concentration of the sample in a smaller spot size, as a result of either increased chromatographic resolution or measures taken after the chromatography has been completed, are helpful in increasing the sensitivity of the detection method, especially in methods that involve spatial imaging of the sample. Detection methods that involve total extraction and removal of the sample molecules from the chromatographic matrix and subsequent concentration in a secondary solvent are less dependent on initial chromatographic resolution.

Since most TLC/MS methods involve some form of extraction of the sample from the chromatographic matrix, the solvents used for this extraction must be able to overcome the attraction between the chromatographic matrix and the sample molecules. For excision of spots and extraction as discrete samples, the eluting power of the solvent and the temperature of the extraction can generally be increased as necessary to accomplish the removal of the sample molecules from the matrix. For evaporation of the sample molecules from the adsorbent into the vacuum of the mass spectrometer, temperatures of up to 300°C may be necessary. For some compounds that bind very strongly to silica, even a temperature of 600°C has been shown to be insufficient for complete sample vaporization. For imaging experiments, in which the shape of the sample spots must be preserved, sample extraction is a more difficult problem, often depending on a balance between extraction and sample diffusion (see Sec. III.B). FAB and LSIMS use a matrix to support the sputtering of sample molecules from the surface; the matrix solvent is used to extract the sample into a more-or-less homogeneous solution. Efficiency of extraction is therefore the most important parameter to be considered. With the use of MALDI as an ionization method in TLC/MS, issues of extraction must be considered concomitantly with the details of crystallization. This procedure is in its infancy, and considerable future development in this area can be expected.

Finally, some practical matters have to be considered. Most mass spectrometer sources have been designed to be as small as possible and feature small-bore gas and liquid inlet lines. TLC/MS methods that involve the separate evaporation of samples into a gas stream or extraction into a secondary solvent can be used directly with these common inlet systems. If the chromatographic matrix and sample material are removed from the chromatographic backing, the sample mixture can be introduced simply as a solid sample on the direct insertion probe into the source of the mass spectrometer. However, if the

chromatogram itself is to be placed under vacuum in the source of the mass spectrometer, the source housing in general must be redesigned to accommodate the larger samples. In custom-built instruments, sample sizes of up to 20×20 cm can be held within the vacuum of the mass spectrometer. The ability to handle large samples minimizes sample handling (always desirable in TLC) or provides the capability for multiple chromatogram loading in the source of the mass spectrometer. There is another position in this discussion. As mass spectrometers become smaller and more portable, the coupling to TLC may no longer involve scanning the TLC plate within the source of a fixed mass spectrometer but rather may involve physical movement of the mass spectrometer itself (or part of the inlet system for the mass spectrometer) over the surface of a much larger TLC plate.

Numerical comparisons of the spatial and time distributions of molecules in various forms of chromatography are useful in describing the operation of TLC relevant to mass spectrometric detection. A brief numerical description was provided above. Here, a comparison based on molecular density is developed. In column chromatography, the sample elutes into the source of the mass spectrometer within a time corresponding to the width of the peak. For a symmetrically shaped peak representing 1 ng of sample and a baseline peak width of 5 s, the average sample flux into the source is 200 pg/s. Assuming an ionization source volume of 0.1 mL (as in an EI or CI source with homogeneous distribution of sample in the gas phase) and a molecular mass of 300 Da, the average source molecular density during the peak elution is therefore 2×10^{10} molecules per microliter. Sample molecules are mixed with residual air and mobile-phase gas, and perhaps a volatile solvent.

In thin-layer chromatography, the sample is held in an (x, y, z) -dimensioned volume that includes constituents of the stationary and mobile phases. Using TLC as the numerical model, assume that the sample is distributed uniformly through the thickness of a high-performance silica layer of 100 μm thickness; the z dimension is therefore 100 μm . Similarly, the assumption of a homogeneous distribution may not be accurate, but any later “extraction” process will render argument of this point moot. For simplicity, consider that the sample spot retains the dimensions of its original application onto the planar TLC surface. The values of x and y therefore depend on whether the sample is spotted or banded (and this ultimately affects detection strategies as well). However, again for simplicity, assume a circular spot of 0.5 mm diameter applied to the surface; the area of the spot is therefore 0.2 mm^2 , and the volume of the (x, y, z) spot is therefore 0.02 mm^3 or 20 μL . (Note that this surface area is much smaller than the vast majority of actual developed spots but illustrates the idealized limiting case.) The sample density, again with 1 ng of sample, is 0.05 $\text{ng}/\mu\text{L}$, and (assuming a molecular mass of 300 Da) the molecular density within the silica gel is 10^{11} molecules/ μL . The sample molecules are not isolated but are held within (and interact with) a complex matrix of phase and phase support materials.

The preceding numerical derivations conclude that the molecular densities (using the assumptions given) are slightly higher in thin-layer chromatography than in column chromatography. If the applied or developed spot size is larger or the silica gel layer is thicker, then the calculated molecular densities become very close, or identical within the limits of the assumptions made in the arguments. Molecular density itself is not a factor in determining the feasibility of the chromatography/mass spectrometry method. Differences in the physical environment and the availability of the sample in time and

space are determinant factors. The total time during which the sample can be made available to the mass spectrometer and the efficiency of physical transport of the sample from the chromatographic environment into the mass spectrometer are issues that are considered in later sections of this chapter.

2. Conditions of Mass Spectrometer Operation Relevant to TLC

The conditions under which a mass spectrometer operates places certain restraints on on-line chromatographic methods. Although ionization can be assumed to be instantaneous, mass analysis is not. As an example, fast-scanning quadrupole and magnetic sector instruments provide scan speeds (for integral mass resolution) of 0.1 s/decade. A mass spectrum measured from mass 1000 to mass 10 would require 0.2 s for scanning the analyzer plus about 0.05 s for system reequilibration. Such scan speeds have not increased significantly in the past few years and are only just adequate for recording several spectra across the elution of a peak from a GC capillary column or microbore LC column. Other mass analysis devices, such as ion traps or Fourier transform ion cyclotron resonance instruments, also have a time function in scanning. Time-of-flight (TOF) mass spectrometers do not scan but do require a pulsed ionization method and time for separate ion packets to pass through a flight tube. These latter instruments have not yet been widely used for TLC/MS, but promising applications are appearing. Time-of-flight mass spectrometers tend to be larger than quadrupole or ion trap instruments, although they are simpler in operation, and many of them are easily equipped with scanning sample stages. The use of MALDI with a TOF instrument is a rapidly expanding field of application. With sample spots in a developed thin-layer chromatogram, where the separation has already been completed, there are no constraints on the operation of the mass spectrometer. Depending on the analytical information required, either low-resolution or high-resolution mass spectra data can be recorded, and both positive- and negative-ion mass spectra can be sequentially obtained from the same sample spot.

The sensitivity of the mass spectrometer is of concern in the TLC/MS sampling. Mass spectrometry is inherently a destructive technique in that molecules must be transformed into ions that are mass analyzed to form the mass spectrum. This is in contrast to, for example, a fluorescence-based detection method, in which the photons absorbed and reemitted do not consume the sample, and for which long integration times can provide an extraordinarily high level of sensitivity. For samples in the molecular weight range of up to several thousand, a sample consumption rate of a few nanograms per second will provide a high quality mass spectrum with most ionization techniques and most mass spectrometers. In terms of TLC, an intermediate extraction step into a secondary solvent concentrates the sample and ameliorates such sensitive concerns. However, in direct imaging analysis (see Sec. III), the in situ extraction and sputtering process must be capable of providing that level of sample flux into the mass spectrometer, especially for the time required to record a spatially resolved image. Compounds with higher than average sputter ion yields, or selected ion monitoring experiments, can be used to decrease the necessary sample consumption rates into the picogram per second range. This sample consumption range is consistent with the sensitivity of modern mass spectrometers. A limiting factor on sensitivity is the percentage of molecules or ions in the sample transformed into ions passed into the mass spectrometer. In EI or CI, only

about 1 in 100,000 molecules are transformed into ions. The same ion production efficiency seems to be prevalent in FB and LSIMS. In MALDI, however, the efficiency seems to be in the range of 1–10% and perhaps higher, with predictable effects on system sensitivity. TLC/MS provides low nanogram detection limits. This limit will drop by a factor of 10–100 as more efficient means of sample molecule ionization are integrated into the practice of TLC/MS.

The requisite pressure for operation of the mass spectrometer can be no higher than about 10^{-6} torr. The chromatographic matrices and development solvents must be chosen with this factor in mind. Most volatile solvents can be removed in a pumpdown cycle that is part of the sample preparation procedure for analysis by mass spectrometry, but those solvents that have a particularly high affinity for the chromatographic matrix may be retained even under vacuum for long periods of time and may force the operation of the mass spectrometer at less than desirable pressure.

The complete MS system must also be examined as a detector in order to assess its fitness for coupling with TLC. In particular, the mass spectrometer must be able to provide an analytical capability that matches the capability of TLC. The informing power of any analytical technique can be defined as the number of binary digits that indicates how much information can be provided by the technique. The informing power for one variable parameter x is mathematically defined as

$$P_{\text{inf}} = R(x) \log_2 S(x) \ln(X_b/X_a)$$

where R is the average resolution of the variable x , and S is the average number of distinguishable steps of values for each measurable quantity. The terms X_a and X_b are the ranges of the measurable quantities. In the case of a quadrupole mass spectrometer with a 1000 Da mass range, unit mass resolution, and an ion intensity range of 2^{12} bits, P_{inf} is equal to 1.2×10^4 bits. Analogously, the informing power of chromatographic techniques can be calculated. In the case of capillary column gas chromatography, assume a 10 min run with 10^5 theoretical plates. If a peak emerges from the chromatographic column every 30 s, then $S(x)$ can be estimated as 20. If resolution of the column is defined as $R(x) = (N/5.54)^{1/2}$, then P_{inf} is equal to 2800. Similar calculations for other column chromatographic techniques provide estimates of informing power from about 1000 for packed column liquid chromatography to about 3000 for supercritical fluid chromatography.

The informing power for TLC must be calculated in a different fashion, because the technique relies on spatial rather than temporal separation. Consider a 100×100 mm two-dimensional TLC layer with spots that are 2 mm in diameter. Assume that a new spot is found every 4 mm. If sample spots with R_f values of 3.1 and 3.2 mm can be differentiated, then resolution is calculated to be 32. However, because the potential area for development is 100×100 mm, $S(x)$ is 5000, which more than offsets the poor resolution. The informing power of TLC is 3600, higher than for most forms of column chromatography. The combination of TLC with mass spectrometry will ultimately place a far more critical demand on the selectivity of the detector, and the ability to acquire and process large amounts of data, than even the most powerful present-day GC/MS systems. In addition, new developments suggest that the limits of detection may ultimately be lower in TLC/MS than in GC/MS, for example, and the broadened dynamic range will

also increase both the informing power and the demands placed upon the instrument control and data processing system.

As described at the end of the previous section, the sample is available to the mass spectrometer during the elution time window in column chromatography, although the sample is not present in a constant concentration. The mass spectra must be recorded during that 5 s period corresponding to the retention time (the value chosen in Sec. I.B.1), and the analyst must wait for that retention time window to record the mass spectra. The sample is in the gas phase or, in the case of electrospray ionization, contained within a liquid aerosol. The sample can be manipulated with relative ease, because the source volume is small and the sample gas and all other gases are thoroughly mixed. The ions that are formed in the ionization source are extracted within about 10^{-5} s into the mass analyzer of the mass spectrometer. The sample is entirely consumed within that 5 s period. Sample molecules that are not ionized are rapidly pumped away, with a total source residence time for the sample of only a few tenths of a second. This short source residence time is essential for the preservation of chromatographic separations.

In TLC, the sample is held within and interacts with the silica gel matrix. An in situ analysis (such as optical spectroscopy) probes the sample in that environment (or at least the part of the sample that is accessible to the spectroscopic method) and illuminates, but does not necessarily consume, the sample. However, mass spectrometry must consume sample to form the ions distributed in the mass spectrum. Therefore, there must be means (a) to release the sample molecules from the silica gel and (b) to transport the sample molecules into the liquid or gas phase for subsequent ionization. These processes require time. To attain the molecular density of column chromatography, all the sample molecules in the sample spot volume must be extracted and transported to the mass spectrometer within (in this example) 5 s. However, at the same time (and in analogy to a visual or optical location of the spots on the plate), if mass spectrometric data are used to image the spot in the xy -plane, then only a small amount of sample can be consumed during each (x,y) interrogation. Further, the sample spot should remain stable and unchanged while it is being imaged. These two goals are fundamentally at odds, and interface designs must balance the goals.

If the interface with mass spectrometry were designed to operate at a set spatial resolution and in only one dimension of imaging (along the axis of solvent development, as is common now), it could be designed to complete an exhaustive extraction within 5 s. Further, that extraction could be completed every 5 s in sequential spots on the TLC plate. This sequence of tasks is the TLC equivalent of sequential retention time windows in column chromatography. In many of the applications described in the following sections, the location of the spots is determined by classical means of visualization, and then the spots in an adjacent lane are individually treated with an extraction solvent outside the mass spectrometer. That particular area of the chromatogram may be cut out and mounted inside the source of the mass spectrometer, or a portion of the plate may be mounted in a holder that allows limited one-dimensional movement. In either case, the parallel between spatial coordinate(s) in thin-layer chromatography and time in column chromatography is imperfectly developed in current instruments and current practices. The extractions take several minutes for each spot (up to 10 min in some reports), and only one spot at a time is extracted, or many are extracted at the same time outside the mass spectrometer with resultant problems in sample diffusion in the xy -plane.

The measurement of the mass spectrum for each spot also consumes time. Using MALDI and a TOF mass analyzer (as in many of the applications described in the other sections of this chapter), mass spectra are averaged together until the signal from the sample rises clearly above the background signals from the matrix. Published applications often do not specify the time required for spectral measurement (because it depends on the amount of sample present in the zone and the efficiency of sample extraction and cocrystallization with the MALDI matrix), but 10–60 s is reportedly required to record the MALDI mass spectrum. There is similarly little discussion of how many discrete mass spectra can be recorded from an individual spot or how many spatially discrete mass spectra are used to define a spot. In a one-dimensional analysis, five or six discrete samples can be taken across a spot (albeit one that may be broadened through external application of the extraction solvent). If two-dimensional imaging is required, then the number of samples increases as the square of the one-dimensional value for equivalent resolution in dimensions x and y . For two-dimensional imaging of equivalent resolution, 25 to 36 discrete (x, y) -encoded mass spectral measurements would be needed.

In addition to the extraction process (and perhaps cocrystallization in MALDI) that must occur in a TLC/MS interface is the subsequent need for physical transport of the sample molecules to a site where they can be ionized. Early instruments that used gas-phase molecular ionization processes such as electron or chemical ionization used thermal vaporization to transfer molecules from the silica gel into the gas phase and then swept the gas into the ionization source of the mass spectrometer. Early uses of a micro flame or cartridge heater evolved into the use of an IR laser to accomplish the same task. The TLC/MS interface to electrospray ionization can use the extraction solvent as a carrier to transport the sample molecules to the ionization source. MALDI ionization can be considered (and advantageously so) to ionize the sample molecules directly from the solid phase of the chromatogram, but the situation is, in fact, more complex and problematic. Cocrystallization of the sample molecules and the MALDI matrix is needed before the matrix performs its functions as an energy and ionization buffer. The extraction solvent carries the matrix through the entire thickness of the silica gel layer. It is unclear how much of the sample migrates preferentially to the surface of the layer, where one could assume that the cocrystallization occurs and the ionizing laser can sample the crystals. Because multiple laser shots are used to create mass spectra that are summed together to form the “measured” mass spectrum, it is clear that each shot samples only a small fraction of the available sample at the surface. Furthermore, crystal homogeneity affects the spectral quality; variations in crystal size decrease the quality of the mass spectra that can be measured. No explicit information about the potential influence of the silica gel on the crystallization process has been presented, although the detrimental effect of the matrix in terms of spectral background ions has been previously noted. Thus, transport issues remain despite the fact that the ionization is directly from the surface of the thin-layer chromatogram. Sample molecules remain in environments that cannot be reached by the ionizing laser, or they are crystallized in such a way that they do not produce acceptable quality MALDI mass spectra.

If the mass spectrometer is to be used as an imaging detector, then the operation of the interface must allow the (x,y) coordinates of location to be correlated with the measured mass spectra. The mass spectrometer is usually used in the single-channel analysis mode,

recording mass spectra sequentially in time, although a multichannel instrument can certainly be used for the analysis of a thin-layer chromatogram. Analytical attributes to be considered therefore include the spatial parameters of the sampling; the accuracy, precision, and range of the chromatogram movement (if any); and the time required to measure a set of (x, y)-correlated mass spectra. Current practice (see Applications chapter in this volume) usually involve the excision of the sample spot from the chromatogram and analysis of the spots one at a time, or movement of the TLC chromatogram in one dimension only with a spatial resolution on the scale of a millimeter between sample spots. The imaging capabilities of the interface are either nonexistent or rudimentary. They may be included in the next generation of TLC/MS instruments as a true imaging interface is developed.

C. Approaches to TLC/MS

Thin-layer chromatography and mass spectrometry can be combined in three ways. In the first experiment, the compound of interest is eluted from the chromatographic matrix and collected, then introduced as a discrete sample to the mass spectrometer. In the second type of experiment, the sample is not separated from the adsorbent; both are introduced into the source of the mass spectrometer at the same time. In the third experiment, the entire intact chromatogram is placed within the source of the mass spectrometer and analyzed in a sputtering or desorption experiment.

In experiments of the first kind, the chromatography is simply a purification step. Once collected from a TLC spot that is identified with some independent method of visualization, samples must still be volatile enough to evaporate into the source of the mass spectrometer. In experiments of the second kind, the spot, also independently located, is scraped from the support and placed on the direct insertion probe of the mass spectrometer. As the probe is heated, the more volatile sample is evaporated into the source while the fairly nonvolatile chromatographic matrix remains in the probe. The method is destructive of both the sample and the chromatogram and again is limited to volatile samples. Particle-induced desorption techniques have made it possible to analyze samples directly from within the chromatographic matrix. These methods include secondary ion mass spectrometry (SIMS), fast atom bombardment (FAB), and laser desorption, including matrix-assisted laser desorption ionization (MALDI) analysis. Again, two approaches have been taken. In the first, sample spots are located independently, excised from the chromatogram, and then bombarded to sputter the sample molecules into the gas phase. In the third general type of TLC/MS coupling, the chromatogram is placed intact within a source housing and a spatially resolved organic map of the surface is measured, although within the constraints of the source dimensions and the raster ranges.

Finally, it should be noted that the concept of using mass spectrometry as a detection method for samples separated by thin-layer chromatography is not particularly new. Kaiser provided an overview of the possibilities in 1969 (5). A number of methods were described in which the sample spots separated by thin-layer chromatography could be evaporated from the chromatogram and routed in a gas stream to either conventional GC detectors or a mass spectrometer. Kaiser notes that "it is a disadvantage of the combinations that the optimum operating conditions of the instruments, which are not

designed for coupling, are readily lost” and that the design of a successful interface can become quite complicated. In a statement that retains its validity many years later, Kaiser notes finally that “equipment manufacturers will, however, not make the necessary modifications until they can be made to realize that direct coupling of methods and instruments is an important aid for the analyst.” The remaining sections in this chapter review the various methods used to couple TLC with mass spectrometry, with emphasis on how such combinations have indeed been of value to the analytical chemist.

II. TLC/MS BASED ON DISCRETE SAMPLE INTRODUCTION

Separation in TLC is spatial rather than temporal in nature, and an ideal TLC/MS coupling would preserve the spatial information inherent in the chromatogram. However, many of the earliest methods described in the literature relied on a separate and independent analytical method for spot location on the TLC plate followed by a discrete analysis of the sample spot material by the mass spectrometer. The sample molecules were evaporated into a gas stream or extracted into a secondary solvent. The extract is then submitted as a sample to the mass spectrometer. The resolution of the separation is not monitored by the mass spectrometer, which serves only to identify sample spots located by another technique. An advantage of such methods is their universal applicability; no modifications to the mass spectrometer are required, because the determination is now the same as would be involved in GC/MS or direct insertion probe work.

Although an experiment that collects individual fractions from a liquid chromatograph and analyzes them by mass spectrometry would not be described as LC/MS, the nomenclature “TLC/MS” has unfortunately been applied to such TLC experiments. A search of the literature to the late 1960s, when mass spectrometry first became generally available for organic analysis, therefore highlights many such “TLC/MS” couplings solely on the basis of the common keywords found in the title or abstract. Many other applications used thin-layer chromatography to prepare samples for mass spectrometric analysis but were not similarly indexed. The following summary provides a brief but not comprehensive use of these TLC/MS methods based on sample evaporation or extraction prior to mass spectrometric identification.

A. Volatilization of Sample Molecules

1. Evaporation of Sample Molecules into a Gas Stream

The article by Kaiser (5) reviews a number of methods that can be used to evaporate sample molecules from TLC spots into a gas stream that culminates in the source of a mass spectrometer. One notes that the methods are identical to those that would evaporate the sample from a TLC spot into a gas stream that leads to any of the detectors used for gas chromatography, so the use of a mass spectrometer as a detector is incidental to the design of the interface. Figure 1 illustrates a method in which a microflame is applied to the back side of a quartz-backed TLC plate, with the gas stream flowing over the sample and finally to the detector. The figure also shows the split in the gas stream between the

gas flow detector (such as a standard flow ionization detector) and the mass spectrometer. Samples must be evaporated from the planar chromatogram without decomposition and must not condense in the transfer lines to the detector. A number of organic compounds, phenols and higher alcohols, for example, were found to decompose on a heated silica gel layer.

An early description of a TLC/MS device can be found in the patent of Parkhurst and Mc-Reynolds [filed in 1974 and issued in 1975 (6)]. In the described apparatus, the TLC plate is placed on a platform close to the source of the mass spectrometer. Various zones of separated components on the TLC plate are heated one at a time, and the desorbed molecules are swept by a gas into the source of the mass spectrometer, which is operated in the normal manner. The focus of the patent application is a means to selectively heat one zone of the chromatogram at a time. For instance, TLC in a circular tube is described in this patent, with a heater coil surrounding the perimeter of the tube and moved along its length. Alternatively, a movable platform is used that brings zones under the irradiation of a fixed high-intensity light source or laser. Finally, a TLC

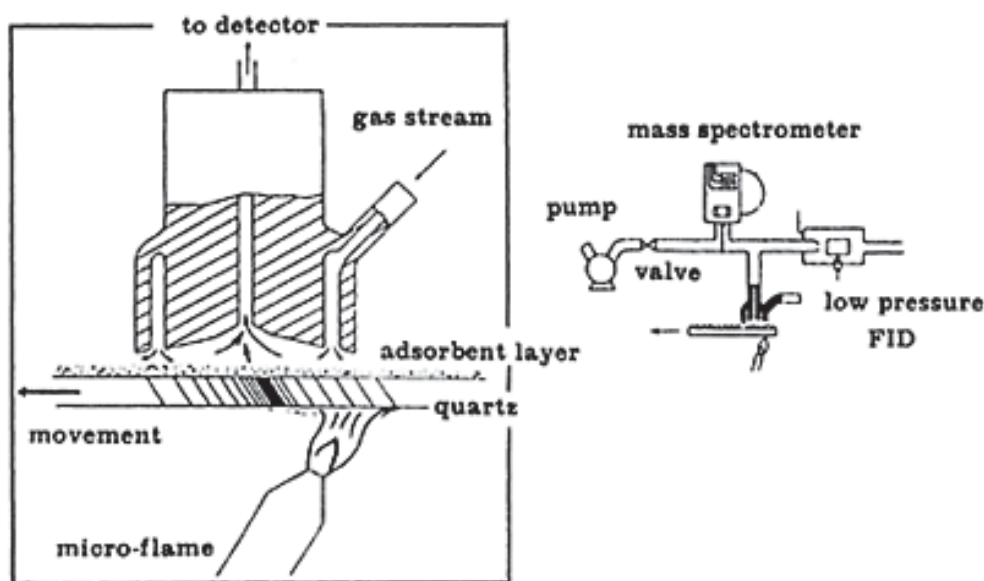


Figure 1 Microflame-based heating technique used for the evaporation of sample from a TLC plate and transfer into the source of a mass spectrometer. (Adapted from Ref. 5.)

plate that incorporates heating elements within the plate structure itself was also described. Again, the need for samples that can be evaporated without degradation into the gas phase is evident.

2. Extraction of Sample Spots from Adsorbents

Because many compounds are not thermally stable, much of the early TLC/MS work involved extraction of the sample spots into a liquid solvent and transfer of the resulting solution to the mass spectrometer. The transfer of material can be such that both the sample and the support are carried through the system, or the sample may be separated from the support and concentrated into the extraction solvent. Analysis of sample compounds alone is covered in this section, and Section II.A.3 covers coanalysis of the sample and support. This section covers TLC/MS methods that involve the extraction of the sample material from the support and subsequent analysis by mass spectrometry. Again, the coverage is illustrative and not comprehensive.

An early application of the extraction TLC/MS method was that of Schwartz et al. (7), who used TLC for separation and high-resolution mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy for the study of metabolites of diazepam in rats. UV irradiation and radiography were used to identify the sample spots of interest on the TLC plate. The samples were eluted from the sample support scraped from plates in the indicated areas and analyzed by mass spectrometry. Quantities of metabolites in the range of 50–500 μg could be characterized, although care had to be exercised in the sample preparation step to differentiate signals from samples from signals from impurities found in the blank extract of the silica gel TLC material.

A number of other investigators have used the TLC/extraction/MS approach. In many of these situations, GC/MS was unavailable or unsuited for the separation of the particular class of compounds under investigation. Derivatization of sample materials to make them sufficiently volatile for GC separation was possible in some cases but was not pursued due to the increased sample handling, lower sample recoveries, and increased chances for sample contamination involved. Applications include the use of TLC/MS in the detection of aflatoxins in contaminated cottonseed meal (8, 9), a study of the lupine alkaloids extracted from species of the plant family Leguminosae (10), of sapogenins from *Digitalis* species (11), of phenolic lipids from *Anacardium occidentale* (12), of the alkaloids extracted from *Ipomoea violacea* (13), determination of amines through the TLC/MS study of their dimethylamino-dinitrobenzoyl derivatives (14), and detection of tetrahydrocannabinol in saliva (15) and of a tetrahydrocannabinol metabolite in urine (16). There have also been a large number of applications in biological and medically oriented studies. Assmann et al. (17) studied the accumulation of oxygenated sterol esters in patients affected by Wodman's disease, a fatal infant disease. Biogenic amines were characterized in tissues as their dansyl-acetyl derivatives with TLC/MS (18). In pharmaceutical applications, an impurity in the anticholinergic drug clidinium bromide was determined by TLC/MS (19). The metabolism of steroids in several different animal species was followed with TLC sample preparation, spot extraction, and high-resolution mass spectrometry (20). The metabolites of phenacetin in urine (21) and identification of a number of drugs given to racehorses has also been accomplished with a combination of thin-layer chromatography and mass spectrometry (22). Metabolites of the carcinogen 7-methyl-benz[*c*]acridine were separated by TLC and high-performance LC and subsequently characterized by mass spectrometry (23).

The stability of organic compounds on thin-layer chromatograms exposed to air has been studied, with mass spectrometry used to characterize the products of degradation. Aromatic thiols undergo oxidation in air and dimerize to the disulfide (24). Arylindandiones, medicinal compounds isolated from various plants, also undergo

degradation in air and light (25). The rates of formation of the dimers can be followed with TLC, with characterization by mass spectrometry.

Two papers describe in detail methods used to transfer material separated by TLC into sample holders suitable for direct insertion probe mass spectrometry. Rix et al. (26) transferred the scraped sample spot into a drawn-out elution column and then eluted the sample through a plug into a separate part of the column (Fig. 2). The concentrated sample solution was then evaporated onto the tip of a standard direct insertion probe. Kohler (27) describes a similar method that can also be used for the collection of samples for subsequent GC/MS analysis.

The logistical requirements of such an analysis are not stringent. Much of this work in the literature is transparent, because the details of such a sample manipulation are within the experimental section of a manuscript, and the work is not referenced or indexed as an application of

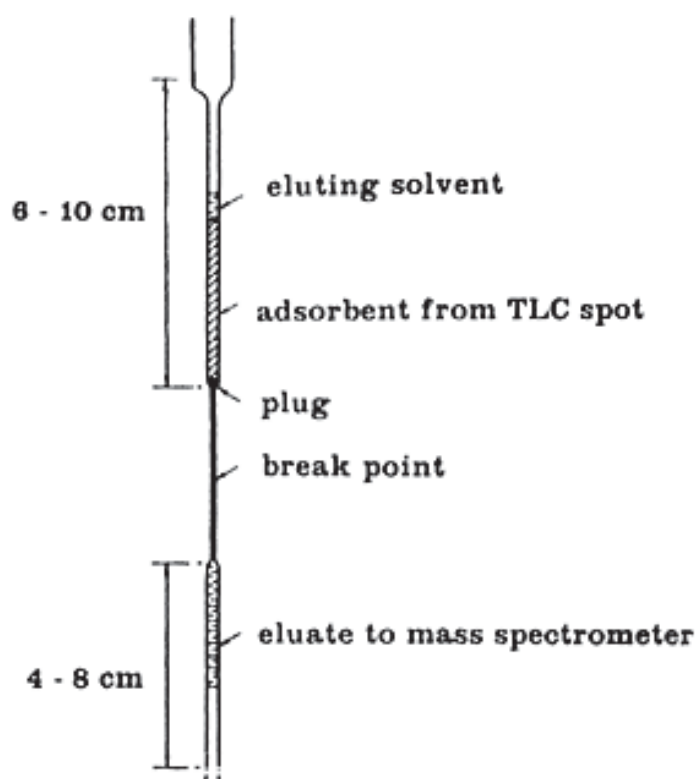


Figure 2 Transfer and elution technique for concentration of the material from a TLC spot into a glass capillary tube for introduction into the source of the mass spectrometer. (Adapted from Ref. 26.)

TLC/MS. The references cited in this section are therefore more historical in nature, showing the development of the method through this logical first step in its development. Work of this sort continues with newer ionization methods, including electrospray

ionization and APCI methods. As a caution, the flow of solvent that contains the sample extracted from a TLC separation should be passed through a fine particulate filter to remove the silica gel particles from the stream directed into the source of the mass spectrometer.

3. Coanalysis of Sample and Adsorbent

If the chromatographic matrix is sufficiently nonvolatile and the sample is volatile, both matrix and sample can be placed on the direct insertion probe of the mass spectrometer. As heat is applied to the probe, the sample evaporates, and either electron or chemical ionization can be used to obtain a spectrum of the compound. Again, an independent method for determination of the sample location has to be used.

Heyns and Grutzmacher reported in 1962 (28) that sample compounds could be evaporated directly from silica gel into the source of the mass spectrometer and that the sample must be present in the spot at a minimum concentration of 1% in order to obtain a mass spectrum of good quality. Some years later, Hutzinger and Jamieson (29) reported that indoles could be detected by TLC/MS using a similar method. Studies of this particular class of compounds was simplified because the indoles formed visible spots upon treatment with electron acceptors and could be evaporated from cellulose chromatographic matrix without decomposition to produce a satisfactory EI mass spectrum of the separate components of the complex. Later work (30) identified several different electron acceptor derivatization reagents that could be used to advantage in the TLC/MS determinations of the indoles as a compound class. The same procedure has been used in a TLC/MS study of the aromatic ethers in essential oils of plant-derived materials. In the study of Forrest et al. (31), compounds such as safrole, eugenol, and isoeugenol could be separated and purified by silica gel TLC, then located by a color-developing reaction with several different chromogenic reagents, and then the sample and the silica gel were introduced at the same time into the mass spectrometer source in the direct insertion probe. As the probe was heated, the sample molecules were evaporated into the vacuum and the silica gel was left behind. The mass spectrum of the ether-reagent complex is observed to be identical to the summed spectra of the individual components, because the complex decomposes during probe heating. With careful control of the heating of the probe, spectra of the sample and the chromogenic reagent could be recorded at different points in a fractional distillation.

TLC/MS has been used in the characterization of hydroxylated chlorobiphenyls (32). Twelve different chromogenic reagents were used in a study of 20 different hydroxylated chlorobiphenyls. The sample spots indicated by the formation of the characteristic color were scraped from the chromatographic support, and the matrix (silica) was introduced into the source along with the sample on the direct insertion probe. Careful heating of the probe produced good quality EI mass spectra of the chlorobiphenyl and the chromogenic reagent, with the latter requiring a higher temperature for evaporation into the source. Dansyl derivatives of hydroxylated biphenyls were also investigated by this TLC/MS method.

The TLC/MS method based on coanalysis of sample spots and chromatographic matrix has been used in the analysis of a variety of drugs. Down and Gwyn (33) used UV light to locate sample spots of phenothiazines, barbiturates, and other drugs such as

caffeine, codeine, and methadone separated on silica layers. Samples and silica were introduced to the mass spectrometer on the direct insertion probe. At probe temperatures of 250–300°C, most drugs produced high quality spectra with little evidence of any thermal decomposition. Some drugs were sufficiently volatile to produce EI mass spectra at a probe temperature of 200°C. Volatilization of 90% could be obtained at a probe temperature of 300°C for most of the drugs studied.

Kraft et al. (34) used polyamide TLC to separate mixtures of phenols, steroids, nucleosides, biogenic amines, and amino acids. With silica or aluminum oxide bases for TLC, many of the samples of interest could not be evaporated into an electron ionization source without excessive thermal decomposition. Samples were located on the polyamide sheet by UV light or spraying with a chromogenic reagent. The spot containing the sample and the polyamide material was carefully removed from the sheet with a spatula and inserted into an electron ionization source via the direct insertion probe. Once the characteristic evaporation temperature for the compound of interest was reached, the spectral signal remained stable for several minutes. Spectra could be reliably obtained with 0.1–3 μg of these samples, with a sample/matrix ratio of 1:1000. Background ions from the polyamide material appear to be limited to lower mass ions that do not interfere with the spectral interpretation.

The TLC/MS method combines the ability to separate small amounts of polar samples with the specificity of mass spectrometric identification of those materials. Fogy et al. (35) used TLC/MS to study degradation products of organophosphorus pesticides. Polyamide 6 was used as the TLC layer material for separation. Samples were located on the TLC plate with a sensitive enzymatic inhibition method. The areas containing the samples of interest were removed from the plate with a spatula and the mixture of sample and support introduced on the direct insertion probe. A temperature of 150°C was sufficient to evaporate the sample into the EI source.

B. Sputtering of Sample Molecules

As outlined earlier, a number of ionization methods have been developed that avoid the need to evaporate the sample into an electron ionization or chemical ionization source. Sample ionization methods such as fast atom bombardment or secondary ion mass spectrometry are now found almost routinely with commercial mass spectrometers. These ionization methods are based on a sputtering phenomenon in which the sample molecules are transferred directly from the condensed phase into the gas phase without the need for a separate and discrete evaporation step. As TLC methods are developed for larger and more nonvolatile sample molecules, the use of these sputtering methods will undoubtedly become increasingly important. An alternative to sputtering with a primary particle beam is the use of a laser beam in conjunction with the application of an energy-absorbing matrix to the surface of the chromatogram. The MALDI TLC/MS experiment is feasible in terms of sample preparation and instrumentation. What remains to be established is the link between the higher mass range capabilities of MALDI and the lower molecular mass compounds usually separated by thin-layer chromatography. For organization, the use of laser desorption will be grouped with the sputtering methods of sample analysis based on particle impact.

1. Extraction of Sample Spots from Adsorbents

Unger et al. (36) described in 1981 the analysis of thin-layer chromatograms by secondary ion mass spectrometry (SIMS); the particular compounds of interest in this study were the quaternary alkaloids found in mushroom tissues, chosen because of their relatively high concentrations in the plant tissue, the already characterized TLC methods for their separation, and the high secondary ion yields for the particular compounds in SIMS. Figure 3 illustrates the quality of the

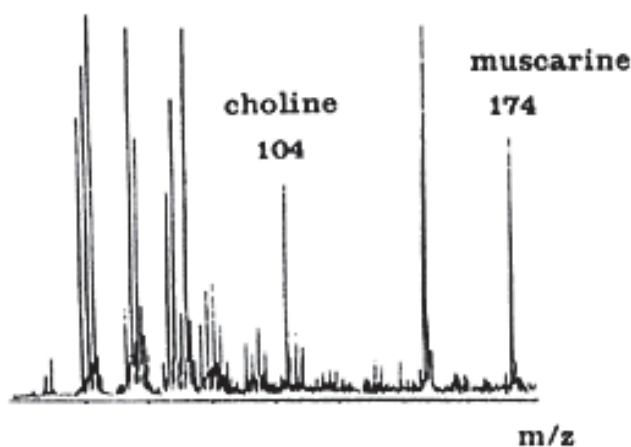


Figure 3 Positive-ion SIMS analysis of the ethanolic extract of a mushroom. (Adapted from Ref. 36.)

positive ion SIMS spectra that could be measured for an ethanolic extract of the sample spots of choline and muscarine on a silver support. This method is described as an indirect method of TLC/MS to illustrate the contrast with the direct method of analysis described in this same paper (and described in Sec. II.B.2). Subsequent methods described in the literature as “direct analyses” are, in fact, extractions of sample material from the TLC absorbent material. Chang et al. (37) described a “direct” method of analysis of TLC spots by FAB mass spectrometry. On examination, the method involves a sample extraction with the liquid solvent used for the FAB analysis. After completion of the TLC separation, the sample spots (the TLC/MS method was described for several different antibiotic compounds) are located on the chromatogram by UV fluorescence, and then the sample and the absorbent are lifted off the chromatogram with double-faced tape, extracted into glycerol, and analyzed (Fig. 4). The integrity of the chromatogram is destroyed, and the sample cannot be recovered after analysis. Tantsyrev et al. (38) described a similar method of indirect analysis that involves a simple extraction of the contents of the spot into a glycerol solvent and subsequent analysis by FAB. In these experiments, simple amino acids were studied by TLC/MS. Both silica gel TLC and paper chromatographic methods were described for the separation of simple mixtures of amino acids.

More complex samples were analyzed by a TLC/MS method that similarly involves extraction of the sample spots with the glycerol liquid matrix typically used in FAB. High-performance TLC plates were used for the separation of mixtures of amine

antioxidants and surfactants (39). Spots for both sample classes were identified by UV light, iodine vapor visualization, or a malonic acid-based amine visualization reagent. Once the sample spot was located, the perimeter was marked with a pencil, and the sample spot was loosened from the plate support with a spatula. The direct insertion probe was tipped with double-faced tape and then placed against the indicated sample spot area on the chromatogram. Thioglycerol (another common FAB solvent) was applied to the tip of the probe and left to equilibrate for 1 min. After extraction was complete, the direct insertion probe was inserted into the FAB source, and the positive ion FAB spectra were obtained in the usual manner. Detection limits of about $20 \text{ ng}/\mu\text{L}$ could be established for the determination of amines in gas oils. A time saving of a factor of 4 was quoted for TLC/MS relative to other analytical methods that had previously been used for these characterizations.

Masuda et al. (40) described the use of TLC/SIMS for the identification of nonvolatile xanthene and triphenylmethane dyes. Aluminum-backed and liquid paraffin-coated TLC plates were used to provide a useful separation of Acid Red, erythrosine, phloxine, and Rose Bengal. A mixture of dithiothreitol and dithioerythritol was used as the matrix of choice in the positive ion SIMS analysis, but direct application of this liquid matrix to the surface of the chromatogram caused excessive sample spot spreading, and satisfactory signals could not be measured unless the total dye in the spot exceeded $5 \mu\text{g}$. These workers describe a method in which the visually located

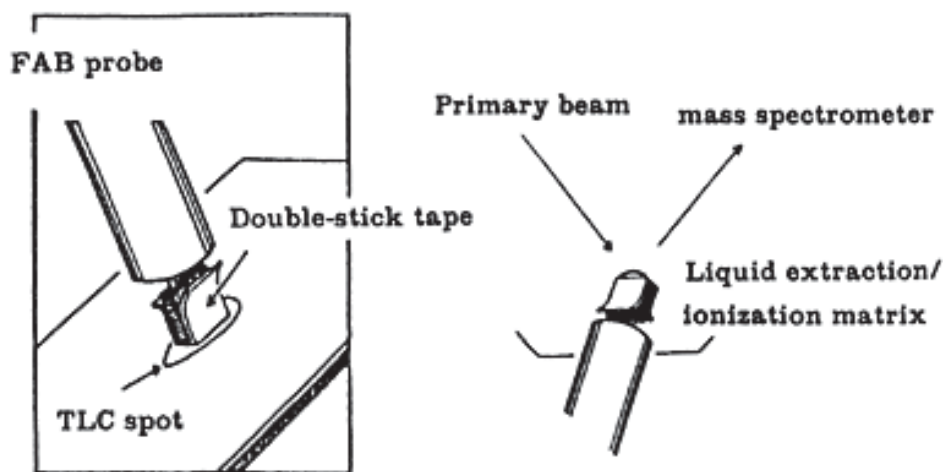


Figure 4 Transfer/extraction procedure for TLC spots for analysis by FAB/MS. (Adapted from Ref. 37.)

spot is encircled with thioglycerol. The thioglycerol concentrates the spot into a smaller area to which the second matrix is then applied. Using this concentration step, satisfactory signals could be obtained for sample spots with as little as $0.1 \mu\text{g}$ of material. Additional work using TLCTLC/MS in the analysis of dyes found in food was published (41). Permitted dyes were determined by a combination of the appropriate R_f value and mass spectra. Dyes not permitted in food could be similarly identified; a limit of detection of $20 \mu\text{g}$ was quoted in Ref. 42.

2. Direct Analysis of Sample and Adsorbent

The FAB experiments described in the previous section use a liquid matrix to ensure a steady secondary ion signal, as is the case with the analysis of discrete samples. The FAB matrix also serves to extract sample material from the chromatogram, whether this is done in a separate extraction outside the mass spectrometer or during bombardment of the excised sample. SIMS used for the creation of spectra from nonvolatile organic and biological samples also used the same suite of liquid matrices and is identical in concept. There are several sputtering methods of ionization that do not require the use of a liquid matrix, and therefore do not involve a liquid extraction of the same compound from the chromatographic spot. TLC/MS applications and techniques of this kind are reviewed in this section.

Unger et al. (36) were the first to describe direct TLC analysis by SIMS without the interdiction of an extraction solvent. Muscarine (a quaternary alkaloid from mushrooms with a high secondary ion yield) could be sputtered directly from a cellulose TLC matrix (Fig. 5). The experiment is based on the relatively low secondary ion yield of the matrix upon bombardment by the primary ion beam and the characteristic signal for the intact cation of the muscarine at m/z 174. The total amount of muscarine present in the TLC spot was about 16 μg . Now SIMS experiments use the liquid matrix typical of FAB experiments, and thus involve an extraction of the sample from the matrix.

Plasma desorption is an ionization method in mass spectrometry based on the passage of a very high energy (MeV) particle beam through a thin layer of sample material, generating sputtered neutral molecules, electrons, photons, and positive and negative ions as a consequence. Krueger (43) described an indirect TLC/MS method based on plasma desorption ionization. Substances separated by TLC are eluted from the adsorbent by a nonaqueous solvent and electro-sprayed onto a thin aluminum foil that serves as the support for the target. Fission fragments generated in the radioactive decay of ^{252}Cf pass through the sample target, sputtering both positive and negative ions from the surface. These ions are accelerated into and analyzed by a TOF mass spectrometer. Krueger claimed a lower limit of detection for compounds such as chloramphenicol and reserpine of 100 ng in the sample spot. Danigel et al. (44) described a larger set of applications

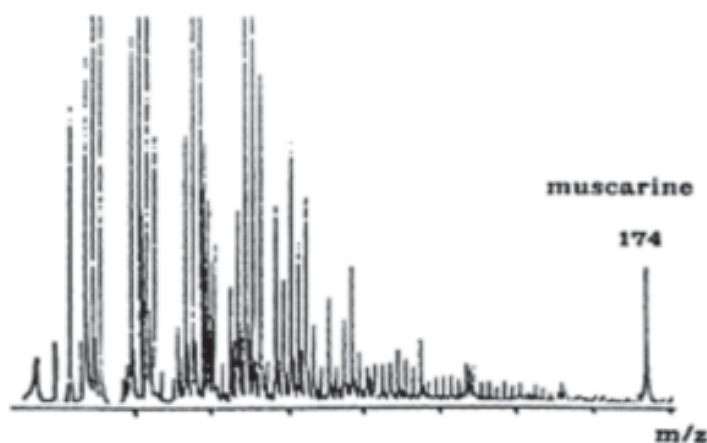


Figure 5 Direct analysis of a TLC spot for muscarine from an ethanolic

mushroom extract by positive-ion SIMS. (Adapted from Ref. 36.)

for the plasma desorption TLC/MS method. Thin-layered chromatographic separations had been developed for several common antitumor drugs (etoposide and teniposide) and their metabolites in response to the excessive time required for the first high-performance LC/MS analytical method. A faster and less expensive method for pharmacokinetic studies was required. In the method described by Danigel et al., a two-dimensional TLC separation is used for the focusing of the sample drugs in a clean sharp line on the chromatographic plate. The area containing the drugs was identified under UV irradiation, and this area was cut out from the chromatogram. A secondary extraction solvent of acetone was used to extract the sample, and the sample was dried, redissolved in chloroform, and electrosprayed onto the target support foil. Measurement of the plasma desorption mass spectrum with the TOF mass spectrometer took between 1 and 10 min, depending on the amount of sample present. An overall savings in time was realized, with TLC/MS capable of analyzing 20 samples per hour as contrasted with the three samples per hour that was typical of the LC/MS method.

Both Krueger and Danigel et al. described the use of a secondary extraction solvent for the removal of the sample from the chromatogram prior to spraying the sample in a thin film on the target foil. If the layer could be made sufficiently thin, ionization by plasma desorption could occur directly without the need for this extraction solvent. In fact, the integrating properties of the TOF mass analyzer are in concordance with this proposed experiment. Alternatively, the matrix can be removed from the backing material and redeposited on a very thin film of a mylar support for direct desorption. Methods might also be developed to remove the backing material in a grid pattern, leaving a very thin silica or cellulose layer stretched over the support grid. High-energy particles would pass through the thin portions of the sample, sputtering material from the matrix without the need for the extraction solvent.

Tetracycline antibiotics have been determined in bovine liver, kidney, and muscle, and in milk by solid-phase extraction followed by TLC/MS with FAB mass spectrometry (45, 46). A reversed-phase C_8 bonded phase silica TLC plate was used. Adjacent lanes of standards provided R_f values for the compounds of interest. This area of the chromatogram was cut into a trapezoidal shape, and additional solvent concentrated the sample in one end of the shape. That portion of the chromatogram was then placed on the FAB probe of a high-performance mass spectrometer. Then the FAB support matrix (thioglycerol) was added to the plate. A detection limit of $0.1 \mu\text{g}$ of sample per spot was reported for most of the tetracycline antibiotics. The trapezoidal slice from the TLC plate used to concentrate the sample for TLC/MS analysis was also used in an application of FAB mass spectrometry to identify and quantitate the drug midazolam (a depressant and anesthetic) in plasma extracts by Okamoto et al. (47).

Oligosaccharides have been determined with a similar method in which the sample bands were cut out from the chromatogram, loaded with solvent, and then sputtered by a primary ion beam (48, 49). The separation was performed on derivatives of the oligosaccharides. A support matrix of tetramethylurea-triethanolamine-nitrobenzyl alcohol was used to extract the sample from the silica and support the generation of negative ion mass spectra. In a variation of the technique, a syringe needle preloaded with

glycerol was touched to the sample spot; a small amount of silica was lifted off the spot and transferred to the stage of the direct insertion probe of the instrument which was also covered with glycerol. Because only a small amount of the silica gel was transferred, extraction was crucial for providing enough sample for analysis (50).

Thin-layer chromatography has been used extensively for natural products characterization, as shown in other chapters of this handbook. However, TLC/MS is only now being applied to this important analytical area. Lemire and Busch (51) used liquid SIMS with TLC to examine some of the alkaloid compounds present in extracts of *Sanguinaria canadensis*. The semisynthetic alkaloid nicergoline was analyzed in a plate cutting/elution experiment with positive ion liquid SIMS (52). A detection limit of 10 ng was complemented by a linear dynamic range of 50–1000 ng for quantitative purposes.

In any screening analysis, the ability to use high mass resolution MS to identify and confirm ion empirical formulas will become increasingly important. High mass resolution has been demonstrated with a multisector (53) and a Fourier transform ion cyclotron resonance mass spectrometer (54). In the latter instrument, MS/MS experiments can be carried out to help characterize the sample ions sputtered from the chromatogram. This very valuable experiment was used to advantage by Monaghan et al. (55) in their TLC/MS analysis of polymer additives separated by silica gel TLC. Lafont et al. (56) examined ecdysteroids from the plant *Silene nutans* and from the eggs of the desert locust *Schistocerca gregaria* using TLC/MS/MS, and deKoster et al. (57) identified a range of rhamnolipids from extracts of *Pseudomonas* microorganisms, using MS/MS to advantage in identifying the structural variation of the lipids. Nucleosides and bases can also be determined after TLC separation and MS/MS characterization (58).

Laser desorption MS has been the most widely used of the sputtering methods in the direct analysis of TLC plates without the use of an extraction solvent. Hercules (59) and Novak and Hercules (60) described a system that uses a commercial laser microprobe to sputter triphenyl-methane dyes from a high-performance TLC plate. Figure 6 illustrates the quality of the spectral data that can be measured for gentian violet and brilliant green. Because the instrument used was equipped with a sophisticated system for sample viewing and positioning, the dyes could be visually located through a sighting microscope, and areas were selected for analysis with a resolution of about 10 μm . Spectral contribution from the TLC plate was minimal, and the location of the organic materials could be specified to about 100 μm . A map of molecular distributions of dyes across a TLC plate determined visually is shown in Fig. 7, along with the masses of the ions found to be sputtered in each area. The use of laser desorption mass spectrometry in direct TLC/MS analysis can be expected to increase rapidly in the near future as the mechanisms of the thermal desorption and sputtering processes become better understood and as means of preparing the sample for efficient transfer of the sample molecules and ions into the gas phase are developed. Dunphy et al. (54) also used laser desorption for the analysis of TLC plates, and both normal-and reversed-phase TLC plates could be satisfactorily analyzed.

Matrix-assisted laser desorption ionization (MALDI) was used for TLC/MS by Gusev et al. (61). Absolute detection limits of 2–4 ng were demonstrated for bradykinin, angiotensin, and enkephalin derivatives. Application of the MALDI support matrix to the TLC plate after separation is completed induces a planar diffusion of 1–1.5 mm. In an interesting application of the MALDI TLC/MS method, the mass spectra of ninhydrin-

stained spots were obtained. Ions corresponding to the ninhydrin adducts with the sample molecules could be observed. A spatially resolved image for bradykinin on a TLC plate in a 2-(4-hydroxyphenylazo)benzoic acid matrix was also reported in this paper.

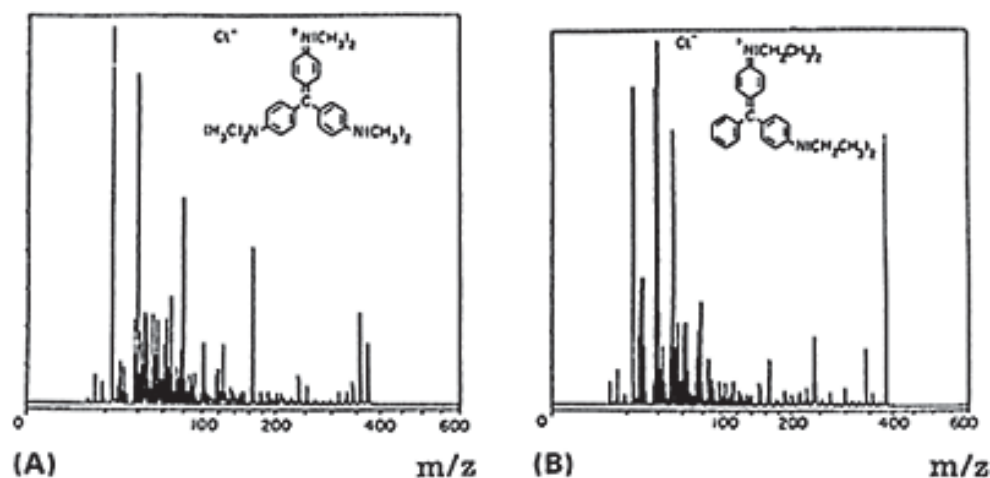


Figure 6 Positive-ion laser desorption mass spectra of triphenylmethane dyes from a TLC plate. (A) Gentian violet; (B) brilliant green. (Adapted from Ref. 59.)

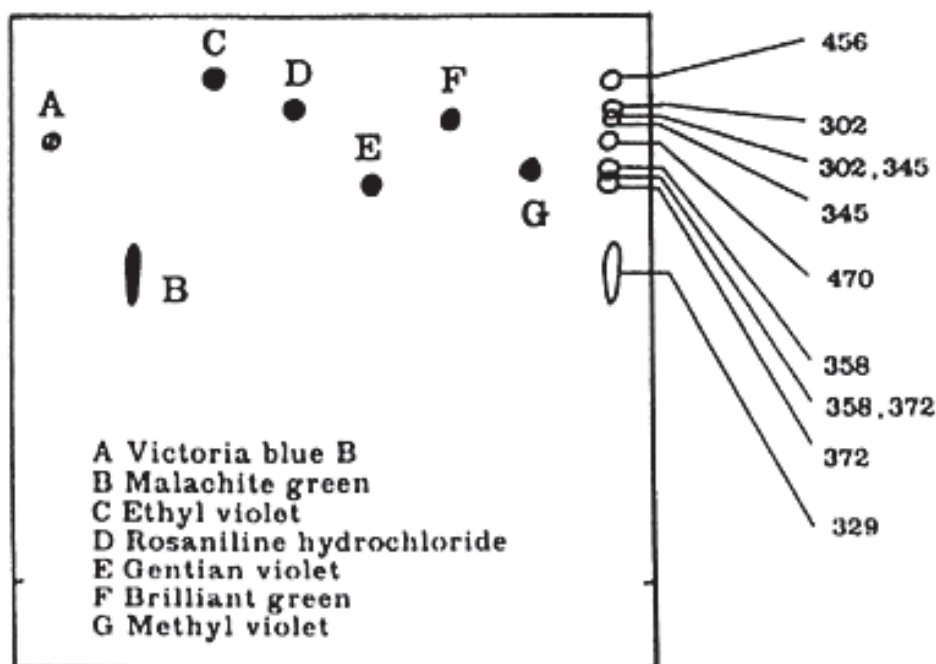


Figure 7 Molecular distributions of triphenylmethane dyes on a TLC plate,

with ions produced by laser desorption indicated. (Adapted from Ref. 60.)

III. TLC/MS BASED ON SPATIALLY RESOLVED ANALYSES

Once the methodology has been developed for the sputtering of organic molecules from surfaces, as detailed in the previous section, the extension of one- and two-dimensional imaging of organic compounds on chromatographic surfaces logically follows. In some cases, instrument sources and sample introduction devices have to be redesigned to accommodate spatial movement of the sample or the probe beam. In other cases, entirely new instruments are constructed that place emphasis on sample manipulation rather than mass spectrometer operation. In both cases, the spatial distribution of the organic compound in a sample spot or band is measured along with the individual mass spectra for each isolated component. The following subsection deals with methods developed in one-dimensional analysis, and Section III.B describes systems developed for two-dimensional imaging analyses.

The means to resolve data into spatially coherent images is readily available. As mass spectrometric data systems become more adept with multidimensional images for other types of mass spectral data, adaptation of these algorithms for the creation of sophisticated TLC/MS data will follow. The only commercial instruments available today provide a one-dimensional motorized scanning probe, and the data handling is exactly analogous to that for GC/MS and LC/MS. It can be predicted that MS/MS data maps will be transformed by far-sighted users into *x*, *y*-resolved data graphics before manufacturers invest time in the development of such hardware. Alternatively, data interchange protocols may provide the easier (but less satisfying) option of transfer of the mass spectral data into another system with complete graphics capabilities. This option, while taking advantage of existing technology, removes the on-line option of using those data in an interactive loop to control the acquisition of additional data.

A. One-Dimensional Systems

1. Plate Scanners Based on Sample Volatilization

Ramaley et al. (62, 63) described a TLC plate scanner based on the thermal evaporation of samples into a gas stream connected to the source of a mass spectrometer, with chemical ionization being used to create ions from volatile molecules. A sophisticated sample movement platform hooked

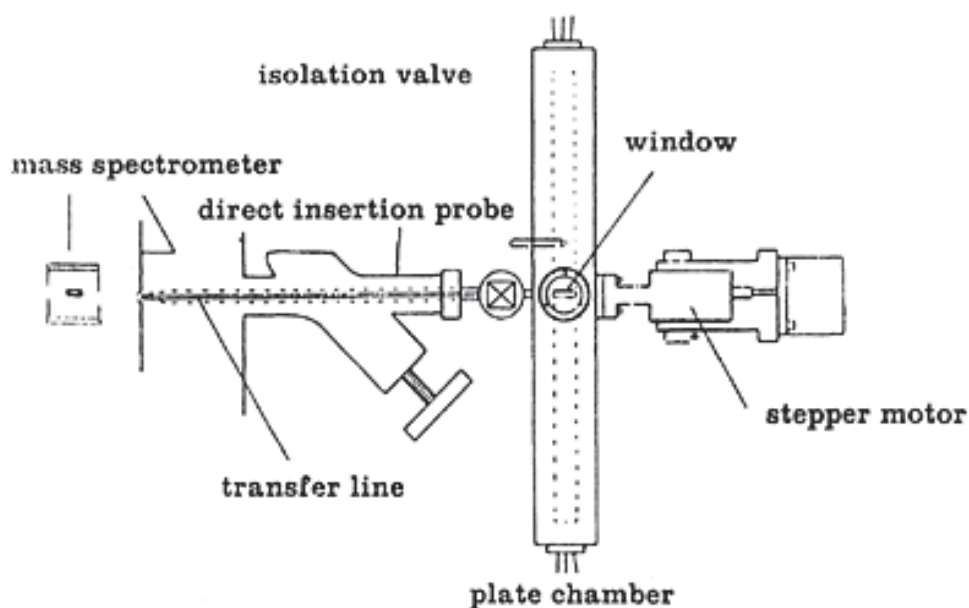


Figure 8 Block diagram of a TLC scanning system. (Adapted from Ref. 62.)

to a stepper motor was used to position the sample spots at the focus of a high-intensity incandescent lamp or a pulsed CO₂ laser. The light beam passes through a window in the scanning chamber, which is held at a pressure of about 1 torr. The chamber is pressurized with the same reagent gas (methane, for example) that would be used in the chemical ionization source. Sensitivity was shown to be about 1 μ g for a broad range of compounds spotted on the plate, with a reproducibility of about 20%. Figure 8 shows a general block diagram of the instrument, and Figure 9 some of the results obtained with this device. The stepper motors were programmed to move the TLC plate at a constant speed through the point of light focus to simulate the elution of compounds from a chromatographic column into the source of a mass spectrometer.

2. Movable Direct Insertion Probes

Tamura et al. (64) described modifications for a commercial mass spectrometer system based on a stepper motor driving a direct insertion probe into the FAB source of a mass spectrometer. The holder at the tip of the FAB probe can accommodate either an aluminum sheet 10×65 mm or a glass plate 7×65 mm. However, because sample movement is in only one plane, only a one-

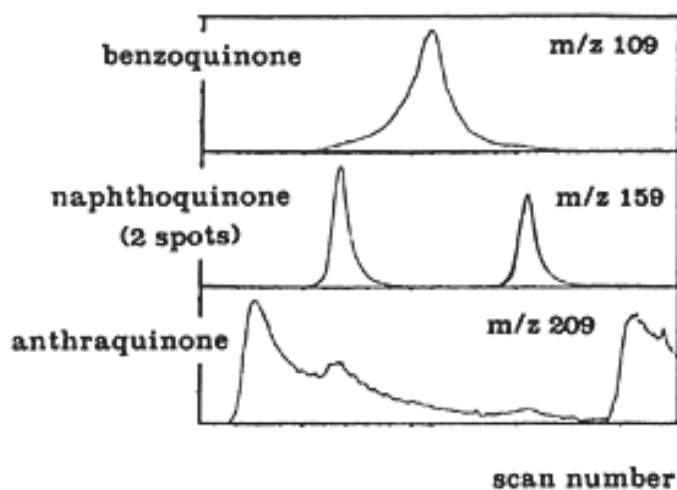


Figure 9 Results for scanning TLC for analysis of aromatic hydrocarbons.
(Adapted from Ref. 62.)

dimensional image of the spots can be obtained. The plate holder can be moved at a maximum rate of 50 mm/min, and the pulses to the stepper motor are controlled in conjunction with the scanning of the magnetic field of the sector mass spectrometer. The operation sequence is movement of the sample, acquisition of the spectrum, then movement of the sample again.

Because a liquid matrix (glycerol or triethanolamine) is applied to the surface of the chromatogram before analysis, this method, like many that have preceded it, relies on efficient extraction of the sample molecules into the secondary solvent and depends explicitly on the ability of that solvent to extract the material without diffusion of the samples in the plane of the chromatographic development. Because all matrices used so far have been liquids, a practical time limit of a few minutes is established before sample bleeding becomes excessive and the chromatographic resolution is reduced. Figure 10 displays the modifications that were made to the direct insertion probe. In principle, the one-dimensional analysis generates data in formats identical to those generated by GC/MS, and the same data processing and display routines can be used by the computer system. No real-time control of the chromatographic movement was described in this publication.

Several Japanese research groups have been active in TLC/SIMS and have described similar systems. This activity results from both the widespread use of TLC in Japan and the competition between instrument companies to devise a commercially viable TLC/MS system. Nakagawa et al. (65) filed a patent application for a chromatographic plate and analysis system in 1983 that consisted of a glass support for TLC with a number of grooves precut into the back surface. Once the chromatographic development was completed, the plates could be broken down into smaller strips that could be mounted directly to a movable direct insertion probe of the type described above. Figure 11 illustrates this device, which was used in the TLC/SIMS characterization of benzodiazepines, steroids, and metabolites of antifungal drugs (66). Several years later, Iwatani and Nakagawa (67) described a scanning TLC/MS method based on SIMS that

used the same type of special glass holders applied to the determination of mass spectra for compounds such as raffinose, small peptides, drug metabolites, and optical isomers of ibuprofen derivatives.

Kushi and Handa (68) described in 1985 a TLC/MS method for the analysis of lipids. Secondary ion mass spectrometry with a liquid matrix of triethanolamine was used for the extraction and ionization of sample spots first located with iodine or Coomassie brilliant blue staining. A piece of TLC plate 5×20 mm in size could be attached to the direct insertion probe, and scanning in one dimension was accomplished by manually inserting the probe into the source of the mass spectrometer. Spectra could be obtained from 1 μ g of a lipid separated on a silica TLC plate with aluminum- or plastic-backed TLC plates. Although not specifically noted in this paper, because a plastic-backed plate is an electrical insulator, some provision for connecting the surface to the plate platform itself must be made to hold the surface at the source potential.

Yamamoto et al. (69) described the combination of TLC with SIMS for the determination of acetylcarnitine and propionylcarnitine in urine. Quantitation was accomplished with a stable isotope dilution method. Kajiura (70) described a TLC/SIMS application to the determination of phospholipid and steroid mixtures, with chromogenic reagents for spot visualization and either triethanolamine or glycerol as the extraction/ionization matrix. A similar application to phospholipids was described the same year by Hayashi et al. (71). Phospholipids as well as antibiotics and small peptides were determined in the scanning TLC/SIMS device described by Shizukuishi et al. (72), associated with the Hitachi Instrument Company. A patent application filed by Hitachi in Great Britain in 1987 (73) describes the coupling of TLC with SIMS. A sample movement system is described in which the areas of the TLC plate between the indicated spots are rapidly transversed so that sputtering is confined to the sample spots of interest. This feature of TLC/MS had been previously described by other workers (see next section).

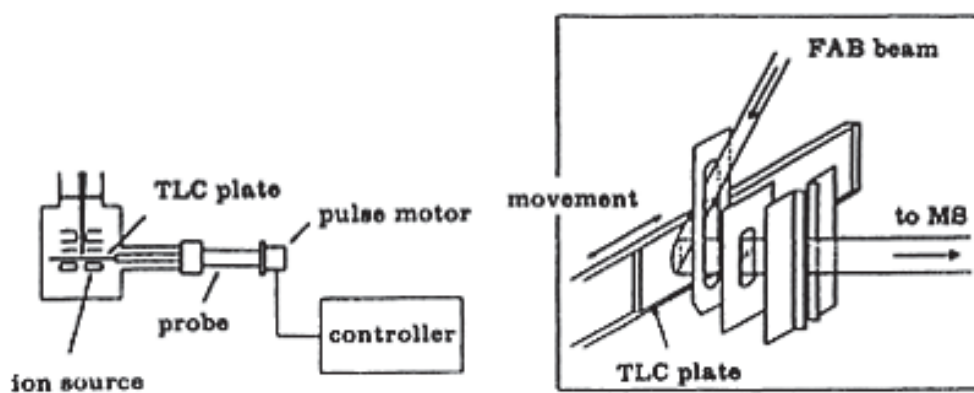


Figure 10 Modifications to a commercial direct insertion probe necessary for scanning TLC/FAB. (Adapted from Ref. 64.)

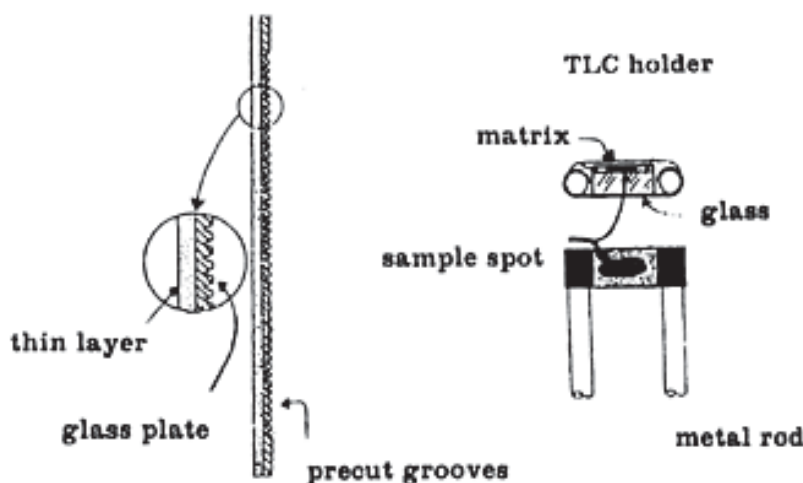


Figure 11 Special scored TLC plate used for scanning TLC/SIMS.
(Adapted from Ref. 65.)

Wilson et al. (74) used an MS/MS instrument equipped with a motorized one-dimensional TLC plate scanner to study a family of ecdysteroids in extracts of the plant *Silene otites*. Plates were cut into strips and attached to the probe, and glycerol solvent was added in preparation for the energetic particle bombardment. Consider the sophistication of the experiment. Mass spectra (and with the instrument used, even high mass resolution mass spectra) are recorded as a function of distance along the TLC plate. The negative-ion mass spectra recorded are characteristic for each of the three predominant ecdysteroids found to be present. In addition to the mass spectrum itself, product ion MS/MS spectra can be recorded for each of the mass-selected $(M-H)^-$ ions for the compounds. The high dimensionality of the data should be apparent, as is the great specificity achieved in identification of a particular compound at a particular R_f value, with a particular mass spectrum (and perhaps with a particular set of exact mass values for those ions), and with a particular set of product ions of particular intensities formed in the collision-induced dissociation experiment. With current instrumentation, all of the sophistication in this system resides with the mass spectrometer. However, higher resolution instruments and MS/MS instruments are dropping rapidly in size and price, and performance and ease of use are much improved. Within a few years, TLC/MS will be complemented with TLC/MS/MS and TLC/high-resolution MS as a matter of course.

Collaborative efforts directed by M.R.Clench at Sheffield Hallam University have produced MALDI/TOF TLC data used for impurity testing in commodity pharmaceutical compounds (74a, 74b). MALDI, as described earlier, is the acronym for matrix-assisted laser desorption ion-ization, and TOF signifies that a time-of-flight mass analyzer is used. Several important points are emphasized in these publications. Mowthorpe et al. (74a) note that the pharmaceutical compounds of interest are of relatively low molecular mass. The energy-absorbing matrix used to prepare the surface in MALDI often provides intense ion signals in the lower mass ranges. Avoidance of mass overlap is possible when both the specified matrix material and the targeted compound for analysis are

known and their spectra are recorded independently. These researchers investigated several means for depositing the MALDI matrix onto the TLC plate, finally choosing an electrospray surface treatment, not electrospray ionization. The issue of reproducibility of the data obtained from localized areas of the TLC spot in which the final sample-matrix cocrystallization may vary was addressed. In a subsequent publication, Cricelius et al. (74b) used TLC, MALDI, and the electrospray matrix deposition method to generate analytical data for an impurity profile for a drug development candidate. The candidate compound was identified, as were three related impurities. The authors also reported on the use of a lock-mass approach to make up for variability in masses measured in the TOF analyzer due to small differences in the nature of the TLC surface itself.

The research group of D.Hercules at Vanderbilt University continues to build on its early work in coupling MALDI with TLC (74c, 74d). They also investigated various methods for the deposition of the MALDI matrix onto the developed TLC plate and methods that could be used for quantitation of the targeted component on a TLC plate. Applications to the determination of cationic pesticides by TLC/MALDI (74e) and specific methods for quantitation down to the picogram level (74f) were recently reported by this research group.

Wilson reviewed state-of-the-art of TLC/MS (74g, 74h) with an emphasis on one-dimensional analyses. The mass spectrometric ionization methods used include electrospray ionization and matrix- and surface-assisted laser desorption ionization (MALDI and SALDI). Both MALDI and SALDI involve ionization directly from the surface of the chromatogram held under vacuum after addition of an energy-buffering matrix. The ionization occurs as the result of surface irradiation by a laser beam, with mass analysis usually accomplished with a TOF mass analyzer. SALDI is a newer ionization process used in coupling TLC with mass spectrometry (74i, 74j) but involves the same one-dimensional measurement approach. Chen (74j) described the in situ determination of organic reaction products using SALDI-based TLC/MS. SALDI is differentiated from MALDI in that the added matrix is thought to mediate the high energy of the desorbing laser through different processes. The matrix in SALDI used by Chen is a mixture of activated carbon powder, glycerol, sucrose, and methanol. The activated carbon is thought to act as the energy mediator, and the glycerol and methanol are transfer and extraction solvents. The sucrose acts as an adhesive (74i) between the matrix and the TLC plate, and the background signals from the SALDI matrix can be lower than those for typical MALDI matrices. The extraction of the sample from the silica gel occurs as the solvent repartitions the sample between the gel and the activated carbon. The sample molecules are released from the activated carbon in a subsequent (presumably) thermal desorption step, aided by the energy from the laser and the transfer of the sample molecules into the vacuum.

Anderson and Busch (74k) reported on the use of electrospray ionization coupled with TLC, and that publication includes a discussion of one-dimensional and two-dimensional interface designs. Electrospray ionization usually produces only molecular ions of the sample compound, with very little fragmentation. Complete structural deduction requires dissociation of the molecular ion, and therefore such dissociations must be induced. Given such a situation, MS/MS (sometimes called tandem mass spectrometry) is often used. In MS/MS, ions are subjected to at least two sequential stages of independent mass analysis with an ion activation step that leads to ion dissociation between them. Tames et

al. (74l) reported a study in which morphine was identified in urine extracts by using a combination of TLC and MS/MS. Organic reaction products were characterized by TLC/MS by Hilaire et al. (74m). This method was also used for confirmation of residues of thyreostatic drugs in thyroid glands using MS/MS after TLC separation (74n).

B. Two-Dimensional Systems

Spots of samples separated by TLC are two-dimensional. Several bands of samples can be run in adjacent lanes on a TLC plate, and scanning along a one-dimensional axis through the center axis of each lane can provide mass spectrometric information about the compounds separated. However, high-performance TLC and many other forms of TLC use two-dimensional development or circular development methods. A full two-dimensional imaging scan is necessary to discern the location of sample spots on the chromatogram and to determine the degree of spot overlap, if any. There have been far fewer reports of two-dimensional TLC/MS than of one-dimensional scanning methods, because the work requires either extensive instrument modifications or the construction of mass spectrometers especially designed for these experiments.

A number of commercial molecular ion microprobes are on the market, but relatively few of them have been modified for use in TLC/MS. To some extent, this is because TLC/MS generally requires the introduction of large amounts of organic solids and liquids into the vacuum system of the mass spectrometer. Most of the microprobe instruments are sold for surface science studies, typically carried out in the pressure range of 10^{-10} – 10^{-11} torr. Not only are the pumping systems generally incapable of handling large amounts of organic vapors, but once “compromised” as a TLC/MS instrument, ultrahigh vacuum cannot easily be reestablished. Increasing pressure from a community of analytical chemists, to echo the comments of Kaiser from Section I.C., should catalyze the efforts of instrument manufacturers in providing instruments capable of routine TLC/MS. Novak et al. (75) used a laser desorption microprobe to produce two-dimensional images of triphenylmethane dyes on a polymer surface (Fig. 12). The sample spot was selected manually through the sighting scope of the laser desorption instrument, individual data points were measured, and the total data set was reassembled into a spatially resolved mass spectrum of the organic dyes as a function of their x, y coordinates on the surface of the chromatogram.

Busch et al. (76) first described a custom-built secondary ion mass spectrometer for the analysis of thin layer chromatograms in 1985. The instrument has been through several revisions since the initial prototype was constructed, including changes in the size of the chromatogram that could be accommodated within the vacuum chamber, in the accuracy of sample placement for acquisition of spatial images, and in the data system used to control the scanning experiment and process the mass spectral data.

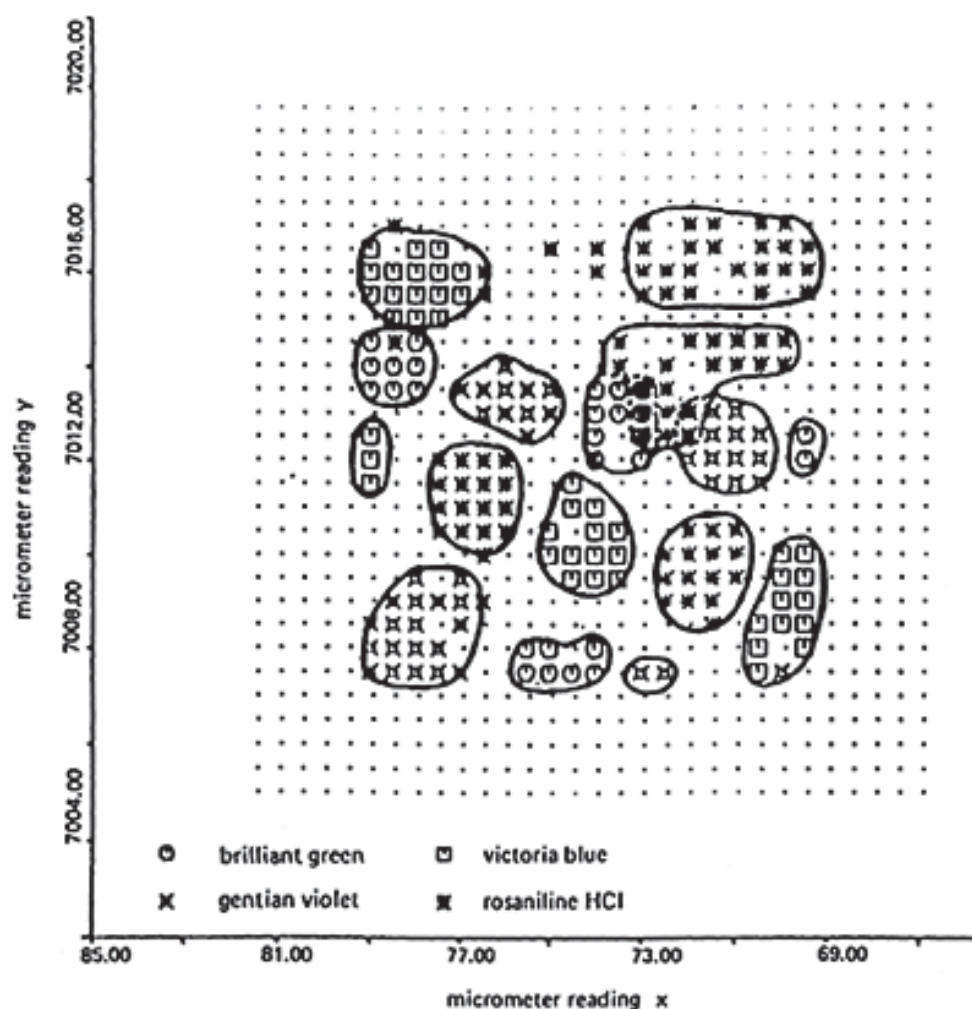


Figure 12 Molecular mapping of triphenylmethane dyes on a polymer surface by laser desorption mass spectrometry. (Adapted from Ref. 75.)

The original instrument was described by Fiola et al. (77), and the original data system by Flurer and Busch (78). As originally configured, the chromatography/instrument could accommodate 25×25 cm TLC plates and could place them at the focus of a primary ion beam with a spatial resolution of 1 μm . The primary ion beam originated in a thermionic cesium ion source of only moderate spatial resolution, but the use of a fine focus liquid metal ion gun (with a focus down to 0.01 mm) was also demonstrated with this instrument (79). In a second-generation instrument, the sample cell was enlarged to accommodate chromatograms up to 20×20 cm, and piezoelectrically controlled xy translators were used to place the sample spots within the point of instrument focus with a spatial resolution of 1 μm (80). The cesium ion gun can be replaced with a flange-mounted fast atom bombardment (FAB) source, a liquid metal ion gun, or a probe-mounted ion gun.

The liquid matrices typically used for FAB and liquid SIMS can be used in studies with the chromatography/SIMS instrument but yield limited time in which the image of

the sample can be recorded without diffusion of the sample spot in the xy plane of separation. A meltable matrix is used, as described by DiDonato and Busch (81), so that the matrix resides on the chromatogram in a solid form just below its melting point; the energy from the beam is sufficient to bring it into a liquid or semimolten state from which a persistent ion current can be measured. Doherty and Busch (82) showed the lack of planar diffusion in the matrix held just below its melting point and the diffusion that occurs as the sample matrix is liquefied. To increase the secondary ion yield for a number of species separated by TLC, a series of derivation reactions were developed that transfer the same molecules into preformed ions, often with surfactant properties in the matrix that is ultimately used for their extraction. The original concept of ionic derivatization was described by Busch et al. (83); methods of sample derivatization that do not increase the size of the samples were developed based on the voluminous TLC derivatization literature. Such derivatizations can be used in TLC/SIMS to increase the secondary ion yield of the separated compounds without an increase in the spot size (84, 85).

A number of applications for the chromatography/SIMS instrument have been described in the literature, including the determination of phenothiazine drugs (86) and quaternary drugs (87) and TLC/MS for coordination compounds (88), phosphonium salts (89), small peptides (90), polynuclear aromatic hydrocarbons (91), geoporphyrins (92), bile acids (93), diuretics (94), steroids (95), and alkaloids from plant extracts (96). In each case, an appropriate solvent must be found that extracts the material from the chromatographic matrix at a temperature and on a time scale compatible with the measurement of the secondary ion image of the surface. Although several general solvents work well for a number of compounds, consideration must also be given to the unique extraction and surfactant effects of each particular solvent-matrix mix, and this is an area of continuing research.

A few examples illustrate the nature of the data that can be obtained from a two-dimensional imaging chromatography/SIMS experiment. Figure 13 shows the image that results when the intact cation at m/z 215 for diphenylethylsulfonium bromide is monitored from a silica gel TLC plate. The primary ions from a gallium liquid metal ion gun were used as the sputtering source. The spacing between the grids is 0.1 mm. The preformed "onium" salts have excellent secondary ion yields; spectra can be obtained for 100 pg of sample material on the TLC surface, and imaging can be completed with about twice that amount of material, depending on the properties of the solvent selected for extraction and ionization.

Figure 14 shows the scan of the $(M+H)^+$ ion of a phospholipid separated from a mixture by TLC outside the analyst's laboratory. The TLC plate was shipped to the SIMS lab, the purity

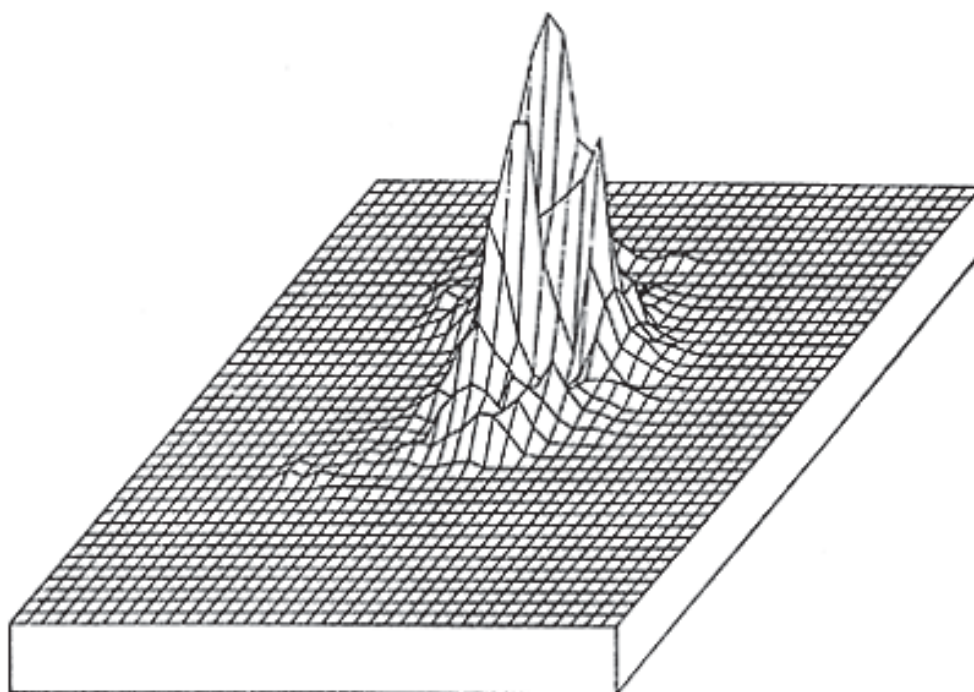


Figure 13 Two-dimensional scanning image of an organic sulfonium salt separated by TLC and sputtered by SIMS.

and spatial profile of the band in question were ascertained, and the plate was then returned to the original owner. Lipids in general also have good secondary ion yields, and microgram quantities of material are more than adequate for imaging analysis. The primary ion beam was generated from a cesium source for the data in Fig. 14; the grid spacing was 0.5 mm.

Because the mass spectrometric information is selective for each component on the surface of the TLC plate, a number of experiments can be carried out with TLC/MS that mirror experiments with other TLC detectors but with greater information resolution. One experiment that has

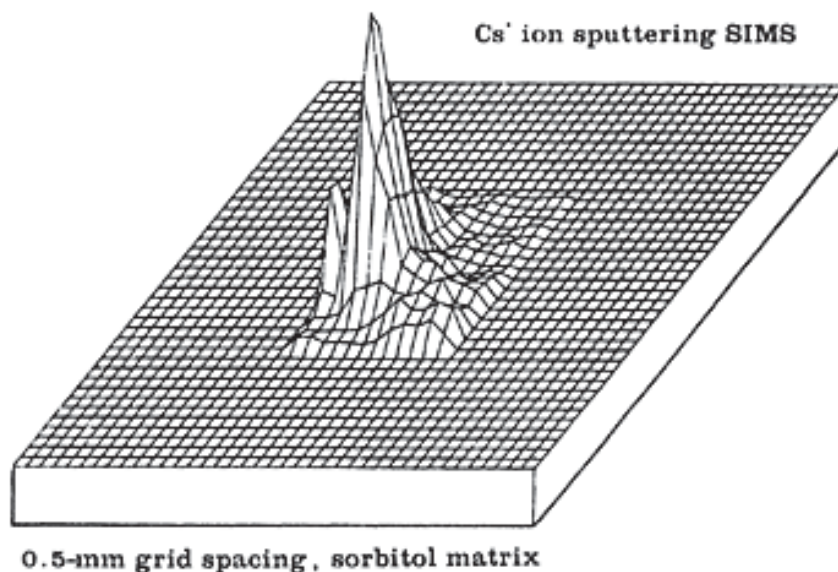


Figure 14 Two-dimensional scanning image of a phospholipid separated by TLC in an outside laboratory, transported to an analytical chromatography/SIMS instrument, and sputtered by cesium ions.

proven to be of value is selected sequence monitoring (90), which allows a search for peptides that contain a selected sequence of amino acids in a mixture of peptides. SIMS mass spectra of peptides typically contain an abundant ion corresponding to the protonated molecule $(M+H)^+$ along with fragment ions generated in predictable dissociations along the peptide sequence. In the selected sequence monitoring experiment, the mass analyzer is set to monitor the mass of an appropriate sequence ion, and the chromatogram is moved in the x and y dimensions. Each peptide that dissociates to form an ion of specified sequence that is characteristic of its mass will produce a local maximum in this plot. Peptides on a plate can therefore be grouped into classes according to their common sequence ions. This experiment can also be used to gain limited information about the sequence of peptides for which the molecular ion mass itself is beyond the range of the mass analyzer. Bradykinin and *d*-phe-bradykinin are two important muscle peptides. Figure 15 shows images of a thin-layer chromatogram containing both of these peptides. Figure 15a was obtained by monitoring the protonated molecule of bradykinin (m/z 1061), and Fig. 15b by monitoring the spatial distribution of the protonated molecule of D-Phe-bradykinin at m/z 1111. A sequence ion common to both peptides at m/z 528 gives the dual-maximum spatially resolved plot shown in Fig. 15c.

Imaging TLC/MS can be accomplished with some models of commercial secondary ion mass spectrometers. These instruments can provide very high spatial resolution and exquisite sensitivity, although they are not usually used for the analysis of organic compounds on TLC plates. Such a sample would be “dirty” and viewed as a source of

contamination in an instrument chamber maintained at a vacuum far lower than is usual in organic mass spectrometers. Some of this concern is unfounded; modern TLC plates, when handled with care, do not delaminate when being transported into the vacuum chamber, and once the volatile solvents are removed in the antechamber, the samples bound to the silica gel do not exhibit an appreciable vapor pressure either. Once these hurdles are overcome, the extraordinary capabilities of these instruments can be used to advantage. Mullis et al. (53) used an imaging TOF SIMS instrument to sputter samples from TLC plates. A spatial resolution of a few tens of micrometers was easily obtained, and a mass resolution of 2100 was measured. Individual silica particles on the surface of the gel could be seen. The mass-resolved ion image documented the distribution of the organic compound in those particles, providing an unprecedented glimpse of the TLC separation at the particle-by-particle scale.

IV. CONCLUSIONS

There are many reviews of TLC/MS (97–99) that focus on various aspects of instrument design or technique applications. As the number and complexity of applications increase, some general aspects of TLC/MS are worth remembering. The advantages of TLC/MS are derived mostly from the well-known characteristics of TLC, extended through the high informing power of mass spectrometry. In TLC/MS, because time is not a factor in the detection system, any spots in the two-dimensional chromatogram can be investigated in any order. This is a tremendous advantage in analysis of mixtures in which the presence or absence of targeted compounds is to be determined and a priority list of compounds can be established. The detection system can then be used first to search for the high priority compounds. A chromatogram can be rescanned many times to increase the sensitivity of the analysis through data processing techniques, even with mass spectrometric detection. Most mass spectrometric measurements are destructive in nature, but FAB and SIMS in particular are surface-sensitive techniques in which the material consumed in the analysis is sputtered only from the top few micrometers of the sample spot. The remaining sample that resides in the underlying bulk can be recovered after the SIMS analysis. The use of SIMS in the detection system therefore also allows experiments in which samples can be repetitively scanned.

There are unique advantages to a mass spectrometric detection system, the most significant of which is the tremendous increase in the amount of information obtained for each spot. There are over 1000 independent channels of information corresponding to unit mass resolution across the mass range of the spectrometer. For each of these channels in the mass spectrum, the y-axis

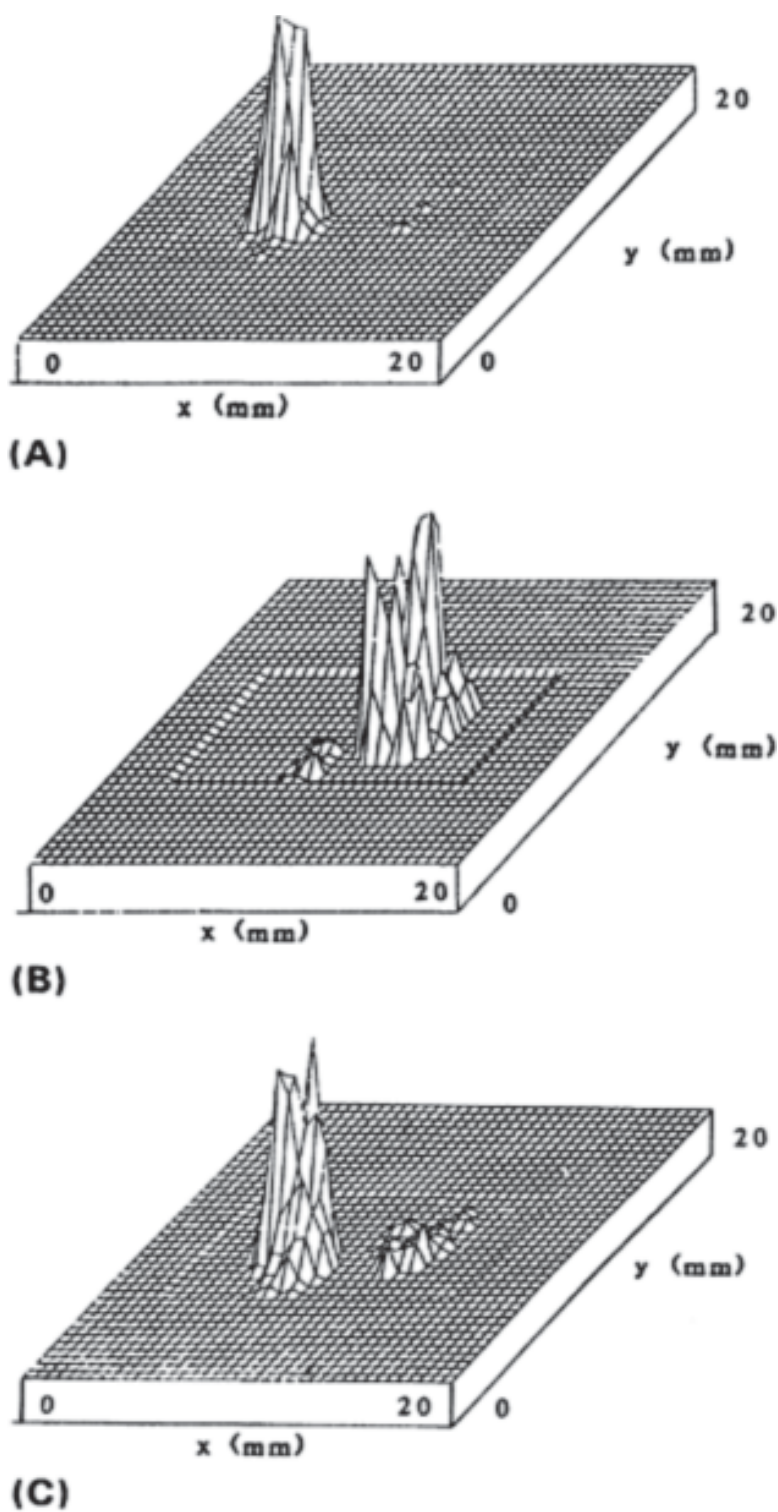


Figure 15 Molecular ion and selected sequence monitoring images for two peptides separated by TLC. (A) Bradykinin, m/z 1061; (B) D-Phe-

bradykinin, m/z 1111; (C) m/z 528.
(Adapted from Ref. 90.)

is the relative abundance of the ion of that particular mass, determined from 0% to 100%, generating typically 100 additional discriminating bits. With experiments such as MS/MS, even more information is recorded, and even higher discrimination can be achieved. A second advantage is the finer spatial resolution possible with the imaging SIMS system compared to even the most sophisticated densitometers. The focusing liquid metal ion gun described in the instrumental section is ultimately capable of giving a spot on the chromatogram of 10 μm diameter; a complete mass spectrum can thus be obtained for each 10 μm x or y movement. The first experiments of this kind for nonchromatographic samples were reported with commercial ion microprobes.

The information from the mass spectrometer is valuable for interpretive purposes but may ultimately be used for on-line control of the scanning experiment itself. The mass spectrum contains ions that indicate molecular weight and structural information, arranged in patterns of mass and relative abundance. The independence of the information for each individual sample compound means that data processing can be used to deconvolute spectra from mixtures of compounds, and the effective resolution of the chromatographic separation can be enhanced. Analysis of the mass spectra with x - and y -dimensions is also used in a feedback mechanism that changes the spatial resolution of the chromatogram movement when spots are present to precisely the resolution necessary to deconvolute the overlapping peaks. A minimum fraction of analysis time is expended acquiring spectra from portions of the gel that do not contain samples. This algorithm, designed to operate with completely unknown sample mixtures, ensures the most efficient use of the system.

Finally, because the separation has already been completed in TLC before detection of the spots is pursued, mass spectrometry is only one of a number of methods that can be used for spot location and sample identification. The list of analytical methods that can be brought to bear upon the analysis of a TLC plate include visual analysis, UV/visible spectrophotometry, fluorescence spectrophotometry, optical microscopic techniques, electron microscopic techniques, ESCA, Auger, reflectance infrared spectroscopy, radioimaging methods, near-infrared analyses, and finally, mass spectrometry in several forms, including SIMS, FAB, and laser desorption ionization. Sample positioning and manipulation are central to each of these methods. A sample ferried between the various instruments is subjected to increased handling and increased contamination and also to the various size constraints of the sample introductions on the various instruments. Assuming that the system should deal with samples of dimensions like the usual 10 $\times$ 10 cm chromatogram, the limiting of sample size for any of the instruments becomes the constraint in sample size range for any of the others, and most instruments will not accept samples of this size, even if the analysis itself is not completed over that range.

The direct replacement of a mass spectrometer detector with a CCD optical camera detector for TLC analysis was described by Busch et al. (100, 101). The sample remains within a positioning chamber, and the positioning hardware remains the same. A global coordinate system pinpoints the sample coordinates and allows correlation between different types of analytical data. The described instrument is a prototype of a general analytical system for the analysis of planar chromatograms that allows the use of several

analytical techniques and incorporates sophisticated image analysis software (102). In this system, the detector instrument modules are added into the system as needed and replaced as necessary. In future developments, two distinct variants of TLC/MS may appear. The first is the accessory TLC/MS that is added to a general-purpose mass spectrometer, such as the one-dimensional movable probes described in Section III.A.2. The second version is TLC/MS in conjunction with a variety of analytical methods in a smaller modular station that emphasizes chromatographic positioning and analysis and the interchangeability of detection systems.

Complex mixture analysis is a proving ground for analytical methods that combine high-resolution separations with equally powerful detection systems. Growing needs for separations of biological mixtures have catalyzed advances in high-performance liquid chromatography, capillary electrophoresis, affinity chromatography, and planar forms of electrophoresis, including agarose and polyacrylamide gel electrophoresis. As with thin-layer chromatography, planar electrophoresis can be coupled to mass spectrometry (PE/MS). There are two aspects of this interface to be considered. The first is the physical manipulation of the electropherogram itself (or the sample extracted from it) into a form compatible with the mass spectrometer's requirements, and the second is the selection of an ionization method. A distinction is again drawn here between methods that attempt to analyze the gel in *x* or *xy* dimensions and those that elute the sample components from the gel and then present a discrete sample solution to the mass spectrometer for analysis. There are ample examples of the latter sort (103–109), including work of Camilleri et al. and other early workers. Work that also involves the preparation of discrete sample solutions includes the investigation of the “freeze-squeeze” method (111, 112), originally described for the rapid recovery of long DNA strands from agarose gels, gel disruption, and homogenization methods (113, 114) used in conjunction with flow-FAB ionization. An early approach to PE/MS (115) used plasma desorption ionization (very fast and very heavy ions derived from a tandem accelerator or present as fission fragments of a radioactive nuclide are used to sputter molecules and ions from a thin sample surface) to determine mass spectra for protein mixtures separated by gel electrophoresis and then transferred to a nitrocellulose membrane with an electroblotting procedure. The use of nitrocellulose membranes and electroblotting procedures is well known within the bioanalytical community. PE/MS has also been accomplished with SIMS to analyze samples separated in paper and cellulose acetate electropherograms, and in these instances no transfer or blotting procedure was necessary (116). With agarose and polyacrylamide gel electrophoresis (PAGE), sample transfer procedures using capillary blots, vacuum blots, and electroblots were further developed (117, 118). These experiments were successful in first demonstrating the spatial fidelity of the transferred material, the two-dimensional imaging capabilities of the PE/MS combination, and the use of mass spectral information to deconvolute overlapping compounds in a single electrophoretic band. Additional work studied various quantitative aspects of sample sputtering from paper and cellulose acetate electrophoretic membranes and the use of digestion reactions of larger peptides on the transfer membrane (119). The report of Nagashima et al. (120) on the cellulose acetate electrophoretic separation and mass spectral characterization of tetrodotoxin is a noteworthy application of this method.

The nature of the membrane onto which the electrophoresed sample is transferred is important. Early work used nitrocellulose membranes, followed by standard biochemical

protocols and the early observation that ion signals for high mass biomolecules from such surfaces were of greater intensity than other surfaces such as metals. Later a number of other membrane surfaces were used in PE/MS, including nylon and poly(vinylidene difluoride) (PVDF) (121–124). Although these materials were new to mass spectrometrists using MALDI, they and a host of other materials were well known in biological applications. The membrane or modified membrane material must be able to blot the sample compound, and it must also be compatible with the matrix molecules used in MALDI. The matrix/sample ratio may often be 1000:1 to 10,000:1. Methods for application of the MALDI matrix, an integral part of sample preparation, are not considered here in detail but are reviewed elsewhere (125).

The development of mass spectrometric detectors for planar electrophoresis followed a course charted previously for the development of TLC/MS. One can reliably predict specific developments in PE/MS in parallel with those of TLC/MS. The scanning capabilities just now becoming evident in PE/MS will be supplanted by imaging capabilities that allow a full two-dimensional characterization of the separated bands. Experimental methods for sample preparation, reaction, and manipulation become noticeably more sophisticated as users realize that the separation matrix itself can be used innovatively as a support and as a tool. Methods will certainly develop that use “chemistry” such as sample digestions and derivatizations. On a planar chromatogram, we can carry out such reactions repetitively, simultaneously, and sequentially. We will also be forced, on the other hand, to develop data systems, imaging systems, and analysis systems that can manage orders-of-magnitude more data than we currently manipulate. As for TLC/MS, we will seek to integrate many different types of spectroscopic data and analytical measurements in one global coordinate system and then to search for correlations and patterns in those data. The distinctions between TLC/MS and PE/MS will eventually disappear as we construct a seamless, automated analytical approach that takes full advantage of the particular values of planar chromatography for analytical measurements.

The first and second generations of specialized custom and commercial instrumentation have been developed, used, and publicized. TLC/MS will always be compared in its analytical performance to other forms of chromatography coupled with mass spectrometry. This is the value of performing the numerical evaluations described earlier. To achieve sample densities in TLC/MS equivalent to those in the GC/MS method chosen for comparison, the entire sample must be extracted and made available for ionization, with preservation of the original spatial dimensions of the sample spot or band application, within 5 s. However, in reality, the 5 s window for complete sample consumption in column chromatography is lengthened into a 5 min window for partial sample consumption in planar chromatography, assuming that the extraction (completed off-line) and cocrystallization make as much sample as possible accessible to the mass spectrometer. If we assume that 5–10% of the sample is so accessible (either directly, as a transfer to some intermediate such as the activated carbon used in SALDI, or through an enrichment device), then the overall factor is at best (60×10 , and only for a one-dimensional analysis) 600–1200 times less sample flux into the source of the mass spectrometer in planar chromatography. The exact value again depends on assumptions in the argument, but this factor is reasonable in terms of reported limits of detection. GC/MS using column chromatography and electron ionization routinely provides limits

of detection in the low nanogram range. Cricelius et al. (74b) provided chromatographic data with a signal-to-noise ratio of 5 for 25 μg of sample on the TLC plates. The lower sample flux into the source of the mass spectrometer is a direct consequence of TLC/MS interface design and, more important, the analyst's implicit approach to how planar chromatogram spots should be detected.

Instrument designs for TLC/MS have involved many types of mass analyzers. Quadrupole mass filters and ion traps offer the advantage of relatively small size. Exact mass measurements are possible with the use of double-focusing sector mass spectrometers or Fourier transform mass spectrometers. Analyses of planar chromatograms with laser desorption and MALDI have typically been completed with a TOF mass spectrometer. Once the ions from the sample are in the gas phase, it might seem that any procedure that accomplishes a mass analysis would be satisfactory. However, as shown above, the ion flux in TLC/MS is usually several orders of magnitude less than in column chromatography. To become competitive with the sensitivity demonstrated by methods that use column chromatography coupled with mass spectrometry, however, and to maintain the imaging capabilities that are desirable, a nonscanning mass analyzer might be best for TLC/MS. The TOF mass spectrometer or an ion storage instrument such as the ion trap or the Fourier transform mass spectrometer are the mass analyzers most suitable for TLC/MS applications as currently practiced (126).

The manner in which TLC/MS is now completed, with a separate and external application of the extraction solvent, a "developing time" of several minutes, and then excision of the sample spot and placement in the mass spectrometer, is primitive and laborious. It is the functional equivalent of collecting sample fractions from a liquid chromatograph, loading the solutions into small discrete vials, and analyzing the samples one at a time with a mass spectrometer. Such an "interface" arrangement has always been possible, but it is not conducive to the synergism of TLC/MS as a continuous coupling of analytical methods. Analytically useful and competitive TLC/MS, although the chromatography is off-line, must be coupled to mass spectrometry through a transparent, automatically functioning interface. The analytical attributes of an "idealized" PC/MS interface are next described.

There are two separable exclusive approaches to the design of an ideal TLC/MS interface. Both approaches have been demonstrated. The imaging interface is a direct analogy to optical spectroscopic detectors now used for planar chromatography. The shape and boundaries of the developed sample spot are determined through a point-by-point examination of mass spectral data. The imaging interface must translate planar (x , y) coordinates in space into a single-channel coordinate (usually time) for mass spectral measurement. There is usually either a compression of data (data are recorded at fixed intervals along only the x -axis of development, for example) or a variable spatial resolution that is also encoded. Because multiple measurements of mass spectra are necessary, it is a requirement that the sample not be completely consumed during each such measurement. Generally, the sample spot could be considered as undisturbed, and exhibiting its native shape and boundaries, if the mass spectral measurement consumes no more than 5–10% (as described before) of the sample. This places an upper limit on the sample flux that can be attained. Following the general assumption that 10 mass spectral scans are desirable to characterize an eluting column chromatographic point, assume that 10 scans are also required to image a spot on a planar chromatogram across

its widest dimension. The grid in the x - and y -directions is 10×10 , for a total of 100 individual sample measurements in this ideal scenario, with consumption of no more than a total of 5% of the sample. [This is a greater number of measurements than reported by Cricelius et al. (74b) but appropriate for the “ideal” interface.] With the example of 1 ng total sample in the spot, 5% sample consumption is a total of 50 pg, with 50/100 or 0.5 pg consumed to provide each mass spectrum. If there is a sample concentration gradient within the spot, there may be more sample available at some (x , y) points and less in others. The need for a nonscanning form of mass analysis becomes clear in this derivation (see below).

Rastering must be accomplished in such an interface to encode the (x , y) information in the mass spectrum. Imaging secondary ion mass spectrometers are commercial instruments used for surface analysis, primarily to determine the spatial distribution of inorganic components. Rastering can be accomplished in several ways but is most commonly done by steering the impinging ion beam onto the surface of the sample. Such an imaging SIMS instrument has been used for imaging TLC spots (53). The issues of sample flux in imaging SIMS are the same as in a potential instrument for PC/MS. Therefore, the instrument used in the study (53) was a TOF-based instrument that provided maximum ion transmission through to the detector. In the application cited, the sample was such that it could be sputtered directly by a primary ion beam without damage. Because no extraction was used, there was no sample spot diffusion on the surface of the chromatogram. The approach is not concordant with the practice of modern TLC, specifically in that usually the sample molecules have to be released from their interaction with the silica gel by using an appropriate extraction solvent. The presence of the extraction solvent provides three areas of complications, as discussed in earlier sections and reemphasized here. The first is that it has the potential to increase sample diffusion on the chromatogram beyond the original developed dimensions, thus compromising separation resolution. The second is that it places a load on the vacuum system of the mass spectrometer, because in the absence of the extraction solvent (or some other matrix) the sample molecules simply revert to their original state of interaction with the silica gel. The third is that the extraction itself should take place quickly (within a few seconds to minimize diffusion) and efficiently (100% of the sample should be available for ionization, even if it is not used).

The second type of TLC/MS interface is the consumption interface. In such a device, at the upper limit, all of the sample within a chromatographic spot would be consumed to produce the mass spectrum, and to bring sample levels to the equivalent of column chromatography the extraction would be complete within 5 s. This would be the total consumption TLC/MS interface. Earlier in this chapter, the sample volume in PC was derived as approximately 20 μL for a circular spot of 0.5 mm diameter and a silica gel layer thickness of 100 μm . Clearly, 20 μL must also be an upper limit on the amount of extraction solvent that could be applied to the spot without causing sample diffusion outside the range of that occurring during the development of the chromatogram. The application of an extraction solvent to the chromatogram is, of course, the converse of the solvent application used to spot the sample onto the chromatogram in sample loading. Small aliquots of solvent are repeatedly applied, with evaporation of the solvent in the intervals between applications. The total amount of solvent used in the application of the sample during spotting is also about 10–20 μL , supporting the converse analogy. The

target scale of sample extraction solvent per spot used in the interface, therefore, should be 10–20 μL , and the time scale should be similarly short.

The argument developed here is that the total consumption interface is the preferred TLC/MS interface to allow the method to reach competitive and meaningful limits of detection. The technological and engineering challenges in designing such an interface are not insoluble. In fact, the appropriate technology has already been demonstrated in other venues and in other applications. Once the defining analytical attributes are realized, it only remains to bring the process to TLC/MS to produce a viable and useful interface (126). The key to successful adoption of TLC/MS into the analytical community will be a simple interface device that transforms the distribution of samples on an xy plane into a sequence of sample molecules in a gas or liquid stream, mimicking a GC/MS or LC/MS analysis. It must be simple and robust, and it must eschew the many unique options and advantages that have long been envisioned for TLC/MS. Today, as chemists examine and assess data, whether the data originated from GC/MS or LC/MS often becomes irrelevant. This must also become the distinguishing characteristic for TLC/MS.

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10

Basic Principles of Optical Quantification in TLC

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I. INTRODUCTION

Quantitative thin-layer chromatography (QTLC) measured by direct photometric scanning has been performed for nearly 50 years. Despite its long history, this procedure has not achieved the reputation of being a very reliable quantitative analytical technique. Relatively large standard deviation has often been mentioned as one reason that QTLC was not acceptable as a reliable quantitative technique. The most important drawbacks were problems in sample application, development, scanning, and data processing. The opposition was unjustified (1). TLC is an openbed technique with many not precisely controllable parameters, which on the one hand contributes to a large dispersion of measurements but on the other hand eliminates systematic errors. High accuracy can easily be obtained by using a large number of applications of the same sample and statistical methods. In addition, certain steps in the procedure can be strictly controlled and even automated. Major improvements in reproducibility, simplicity, and speed are obtained with automatic sample applicators, controlled development and drying conditions, and sophisticated computer-controlled scanning modes with the use of image processing.

The possibility of simultaneous development of up to 74 samples on one HPTLC plate makes planar chromatography one of the most informative microanalytical techniques. Many more expensive and sophisticated combination techniques such as gas chromatography/mass spectrometry (GC/MS), high-performance liquid chromatography/MS (HPLC/MS), HPLC/inductively coupled plasma-MS (HPLC/ICP-MS), and capillary electrophoresis/MS (CE/MS), can generate even more data per second than planar chromatography and can collect data from different detectors at the same time but from only a single sample. Different samples can be compared only by use of software that enables collection and post-run parallel presentation of results. Samples can be evaluated together, but data are collected at different times and only in the case of very robust measurement conditions can data be compared.

Among the users of chromatography around the world today, it is possible to see renewed and increasing interest in TLC. Analysts have seen that sophisticated and specifically oriented techniques cannot be properly used if they are not planned according to the results obtained by prescreening using cheaper, less sensitive, but more informative techniques such as TLC.

The production of uniform TLC plates with different types of layers, instrumentalized programmable applicators and development systems, and the use of sophisticated, inexpensive computers, scanning devices, charge-coupled device (CCD) cameras, and printers open up new possibilities for reliable quantitative TLC.

Various modes of quantification in TLC are indicated in Fig. 1. In the simplest mode, substance is eluted from the plate and quantified with a spectrophotometer. Today elution is not often used for quantitative measurement, but it is very convenient for identification of compounds in separated spots on TLC plates with mass spectrometry (2, 3). Direct in situ modes of quantification, using a densitometer, CCD camera, or flatbed scanner, are used for routine work. Most often,

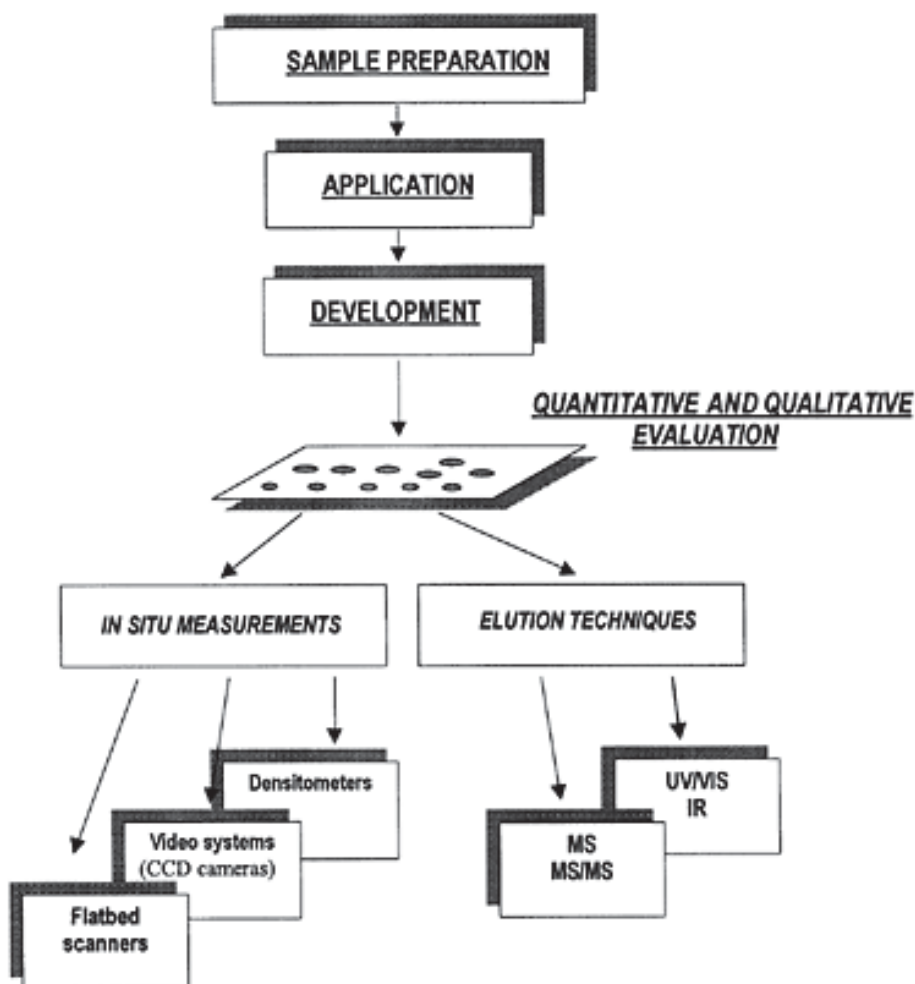


Figure 1 Operations in quantitative TLC.

plates are scanned with densitometers equipped with sensitive photomultipliers. Information from a plate comes in the form of a unique signal, integrated in the analog mode from a relatively large scanning slit, which moves at a speed of a few millimeters per second (in more modern equipment, it can even increase by up to a few centimeters per second). The signal from the illuminated or nonilluminated side is collected, digitized, and processed using a personal computer.

Thin-layer chromatographic plates can also be scanned with flatbed scanners (4, 5) and CCD cameras equipped with video chips that offer more than a million small detectors (pixels). These techniques are not as sensitive as densitometry, but the data acquisition from a whole plate is very rapid and scanning parameters are easily adapted to the particular plate conditions. The signal from each pixel is digitized and fed into a powerful computer. Then, after several very quick scans, the evaluation is done with the use of statistical methods. It appears that both modes have a future and will be used in conjunction in absorption and fluorescence measurements. Some analysts want to compare the two scanning procedures, but we must be careful with this comparison. Today, image-analyzing systems are not yet properly used in TLC; analysts (and even instrument manufacturers) think that both data acquisition methods are the same or that there is only a slight difference, which depends on the form of sensor. From our experiments, however, we can say that it is not so simple. There are basic theoretical and practical differences between the data acquisition modes that have an important influence on the validity of the results of each of these two scanning techniques.

II. BASIC PRINCIPLES OF QUANTIFICATION

A. Radiation Transfer Equation

All optical methods for the in situ quantitative evaluation of TLC chromatograms are based on measuring the difference in optical response (intensity of diffusely reflected or diffusely transmitted light) between blank regions of the stationary phase and regions with a separated substance, which can be measured either on the illuminated side (reflectance) or on the opposite side (transmission). The principle is similar to measurement in regular absorption spectroscopy; however, the relationships are much more complex, because TLC sorbents consist of tightly packed particles that scatter the incident beam.

When a parallel beam irradiates a flat layer of thickness z , two phenomena arise on the illuminated and nonilluminated sides. The first phenomenon is the regular (specular) reflection from the smooth parts of the surface, and the second is the diffuse reflectance from the opaque parts of the layer. These two extreme cases require different spectrophotometric approaches. In the first case, it is possible to obtain absorption spectra and the most important optical constants, the refractive and absorption indexes, by applying Fresnel's equations; quantification is done with the Beer—Lambert law. In the second case the angular distribution of diffusely reflected radiation is isotropic and the density of the radiation is independent of direction. When scattering exceeds adsorption, a radiation transfer equation is valid.

On the nonilluminated side of a plate, measurements can also be separated into two extreme cases. In the case of a very small amount of scattering, absorption spectra and the concentrations of compounds in bands are obtained with the Beer—Lambert law

$$I = I_0^{-abc} \quad (1)$$

where

I =intensity of attenuated beam

I_0 =intensity of incident beam

a =absorptivity coefficient

b =length of optical path

c =concentration

When the scattering is strong, it is impossible to obtain clear transmission spectra, and the concentrations of absorbing compounds in chromatographic spots must be determined with the use of special equations. No rigorous theory of multiple scattering has been proposed, but many attempts have been undertaken to develop a phenomenological approach to absorption and scattering. All theories are based on an infinitesimal layer in which the radiation field is divided into two or more radiation paths. Differential equations are then established for reflectance and transmission. The equations are integrated over the total thickness of the layer, resulting in relatively simple relationships. The agreement between experimental data and calculated values is acceptable when suitable conditions exist. The change in intensity of a beam of radiation of selected wavelength in a path length dz within the medium is given by the radiation transfer equation

$$\frac{-dI}{K\rho dz} = I - \frac{J}{K}$$

where

dI =change in intensity of the radiation flux

K =attenuation coefficient corresponding to the total radiation loss due to adsorption and scattering

ρ =density of the medium

J =scattering coefficient

dz =change in optical path length

This equation is a differential equation, because J/K , the source function, depends on the intensity of the radiation at each point. A solution of this expression can be obtained only by approximation. In the exact solution, the equation requires the division of the radiation field into a large number n of linear differential equations. The detailed solution was presented by Chandrasekhar (6). Such a rigorous solution is practically never used for the calculation of isotropic scattering in the thin layer.

Numerous researchers have developed their own simplified solutions to the radiation transfer equation. The first solutions were Schuster's equations (7), in which, for simplification, the radiation field was divided into two opposing radiation fluxes ($+z$ and $-z$ directions). The radiation flux in the $+z$ direction, perpendicular to the plane, is represented by I , and the radiation flux in the $-z$ direction, resulting from scattering, is

represented by J . The same approximation was used by Kubelka and Munk in exponential (8) and hyperbolic (9) solutions. In their exponential solution, a flat layer of thickness z , which scatters and absorbs radiation, is irradiated in the $-z$ direction with monochromatic diffuse radiation of flux I . In an infinitesimal layer of thickness dz , the radiation fluxes are going in the $+z$ direction J and in the $-z$ direction I . The average absorption in the layer on path length dz is K , and S is the scattering coefficient. Two fundamental equations follow directly:

$$\frac{dI}{dz} = -(K + S)I + SJ \quad (3)$$

and

$$\frac{dJ}{dz} = -(K + S)J + SI \quad (4)$$

These equations can be interpreted as follows. The intensity of the light that travels in the direction of transmission decreases by absorption K and scattering S and increases by scattering from the light traveling in the opposite direction J . Light that travels in the direction of reflection J behaves in the same way but in an opposite direction.

Kubelka and Munk derived solutions for Eqs. 3 and 4. The result is the well-known Kubelka-Munk equation, Eq. 5. It relates the diffuse reflectance R_∞ of an infinitely thick, opaque layer and the ratio of the absorption and scattering coefficients, K/S , and has become the fundamental law of diffuse reflectance spectroscopy.

$$\frac{K}{S} = \frac{(1 - R_\infty)^2}{2R_\infty} \quad (5)$$

If we assume that the scattering coefficient of the sorbent does not change in the presence of chromatographic spots, the Kubelka-Munk equation can be transformed in the form

$$F(R_\infty) = \frac{(1 - R_\infty)^2}{2R_\infty} = \frac{2.303\epsilon c}{S} \quad (6)$$

where ϵ is the extinction coefficient and c is the molar concentration of the sample.

Equation 6 presents a solution for a layer of infinite thickness with homogeneous distribution of scattering and absorbing centers. Therefore, it is not very applicable in quantitative TLC, where the thickness of a layer is about 0.1–0.2 mm and absorbing molecules of sample are assuming a gradient in-depth distribution inside the sorbent.

In 1948 Kubelka proposed an explicit hyperbolic solution (9). Agreement between the experimental data and calculated values is very good, and his equations still represent a relatively simple approach to the solution of diffuse reflectance R_0 and transmittance T_0 , adequate for most densitometric applications:

$$R_0 = \frac{\sinh(bSd)}{a \sinh(bSd) + b \cosh(bSd)} \quad (7)$$

$$T_0 = \frac{b}{a \sinh(bSd) + b \cosh(bSd)} \quad (8)$$

where

$$a = \frac{S + K}{S} \quad \text{and} \quad b = \sqrt{a^2 - 1}.$$

where K =absorption coefficient, S =scattering coefficient, and d =layer thickness.

The presented equations and suggested solutions show how complicated is the relationship between the concentration of a sample and the intensity of diffusely reflected light. Equations 7 and 8 represent a simple phenomenological approach to the problems of diffuse reflectance and transmittance and are very suitable for the calculation of concentrations in QTLC (10, 11).

Continuum theories are not satisfactory for powder-type layers such as TLC plates. The scattering and absorption characteristics of a medium are reflected in two constants, K and S , but the influence of the particle size as well as that of the nonuniform vertical distribution of the absorbing molecules inside the sorbent layer are completely ignored.

B. Discontinuum Theory

Bodo (12), Melamed (13), and Johnson (14) developed well-known discontinuum theories for the determination of absolute optical constants from the properties of individual sample particles.

In 1975 we started to study the relationship between the concentration of a substance in the spot and the signal in reflectance (remission) and transmission measurements, using the discontinuum theory and specially prepared multilayered models (15–19). In the first step, intensities of reflected and transmitted light were calculated according to a prepared theoretical model of a TLC plate. A chromatographic band was placed in different sublayers whose thickness equaled the mean particle diameter. The total reflectance, R , and transmittance, T , were obtained by summing all transmitted and reflected fractions of all sublayers. In the second part, real models (Fig. 2) were prepared from various kinds of layers (papers and TLC sorbents), and the effects of the nonuniform concentration of the depth distribution (in the z -direction) were investigated. Finally, the results from the theoretical models were compared with the values obtained with the real models.

Reflectance and transmission of each sublayer are determined by the equation proposed by Bodo, who assumed that the fraction α of the incident radiation is reflected from the individual layer and is attenuated by absorption to the fraction $(1 - \alpha)e^{-Kd}$. In our calculation K represents

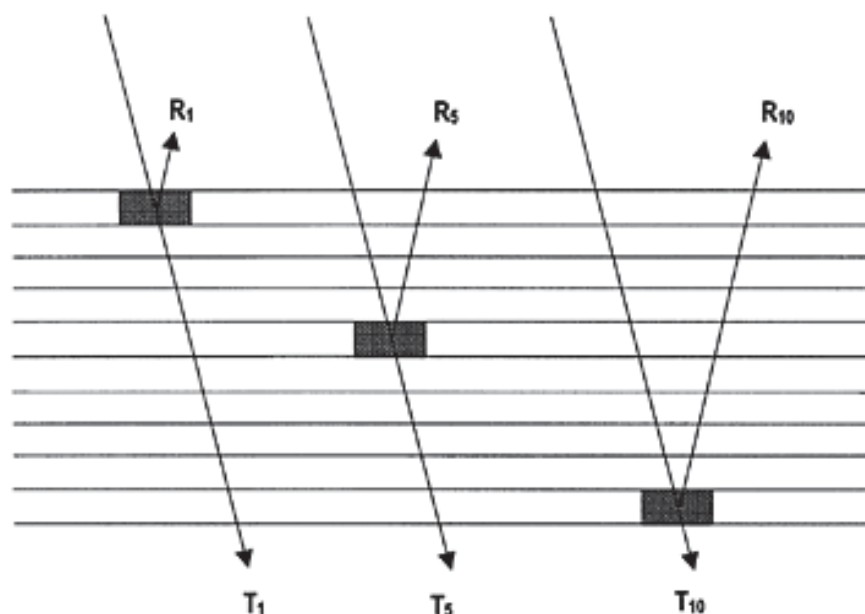


Figure 2 The multilayer model used in the calculation. The positions of chromatographic spots at the top (near side), in the middle, and at the bottom (far side) of the layer are shown.

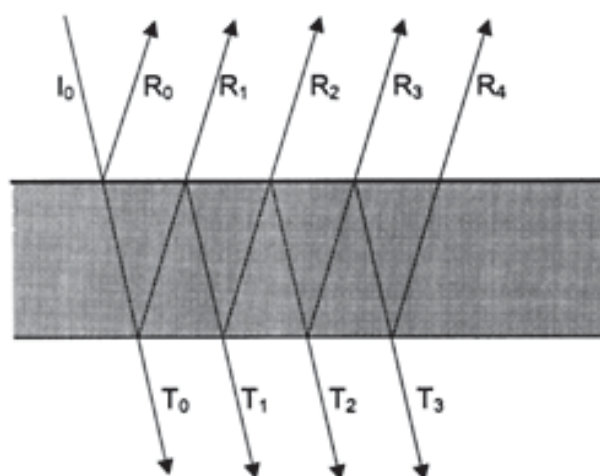


Figure 3 Transition of an incident beam I_0 , through a thin layer. Scattered light is not yet diffused, but it will be after the transition of many such layers. Geometrical series for both the reflected (R) and transmitted (T) radiation fluxes.

the sum of the absorption of a sample and the absorption of a layer L , and d is the particle size (layer thickness). On the bottom of the layer, the fraction $1-\alpha$ of the radiation, still present, is again reflected, so that the fraction $(1-\alpha)^2 e^{-Kd}$ passes through, etc. We thus obtained geometrical series for both the reflected and transmitted radiation fluxes (Fig. 3).

$$R = \alpha \frac{e^{Kd} + (1-\alpha)^2 e^{-Kd}}{e^{Kd} - \alpha^2 e^{-Kd}} \quad (9)$$

$$T = \frac{1 - \alpha^2}{e^{Kd} - \alpha^2 e^{-Kd}} \quad (10)$$

In our calculations, two different values of α are used: α_1 for parallel radiation and α_2 for diffuse radiation. We assume that the radiation beam is parallel to the sample surface rays and that the reflected beams are more or less diffuse. Experimentally, it was determined that the fraction of reflected flux was usually greater for diffuse radiation than for parallel radiation (20), $\alpha_2 = w\alpha_1$. From the experiments with diffuse quartz (21), α_1 is 6% if λ is greater than 380 nm. Because the layer is subject to radiation at all possible angles, the average path length of the radiation within the layer is not equal to the layer thickness but must evidently be greater. Calculations show that the mean path length of diffuse radiation is twice the geometrical layer thickness.

To obtain the remission and transmission of the whole layer of sorbent, we consider only the first and second sets of transmitted and reflected radiation. In transmission, we consider the contribution of the incident light and of all the secondary beams that proceed from the reflection of the incident beam, Eq. 11. For remission, we use only primary reflected incident light and secondary reflected beams of already reflected incident light, Eq. 12.

$$T_0 = \sum_{i=1}^n T_i + \sum_{x=1}^{n-1} r_x A_x \left(\sum_{i=1}^x T_i \right) \left(\sum_{m=x+1}^n t_m \right) \quad (11)$$

$$R_0 = R_1 + \sum_{x=1}^{n-1} A_x B_x T_x r_x \left(\sum_{i=1}^x T_i t_i \right) + \sum_{x=1}^n R_{x+1} \left(\sum_{i=1}^{n-x} t_i * T_i \right) \quad (12)$$

where

$$A_x = R_{x+1} + \sum_{y=x+2}^{n-1} R_y \left(\sum_{k=x+2}^y T_k t_k \right)$$

$$B_x = r_x + \sum_{y=x}^{n-1} r_y \left(\sum_{j=x}^{n-1} t_j \right)$$

T =transmission of parallel light

R =remission of parallel light

t =transmission of diffuse light

r =remission of diffuse light

The use of only two sets of radiation fluxes in the final calculations is sufficient, because the values of the third set, which are very small, have no influence on the final results but serve only to make tedious the development of the calculation algorithm.

Our results obtained using multilayer models and a CCD camera, showed that higher order sets of radiation fluxes must also be taken into account as the result of the illumination of a whole plate. Inside an illuminated layer, the intensity of diffuse light is much greater because a higher number of reflected beams are coming from all parts of the layer, which are informative and detectable. In classic densitometry, scanning is performed inside a black box and only an incident beam with a small diameter is used. When the beam hits a sorbent it scatters, and part of the scattered light is lost inside a layer. Results show that image-analyzing systems are much more informative about the conditions inside a layer than densitometers, due to the bigger illumination field and larger number of scattered beams.

Using Eqs. 11 and 12, the relative changes of the diffuse reflected and transmitted light throughout the layer (consisting of 10 sublayers) were calculated (a chromatographic band was placed at a different R_f value on each of the sublayers of the model). A very small absorption coefficient, $a=0.0001$, was used for the sorbent. Such a model presents a densitometric measurement on a silica gel layer in the visible spectrum, because silica gel absorbs practically no light above 400 nm (22). The concentration of the spot within a single layer was uniform. Mathematically obtained results show the great influence of the vertical depth of the sample in remission and practically none in transmission.

In reality, distributions of spots inside the layer are not known. Special models are prepared, measured by a densitometer, and evaluated. Measured and calculated values provided insufficient insight to explain the effect of the vertical concentration gradient on the results of reflectance and transmission measurements, which were later confirmed by using photoacoustic spectroscopy (26–32). The small disagreements between calculated and experimentally obtained relations were the result of incorrectly estimated optical parameters. The values of α , L , and d used in calculations were obtained from the literature and from our own optical experiments.

A considerable number of models were prepared from different kinds of papers and sorbents used in TLC, and the effect of the nonuniform concentration distribution $c(z)$ was investigated. The relative values of remission and transmission of the models were calculated, and results were compared with the values obtained with the real models. Table 1 and Fig. 4 contain results of the model prepared using 10 layers of silk paper. Inserted strips of colored paper represented the spots in different positions (layers). The absorption of the colored paper (silk paper was taken as a blank) at 550 nm was 0.3 A.U. The standard reflection ($R_0=100\%$) was determined using Al_2O_3 powder. The R_0 value of the model was found to be 75%. At first sight, the increase in the signal (ΔT) with increasing depth of the sublayer seemed unusual, but it was confirmed by our measurements. It is known from previous experiments that the path of diffused light in the layer (or sublayer) is longer than the path of the specular incident beam, $\alpha_2=2\alpha_1$.

To find a simple solution to our multilayer model, we also tried some other mathematical approaches, such as Markov chains and transition matrices (19). The obtained results closely tracked the experimental results as well as the results obtained with the multilayer models and proposed calculations using our formula and the Bodo equations.

Table 1 Multilayer Model Consisting of 10 Sheets of Drawing Paper^a

Layer	Remission		Transmission	
	Measured	Calculated	Measured	Calculated
1	100.0	100.0	100.0	100.0
2	61.0	61.8	101.0	104.1
3	36.2	37.9	102.0	106.2
4	20.9	22.9	103.0	107.3
5	13.6	13.4	105.0	107.7
6	5.6	7.6	105.0	107.7
7	3.4	4.1	103.0	107.3
8	1.7	2.1	102.0	106.2
9	1.1	0.9	102.0	104.1
10	0.0	0.2	101.0	100.0

^a $K_0=0.001$ A.U., $K_1=0.3$ A.U., $\alpha=16\%$, $r_0=0.75$, wavelength=550 nm. Signal normalized to the level of the spot in the first layer.

C. Measurement of Fluorescence

In situ fluorescence measurements are a favorite tool for quantitative and qualitative determination in TLC, especially when very low concentrations have to be measured. Fluorescence is the reemission of absorbed energy, which occurs as the excited molecules of a sample return to their ground state. The reemitted light is somewhat longer in wavelength than the absorbed light be-

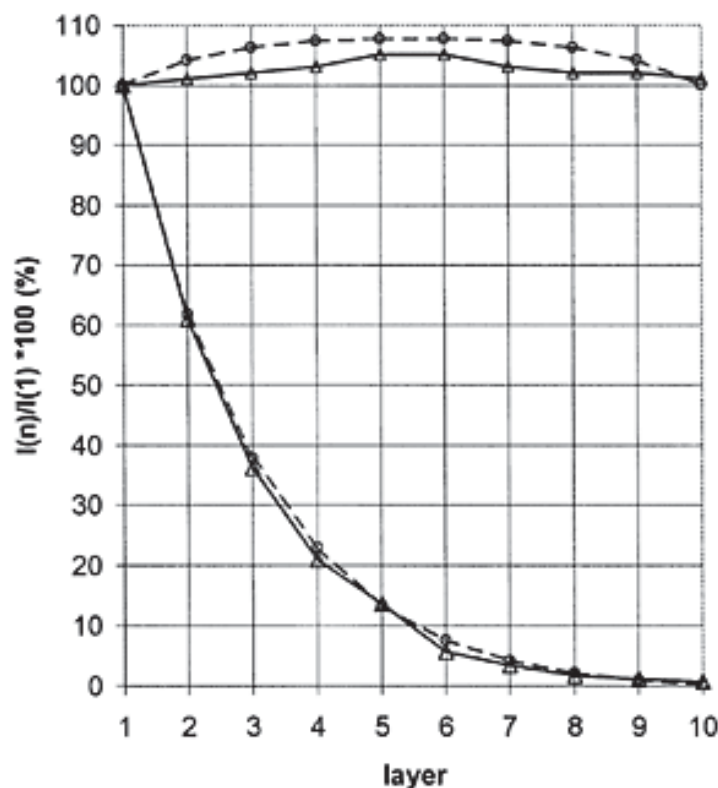


Figure 4 Multilayer model consisting of 10 sheets of drawing paper. Calculated values (R and T) are presented with dashed lines and measured values with bold lines. Scanning and calculation parameters are $K_0=0.001$ A.U., $K_1=0.3$ A.U., $\alpha=16\%$, $r_0=0.75$, wavelength 550 nm.

cause some of the energy is transferred to vibrational motion. In fluorescence, absorption and the reemission take place in a very short period of time (10^{-12} – 10^{-9} s). In the case of fluorescent analysis, measurements are carried out at a wavelength different from that of the illuminating wavelength. This represents the fundamental difference between conventional absorption measurements and fluorescence measurements.

There are many different ways in which the fluorescence intensity of a compound in a chromatographic spot can be calculated (23–25). Most frequently, the Beer–Lambert law is used, because it is simple and accurate. If I_0 is the intensity of the incident beam and I is the intensity of the light at the nonilluminated side of a plate, then $I_0 - I$ is the amount of light that has been absorbed by the layer. Part of this absorbed light, which is given by Φ , the quantum yield, is then reemitted from the layer, but with a wavelength different from that of the exciting beam. The intensity of the transmitted light is obtained from the equation

$$I = I_0 e^{-abc} \quad (13)$$

where a =absorptivity, b =length of cell path (thickness of TLC layer), and c =concentration.

$$F = \Phi I_0 (1 - e^{-abc}) \quad (14)$$

If the concentration $c \ll 1$, then the fluorescence can be written in linear form as

$$F = \Phi I_0 abc = Kc$$

This solution is very useful, because it can give a simple but correct answer to a lot of questions about fluorescence. Nevertheless, to apply this method, some very important simplifications must be made. For instance, the problems of scattering and absorption of the layer itself have been neglected, and uniformity of concentration in a spot has been assumed. The most important result of Eq. 14 is that a linear relationship can be used when the concentration of fluorogen is low.

The influence of the concentration gradient on the intensity of fluorescence was investigated by using a multilayer model of silk paper on a quartz plate. An equal amount of fluorogen was applied on each sublayer, and fluorescence was measured from the illuminated and nonilluminated sides at different wavelengths. The intensity of fluorescence due to sample position was also calculated from the multilayer model. First, the fluorescence intensity inside a layer, which cannot be measured because it is impossible to insert a photodetector in a layer, was calculated. Then the intensity of the light emitted at the near far sides of the TLC plate was evaluated. Two different calculation procedures were used to solve this problem. In the first, a real multilayer model with well-defined sublayers was used. The remission and transmission of each sublayer were obtained from Bodo's equations (12). In one of these layers, n , a known amount of a fluorogen was placed. The results of this tedious calculation were published (19), but in practical work the second procedure, a much easier Kubelka-Munk hyperbolic solution, was used to calculate remission and transmission.

The model is shown in Fig. 5. A spot lies in sublayer n , and the model has N sublayers. The intensity of an incident beam entering sublayer n is given by Eq. 15. T_{n-1} is the transmission of $n-1$ sublayers. The intensity of the incident beam that penetrates the layer containing the fluorogen is $I_0 T_{n-1}$, and the part of it that is absorbed is

$$T_{n-1}(1 - T_n)$$

The light that is not attenuated in the spot proceeds into a layer that consists of $N-n$ sublayers. Part of this light is reemitted, and its intensity is

$$T_{n-1} T_n R_{N-n}$$

This light enters the spot again, and additional light is absorbed:

$$R_{N-1} T_{n-1} T_n (1 - T_n)$$

The total amount of light absorbed in a layer containing a fluorogen is given by

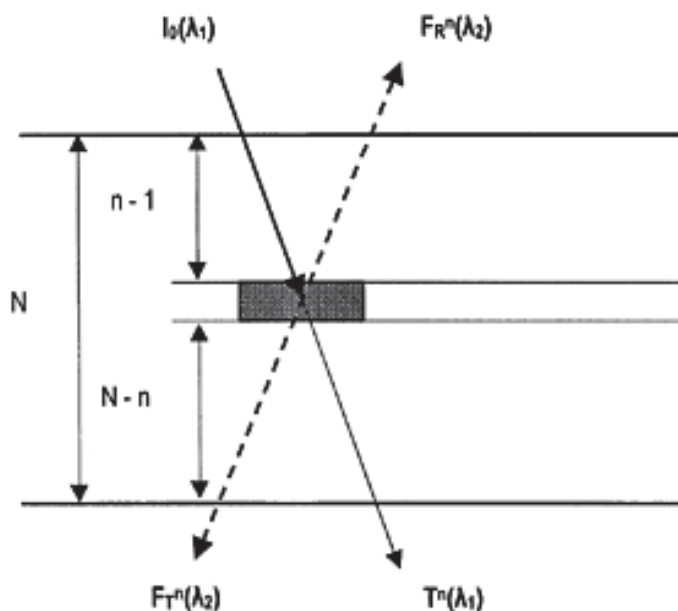


Figure 5 The model used in the calculation of fluorescence intensity. N is the total number of sub-layers; n is the layer where the spot of fluorogen is located.

$$A_n = T_{n-1}(1 - T_n) \frac{1 - R_{N-n}T_n}{1 - R_{n-1}R_{N-n}} \quad (15)$$

The factor $1/(1-R_{n-1}R_{N-n})$ arises because of the scattering between layers. If the difference between the amounts of light absorbed by the fluorogen and the sorbent and the amount of light absorbed by the sorbent is $A_n - A_{n0}$, and the quantum yield is Φ , then the equation for the intensity of fluorescence in sublayer n is

$$F_n = \Phi I_0 (A_n - A_{n0}) \quad (16)$$

where

Φ =quantum yield

A_{n0} =light absorbed by fluorogen and sorbent in layer n

A_n =light absorbed only by the sorbent in layer n

I_0 =intensity of the excitation source

To calculate the intensity of excited light that can be measured from the illuminated side, it is necessary to consider the absorption and scattering of a sorbent at the wavelength of the reemitted light, that is, λ_2 . To distinguish transmission and remission at λ_2 from that at λ_1 , lowercase letters t and r are used. It is necessary to take into account

that one-half of the excited light is traveling in the near-side direction and one-half in the far-side direction. At the near side, the fluorescence is given as

$$\frac{1}{2} F_n t_{n-1}$$

The light traveling in the far-side direction is partly remitted, and at the near side it is possible to detect this part of the light also:

$$\frac{1}{2} F_n r_{N-n} t_n t_{n-1}$$

The total fluorescence is therefore given by the equation

$$F_{\text{rem}} = \frac{1}{2} F_n t_{n-1} \frac{1 + t_n r_{N-n}}{1 - r_{N-n} r_{n-1}} \quad (17)$$

Together with the excitation light, λ_2 , the incident beam, λ_1 , is also reemitted from the sorbent. A cutoff filter or a monochromatic filter is used to attenuate the excitation beam. When an edge of the cutoff filters is too close to an excitation wavelength or the monochromatic filter used is too wide, then part of the incident beam gives rise to absorption phenomena, which produce very serious faults. In the remission mode of measurement, these phenomena are not easily detected. Values of fluorescence at the near side can be derived by using the equation

$$F_{\text{measured}}^n = F_{\text{rem}}^n - r^n \quad (18)$$

where F_{rem}^n is the intensity of fluorescence at the near side of the TLC plate and r^n is the absorption of the sample lying in sublayer n , within the limits of transmittance of the cutoff filter, measured in remission mode.

In the transmission mode, the same equations as in the remission mode can be assumed, but the directions of the beams have to be changed. The intensity of fluorescence at the far side is given by

$$\frac{1}{2} F_n t_{N-n}$$

and the total intensity is given by

$$F_{\text{trans}}^n = \frac{1}{2} F_n t_{N-n} \frac{1 + t_n r_{n-1}}{1 - r_{n-1} r_{N-n}} \quad (19)$$

In the same way as with remission measurements, an improper cutoff of the monochromatic filter gives rise to incorrect results; in transmission mode, however, the influence is much greater. In an extreme case of improper selection, it is possible that absorption predominates over fluorescence and the sum total of the signals is negative. Equation 20 gives measured values of fluorescence at the far side:

$$F_{\text{measured}}^n = F_{\text{trans}}^n - t^n \quad (20)$$

where F_{trans}^n =intensity of fluorescence at far side of TLC plate and t^n =absorption of sample lying in sublayer n , within the limits of transmittance of the cutoff filter, measured in transmission mode.

Calculated values of fluorescence are shown in Fig. 6. Values of absorption in the remission and transmission modes are calculated using the following simplified equations.

Transmission:

$$t^n = t_{n-1}t_n \frac{t_{N-n}}{1 - r_{n-1}r_{N-n}}$$

Reemission:

$$r^n = r_{n-1} + t_{N-n}^2 \frac{r_n + r_{N-n}t_n^2}{1 - r_{n-1}r_{N-n}}$$

The factor $1/(1-r_{n-1}r_{N-n})$ is introduced to take into account the scattering between the layers.

A multilayer model gives a better understanding of fluorescence measurements. Only molecules lying on the illuminated side of a layer can produce a signal, because the absorption (attenuation) of the incident light in the UV range, which is normally used for excitation, is so great that the molecules from the nonilluminated side cannot be excited at all. In this case there is no difference between remission and transmission measurements, and it is impossible to avoid the influence of the concentration gradient on the final results. The transmission mode is more sensitive to the selection of measurement conditions. The use of a broad monochromatic filter or an improper cutoff filter can produce very great errors. An example of incorrect selection of cutoff filter is shown in Fig. 7. The measured substance absorbed the incident light at selected wavelengths, the absorption signal was mixed with the reemitted light of a slightly different wavelength, still in the selected range, and the two signals with different signs were combined. It is possible to see that at the fluorogen in the middle of the layer the absorption overcomes the fluorescence.

In transmission measurements, when the scattering of the light in a TLC plate is great and the attenuation of the incident light is not very strong, the signal from the second inner sublayer is greater than the one from the first sublayer near the illuminated side. Nevertheless, the intensity of the excited light in the first sublayer is greater. This is due to the fact that the light reemitted by the spots lying on top of the surface cannot be reflected toward the far side to the same degree as the light reemitted by the spots lying inside the sorbent.

Summarizing the results, we can conclude that the remission mode of fluorescence scanning is much better than the transmission mode. It is not possible to simplify calculations with the use of the same scattering coefficient for the excitation beam and the reemitted light; and it is necessary

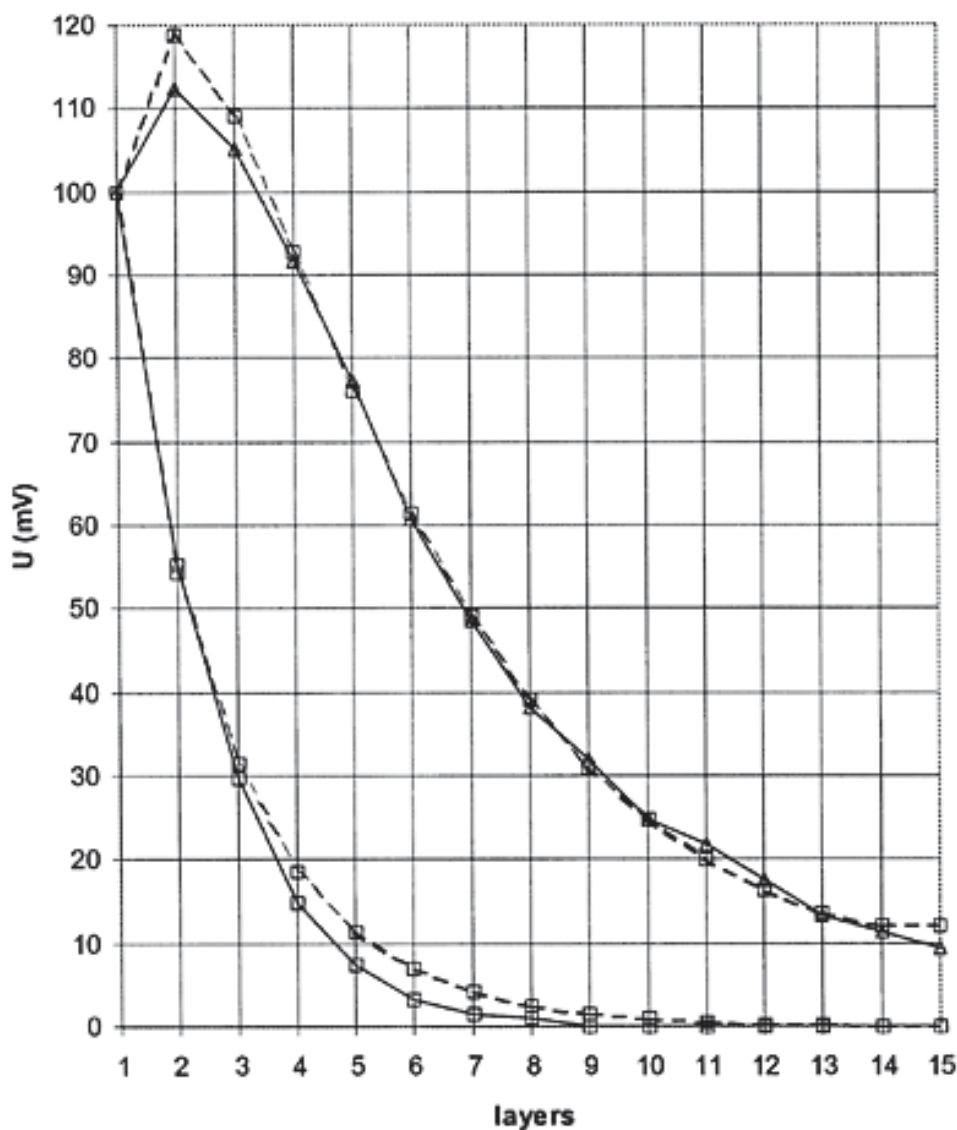


Figure 6 Calculated and measured values of fluorescence intensity at the far and near sides of a TLC plate. Parameters used: N (number of sublayers)=15; K (absorption coefficient of sorbent at λ_1)= 0.26 A.U.; K_1 (absorption coefficient of spot at λ_1)=0.50 A.U.; K_2 (absorption coefficient of sorbent at λ_2)=0.001 A.U.; S_1 (scattering coefficient at λ_1)=0.10; S_2 (scattering coefficient at λ_2)=0.42, λ_1 =365 nm, λ_2 =527 nm.

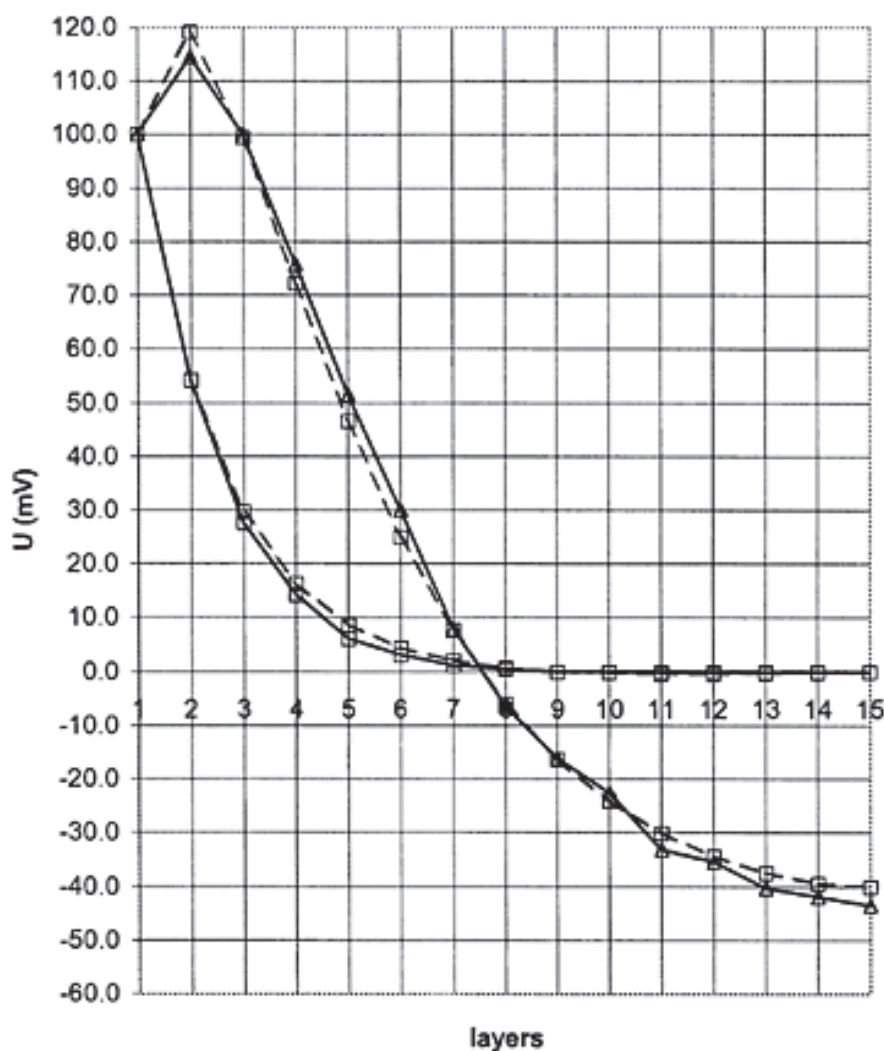


Figure 7 Calculated and measured values of fluorescence intensity at the far and near side of a TLC plate when the influence of absorption or remitted light (λ_2) must be considered.

Parameters used: N (number of sublayers)=15; K (absorption coefficient of sorbent at λ_1)=0.25 A.U.; K_1 (absorption coefficient of spot at λ_1)=0.50 A.U.; K_2 (absorption coefficient of sorbent at λ_2)=0.0001 A.U.; K_3 (absorption coefficient of spot at λ_2)=0.01; S_1 (scattering coefficient at λ_1)=0.15; S_2 (scattering coefficient at λ_2)=0.25.

to avoid the effects of the vertical concentration gradient (secondary chromatography) when small standard deviations in the measured values are required.

D. Depth Profiling of TLC Plates by Photoacoustic Spectroscopy

In 1996, we started investigations of nondestructive depth profiling of TLC plates. The results obtained by the use of different photothermal techniques, i.e., photoacoustic Spectroscopy (PAS), photothermal beam deflection, and radiometry, showed that PAS is the most suitable for characterization of TLC plates (26). PAS was therefore used for all our further investigations of vertical concentration profiles of compounds on TLC and HPTLC plates. Although PAS had been used previously for the qualitative and quantitative spectroscopic analysis of TLC plates, the first results of photoacoustic depth profiling of TLC plates were published by our group in 1997 (27, 28).

Photoacoustic detection relies on the detection of the pressure variations (pressure waves) due to the heating of the gas adjacent to the sample. In other words, we are detecting the pressure waves (sound) generated by the absorption of radiation in a periodically irradiated sample. The sample is enclosed in a photoacoustic cell and excited through the cell's windows. A microphone acoustically coupled with the cell picks up the modulated signal. In 1981 Helander explained the capability of PAS for in situ and nondestructive depth profiling of solid samples. This unique feature is due to the fact that the magnitude of the induced photothermal effect depends on the concentration and thermal diffusivity of a compound. As described by Bein and Pelzl in 1989 (30), the plot showing the dependence of the photoacoustic (PA) signal on the modulation frequency provides information about the depth profile of the analyzed compound.

As mentioned above, variations in the vertical distribution of samples and standards have a big impact on densitometric reflectance measurements. The effect of inhomogeneous depth distribution of compounds on quantitative TLC was studied on 5×5 cm TLC and HPTLC (Merck) plates coated with 250 or 200 μm of silica gel using the separation of dyes as a model. Camag test dye mixture III was applied to the plates, and the plates were developed using toluene and then dried. Contents of the test dye were Ciba F II, indophenol, Ariabel red, Sudan blue II, Sudan IV, and dimethylaminoazobenzene, giving violet, yellow, red, blue, and black spots and another violet spot on the developed TLC plate. The PA signals were analyzed using the theory for a two-layer model (30). Our model consisted of the sorbent with glass as the supporting material. Thermal diffusivity values (α) of the spots on TLC plates were obtained by curve fitting normalized phase lags of PA signals. Different thicknesses of the sample, corresponding to the thermal diffusion length (μ), given as $\mu = (\alpha/\pi f)^{1/2}$, were probed by varying the frequency (f) of laser beam modulation. For each frequency the normalized PA signal was calculated. The PA signals originating from different layers of a TLC plate were calculated by subtracting the values obtained at higher modulation frequencies from those obtained at lower modulation frequencies. From these two modulation frequencies and the previously obtained thermal diffusivities of each spot, we calculated the depth and thickness of each layer. Depending on the available range of modulation frequencies used in the PA measurements, the thickness of the probed layers varied from 23 to 37 μm . All results presented here have been corrected for differences in layer thickness.

The results of photoacoustic investigations showed that all compounds exhibit nonhomogeneous concentration profiles in the vertical direction but tend to concentrate in the upper 25% of a 250 μm thick layer. The observed differences in the vertical concentration distribution of different compounds (in yellow and violet spots) on the same TLC plate (Figs. 8 and 9) indicate that the effects of secondary chromatography depend strongly on the properties of the compounds in each spot. The situation is different in the case of HPTLC plates, where even the compounds in violet spots tend to concentrate in the upper 0–37 μm of the layer (Fig. 10). Differences in the vertical distribution of compounds inside the sorbent of TLC and HPTLC plates can be explained by 50 μm differences in the layer thickness. In the case of thinner layers (HPTLC plate), the evaporation of the mobile phase is faster and causes faster movement of the substance to the surface of the plate. Differences in PA signals from the same depths observed for spots of the same compound from different tracks indicated that nonuniformity within one TLC plate could be the source of erroneous quantification of densitograms. Additionally, when monitoring only the surface of the TLC plate, as by reflectance densitometry, and by photoacoustic spectroscopy when the probed sorbent layer is too thin, such an irregular vertical concentration distribution can result in nonlinearity of the calibration curves (top curve in Fig. 11). Taking into account all the considerable irregularities in the vertical concentration distribution by photoacoustic probing of thicker sorbent layers leads to improved linearity of calibration curves (bottom curves), compared to those obtained by reflectance densitometry.

In recently published papers (31, 32), we reported the effects of the drying process on the vertical distribution of compounds inside the sorbent on TLC and HPTLC plates. We investigated the influence of drying in a dryer, in a stream of warm air, and in the ambient air. The results obtained by PAS studies from different TLC and HPTLC plates were in good agreement with those obtained by reflectance densitometry. The results of PA measurements of the same plates gave the highest PA signals in the top 37 μm of the layer for most of the tracks on all three HPTLC plates (Fig. 11). Significant differences in PA signals were observed under different drying conditions in the top 62 μm of the sorbent. Differences in the depth distribution of compounds

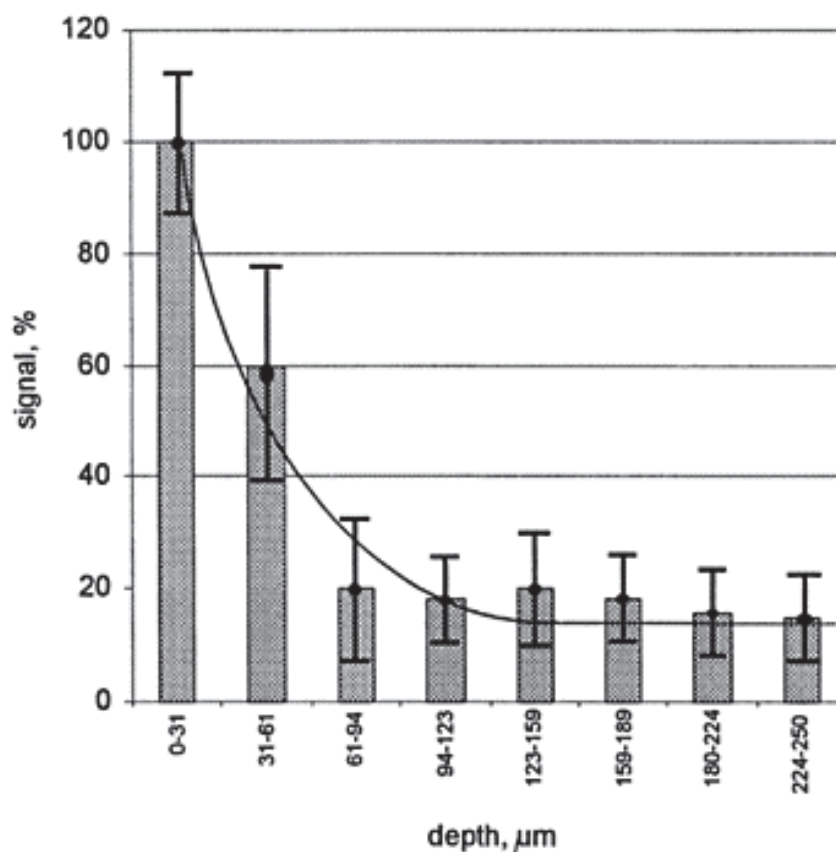


Figure 8 Depth distribution of the compound in yellow spots of equal concentrations. Four tracks on one TLC plate were measured.

are especially remarkable among five tracks from an HPTLC plate dried in a stream of warm air. A comparison of the response areas obtained for five spots of each color from the three HPTLC plates dried in ambient air, in a stream of warm air, and in a dryer showed the highest RSD values for the plate dried in a stream of warm air (Table 2). The RSD for drying in a dryer is significantly lower than the RSD values for drying in ambient air or in a stream of warm air. This can be explained by the nonuniformity of drying conditions across the HPTLC plate while drying in a stream of warm air. As expected, among the selected drying techniques the most uniform conditions are achieved in a dryer.

Summarizing the results of our investigations, we can conclude that secondary chromatography and consequently both the vertical and radial concentration distributions of compounds in the sorbent depend on drying conditions as well as on the properties of investigated compounds and the type of chromatographic plate (TLC or HPTLC). Drying in the dryer was demonstrated to give the most reproducible results, and drying in a stream of warm air, the least reproducible results.

III. IN SITU QUANTIFICATION OF TLC PLATES

Data acquisition is one of the most error-prone steps in quantitative evaluation. The amount (or identity) of substances separated with TLC can be determined directly on a plate by measurement of UV/Vis absorption, fluorescence, or fluorescence quenching. Spectroscopic methods of detection were used at an early stage of chromatography; substances separated by paper chromatography and by TLC were investigated by reflectance and transmission spectrometry some 50 years ago. From the pioneering work of Salganicoff, Polak, Goodall, Goldman, and Ebel, modern in-

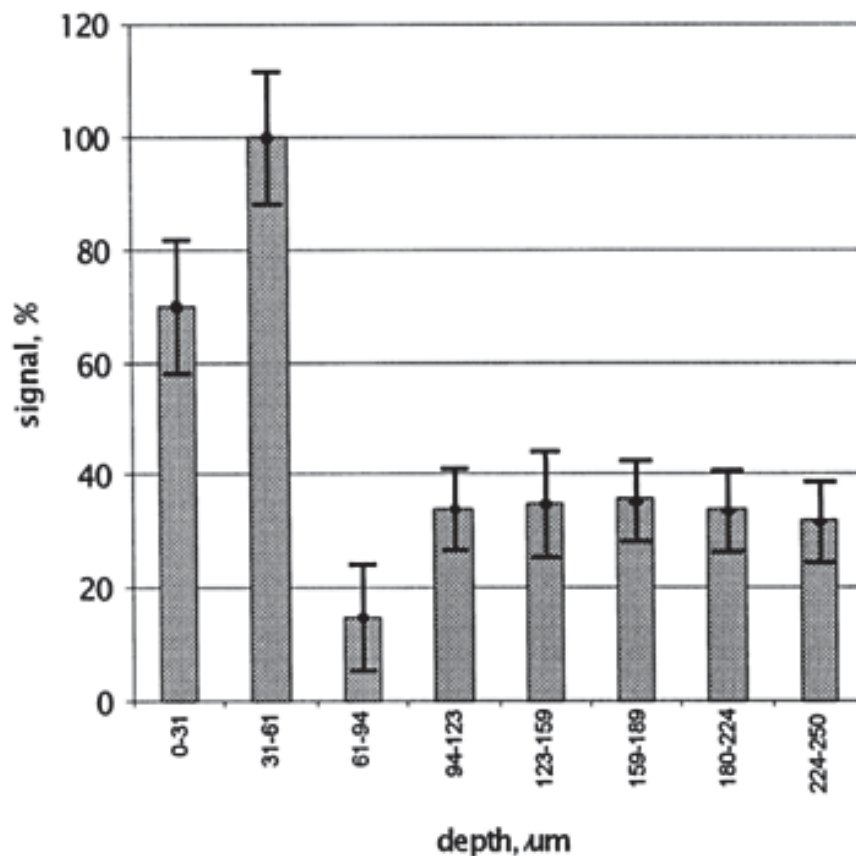


Figure 9 Depth distribution of the compound in violet spots of equal concentrations. Four tracks on one TLC plate were measured.

struments, called densitometers, were developed. Today in situ quantitative and qualitative evaluation of developed TLC plates is performed with modern computer-controlled densitometers, image-analyzing systems (with CCD cameras), and, in semiquantitative mode, even with low-priced flatbed scanners.

A. Densitometers

1. Reflectance Mode

The principle of a reflectance measurement is shown in Fig. 12. This technique can be applied in the UV/Vis spectral range and is also suitable for fluorescence and fluorescence quenching modes. The fact that it can be applied in the UV range on inexpensive supports such as glass plates is a very important factor for routine work, because many substances absorb light in the UV range. Different lamps must be used as light sources to cover the entire UV/Vis range. Halogen and tungsten lamps are suitable in the visible spectral range (400–800 nm) and deuterium (190–400 nm) and xenon lamps in the UV range. The high-pressure mercury vapor lamp, which provides high energy at defined wavelengths, is used mainly for fluorescence measurements but can also be used for absorption measurements if it has an emission line at the wavelength required.

Monochromatic light is generated by monochromators; modern instruments have gratings, but in some old systems it is still possible to find prisms. In low-priced instruments dedicated to special applications, monochromatic filters are often used. The diffused light is measured by photomultipliers, photodiodes, or photoresistors. Photomultipliers are very convenient for densitometry because of their broad wavelength range and the linear relationship between output current

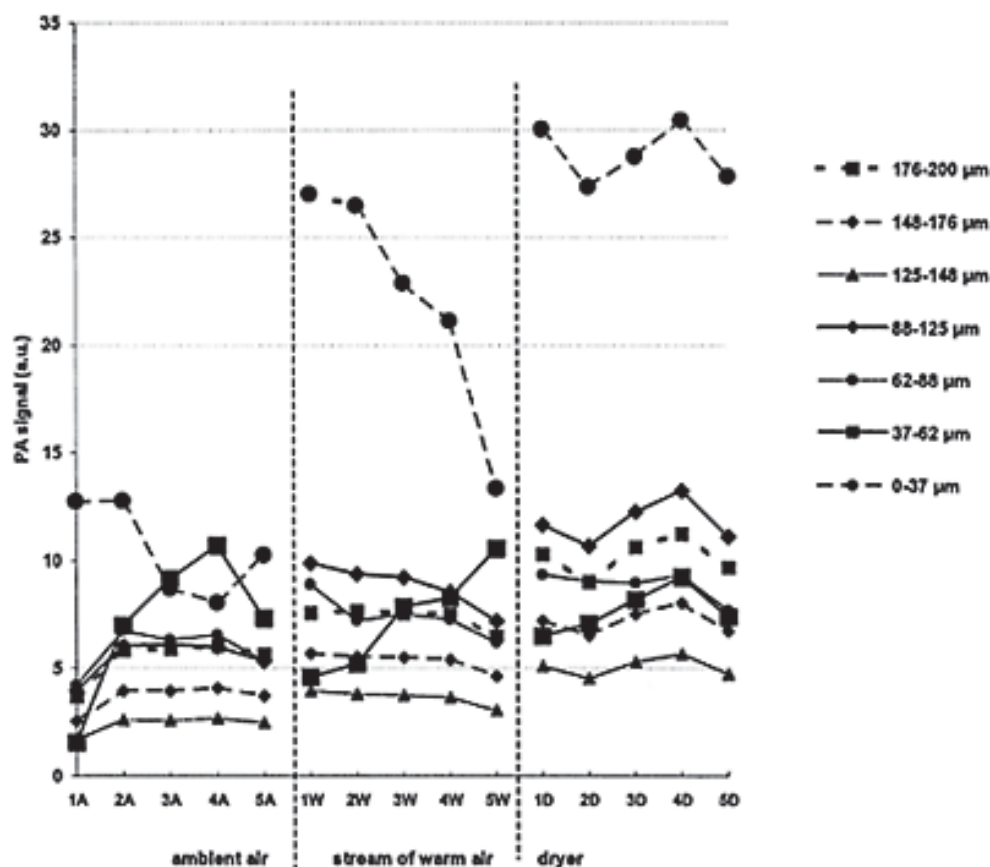


Figure 10 Depth distribution of the compound in violet spots on HPTLC plates dried in the ambient air (A), in a stream of warm air (hair dryer) (W), or in a dryer (D).

and the energy of excitation within a wide range of operating conditions; but they are expensive and large and cannot be used as multisensor detectors.

The detector output is converted into a suitable signal and amplified. In addition to analog scanning, which was typical in previous systems, modern instruments are equipped with analog-to-digital converters, and data are digitized and processed by computers. Today, in most cases, scans are taken in the reflectance mode. The analyst can work in a range of 190–800 nm regardless of the quality of the sorbent. The baseline drift, caused by variation in the thickness and uniformity of the layer, is small, and the signal level is relatively high. A big disadvantage of the reflection mode is the strong influence of the vertical depth distribution of the compound in the measured spot on the signal. Furthermore, differences in the vertical concentration profiles of samples and standards can cause erroneous interpretation of the results. Such variations can also result from improper treatment of a plate after development, e.g., nonuniform drying.

2. Transmittance Mode

The principle of transmittance measurements (transmittance mode) is shown in Fig. 13. This technique is particularly useful for measurement of the absorbance of a substance in the visible spectral range. A photometric detector measures the intensity of transmitted light on the nonilluminated side of a plate. The signal is a function of the number of absorbing molecules in the layer. The Beer—Lambert law cannot be used to calculate the concentration of the substance, because scattering in the layer makes a significant contribution. The best solution is the hyperbolic equation proposed by Kubelka and Munk. Fluctuations in transmission resulting from different vertical concentration profiles of samples are small, and errors caused by concentration gradients are negligible.

Background interference plays a more dominant role in transmission than in reflectance, leading to less favorable signal-to-noise ratios in the transmittance mode. The baseline is very sensitive to the optical properties of the absorbent and to the changes in the layer thickness. Compared to reflectance, light intensity in transmittance is weaker by a factor ranging from 5 to

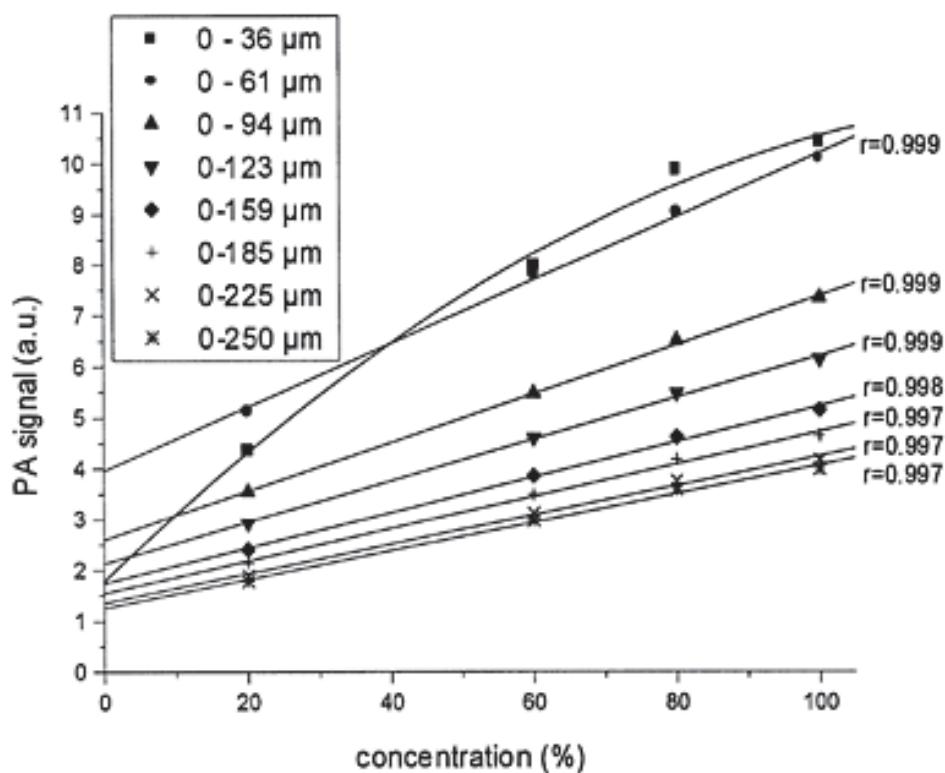


Figure 11 Calibration curves for the yellow spots obtained by the PAS when probing different thicknesses of the sorbent.

Table 2 Peak Areas Obtained by Densitometric Measurements of Spots (n=5) on HPTLC Plates

Spot color	RSD (%)		
	Drying method		
	Ambient air	Stream of warm air	Dryer
Red	2.47	5.56	1.38
Blue	1.32	5.58	1.16
Violet	3.50	3.86	0.84
Yellow	1.11	3.86	0.91

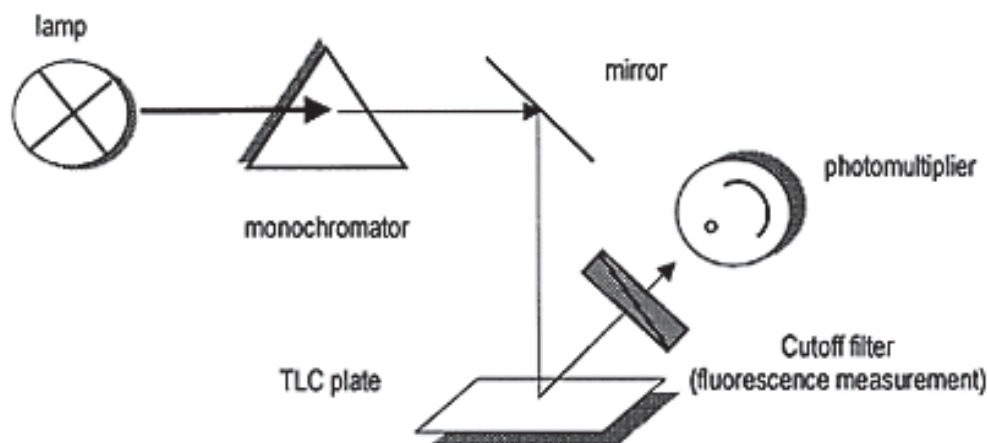


Figure 12 Reflectance mode of scanning (remission).

10. Nevertheless, the transmission mode would appear to be more sensitive than the reflectance mode because all the molecules in a spot influence the signal, not just the molecules close to the surface as in the reflection mode.

B. Digital Cameras

The first results on quantitative evaluation of TLC plates with an image-analyzing system presented at the Third International Symposium on Instrumental High Performance Thin-Layer Chromatography in 1985 in Wiirtzburg (33) were not accepted with special interest. These results were obtained with a Micro D camera Hamamatsu with 128K pixels and processed with an Apple II computer (64K RAM) and a screen with 290×170 pixels. However, this was the beginning of image analyzing in planar chromatography. Even with this simple instrumentation it was possible to demonstrate the power of these new scanning and quantifying procedures.

In the last 15 years or so, scanning of TLC plates with image-analyzing systems, especially with CCD cameras, has become popular. The advantage of video systems is that they enable the simultaneous on-line acquisition of information on a whole TLC plate and a large number of digitized raw data (pixels) that can be processed with a computer. It is possible to construct programs that detect and eliminate chromatographic and scanning errors. Method sensitivity is improved with longer scanning time, and the signal-to-noise ratio is improved by the accumulation

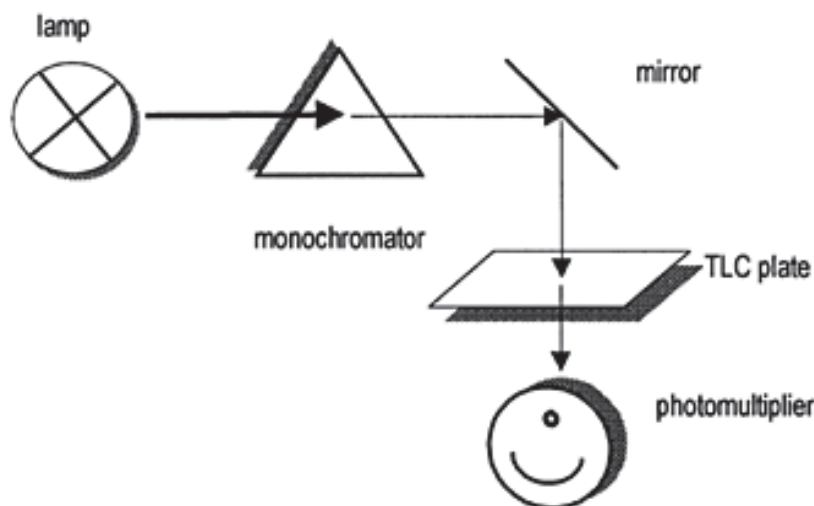


Figure 13 Transmission mode of scanning.

of several scans (frames). Because the whole plate is processed at the same time, it is possible to simultaneously evaluate and compare data from all the tracks. In this case the biggest advantage of TLC—the parallel development of standards, control samples, and samples—is fully supported.

The main difference between densitometry and image processing is hidden in the illumination mode. In densitometry a small slit with a relatively high intensity incident beam is used, and most of the light is reflected or lost inside a layer; meanwhile video systems illuminate the entire plate, and in this case more diffusely reflected light is captured. Because this light gives information about the number of molecules inside the layer, the image processing techniques are not so sensitive to the depth concentration gradient.

However, image-analyzing techniques are not perfect. It is difficult to prepare homogeneous illumination during image acquisition; this is the main source of quantification errors, and scanning conditions are limited. In routine work, we can use measurement only in the visible range and certain wavelengths (254 and 366 nm) in the UV range. It is not possible to record spectra from separated spots. Recently Ebel and Henkel (34) presented an interesting paper describing basic algorithms of image processing in TLC.

In many laboratories, TLC is used as a semiquantitative method that is very suitable for research and routine work. Usually, chromatograms are quantified visually, using external standards spotted on the same plate. There is one critical point in this determination: Visual inspection and quantification of plates depends too much on individual decisions made by an analyst and is therefore too subjective. To document the results and make them more reliable, digital images or photos should be taken and stored.

Today it is not difficult to construct your own image-analyzing system from commercially available electronic components. In our laboratory such a system is used for the development of new algorithms and for quantitative methods. We study the relationships between peak positions and the intensities of spots in the images taken in the remission and transmission modes. Results are compared with densitometric

measurements and with other analytical methods, e.g., HPLC, capillary electrophoresis, or UV/Vis spectroscopy. These results rank image-analyzing systems above scanners. According to our knowledge, this is the result of total plate illumination, which offers real measurements in diffuse light. The difference between scanners and CCD cameras is seen when quantification of plates with a concentration gradient inside a sorbent, or a large concentration range, is being carried out.

The goal of an imaging system is to provide sufficient image quality to enable the extraction of the desired information about the object from the image. The most important components of an image-analyzing system are the image acquisition device, illumination system, frame grabber, and software for data acquisition and quantification. Knowledge about these components and their influence on the final results is crucial if we want to get the best possible results. When deciding to buy or construct and use an image-analyzing system, we should take care to understand some basic terms such as resolution, noise sources, signal-to-noise ratio, sensitivity, coatings, down-converters, and back-thinned CCD (35). Additionally, it is important to integrate components that have the same level of performance. For instance, it is wasteful to display the image from a high-resolution camera with a high-resolution imaging lens on a low-resolution monitor. Although the signal resolution from the camera is excellent, the monitor is not able to display it fully.

The components of an imaging system (lens, camera, monitor, capture board, illumination) influence the image quality parameters: resolution, image contrast, perspective errors, geometrical errors (distortion), and depth of field. Resolution is influenced by the lens, camera, monitor, and capture board; image contrast is influenced by the lens, camera, and illumination; perspective errors are influenced by the lens aperture; geometrical errors (distortion) and depth of field are influenced by the lens.

We should never forget that all the PC and software improvements are meaningless if we cannot obtain a quality image. Therefore, high-resolution CCD cameras for digital image capturing are needed. "High resolution" refers not only to the resolution of features that are not well separated but also to the gray level or number of colors that can be distinguished. Spatial resolution (the actual number of pixels), which determines the amount and detail of information captured in the image for display, analysis, and quantification, is also very important. One of the problems in the field of video scanning is still the insensitivity of CCD cameras in the UV spectral region. Even different organic and inorganic coatings, so-called downconverters, did not solve the problem, although they have considerably improved the possibilities of measuring in the UV spectral range. It is already possible to buy a back-thinned CCD camera—e.g., Hamamatsu C8000–20 NR (170–1200 nm) and C8000–20 VUV (below 170 nm) CCD cameras—that have much higher quantum efficiency (from 50% to 80%) than other cameras in the UV spectral range. Unfortunately, the price of such a camera is extremely high, but it will surely drop within the next few years.

Monochrome cameras have higher resolution, better signal-to-noise ratio, better light sensitivity, and greater contrast than similarly priced color cameras. Color imaging requires more processing and does not yield significantly more information about the object, but it might be very important for identification of separated substances in planar chromatography (e.g., finger-prints of extracts of medicinal plants). However, when a high-resolution color image is necessary, it is beneficial to use a three-chip (also called 3-

CCD or RGB) camera. By utilizing three CCD sensors, these cameras offer greater spatial resolution and dynamic range than single-chip color cameras. The image is directed to each sensor by a prism and is then filtered to provide independent red, green, and blue signals.

1. Illumination Systems

Illumination is the key to a successful imaging system, but the effects of illumination on image quality are often underestimated. Proper lighting can increase the image contrast and resolution, improving the overall performance of the system. This can include the illumination setups as well as filtering, etc. The desired image quality can often be improved by upgrading a system's illumination rather than investing in higher resolution detectors, imaging lenses, and software. Proper light intensity in the final image is directly dependent upon the components selected. Every component (e.g., imaging lens aperture, etc.) affects the amount of light incident on the sensor and therefore the system's image quality. The camera's minimum sensitivity is also important in determining the minimum amount of light required in the system. In addition, CCD camera settings such as gain and shutter speed affect the sensor's sensitivity. Additionally, the lighting system should enable uniform illumination of the TLC plate. Unfortunately, none of today's commercially available image-analyzing systems for planar chromatography provide this. Therefore, we have to be aware that uneven illumination of separated substances with the same R_f value on different parts of the plate might be a source of error in quantitative TLC.

As users of the image-analyzing systems we have to be aware of the importance of color calibration of the CCD camera, monitor (screen), and printer. To achieve reproducible results, the calibration of any printer or screen colors should be left unchanged. An additional limitation of the currently commercially available image-analyzing systems is the illumination in the UV spectral region, which is limited to wavelengths of 254 and 366 nm, which is not enough.

2. Software

Another important part of an effective image-analyzing system is software. There are at least 20 different versions of software for quantitative evaluation of thin-layer chromatograms with image-analyzing systems written by different companies around the world. As end users, we can say that most of the software is written by the specialists, who are not familiar with TLC. This is one of the reasons software is too complicated (in other words, not user-friendly) or does not enable us to take advantage of TLC coupled with image analysis. Additionally, the concept of available software is not useful for two-dimensional TLC, and also circular and anticircular mode. Software that implemented ideas of the users (e.g., the possibility to find tracks and spots or bands automatically) could enable one to make the analysis much faster, which is especially important in routine analysis.

Renger suggested a real image-analyzing system based on modern fuzzy logic pattern recognition that should be able to use and evaluate the information available, including overlapping, poorly resolved peaks, if a sufficient number of calibration samples are

available (36). However, currently commercially available image-analyzing systems for applications in TLC can use only a small part of the multidimensional information saved on the image of the TLC or HPTLC plate. Nevertheless, we have to be optimistic, because there are some new ideas, with new algorithms for image processing, on the horizon, including new algorithms for image processing, which were recently proposed by Ebel and Henkel (34).

C. Flatbed Scanners

Thin-layer chromatographic plates with visible spots or bands can be scanned with flatbed scanners such as those normally used in offices. Such scanners are not expensive and are very suitable for the documentation of TLC plates. Images are stored in digital form, and tracks and spots can easily be detected and quantified with software such as Videodensitometer Sorbfil 1.1 (Sorbfil, Krasnodar, Russia). For quantifying intensive spots, the reproducibility and accuracy of this low-price scanning procedure are comparable with the results obtained with more expensive image-analyzing systems and densitometers because there is no problem with the uniformity of illumination of the TLC plate.

Quick and low-priced quantitative and qualitative evaluation of TLC plates with a digital flatbed scanner in the visible spectral range is the method of choice in many cases. Raw data taken from a TLC plate and stored in bitmap format can be quantitatively evaluated. If data are quantitatively evaluated with a low-priced flatbed digital scanner and processed with a universal TLC software pack, then we have an analytical system with an incredible price/performance ratio. A flatbed scanner can be obtained in a local computer shop. Compact and inexpensive, Sorbfile software, with a program size of less than 2 MB, can be installed in any computer with an NT or Windows 95, 98, or 2000 operating system. This means that semiquantitative TLC can be used in every laboratory as well as in schools, farms, vineyards, hospitals, etc. Results obtained with a flatbed scanner and processed with Sorbofil software are shown in Fig. 14. They show the potential power of this type of semiquantitative analysis.

A similar system with the UMAX UC1260 scanner and MagicScan v. 2.4.1 scanning software (UMAX Data Systems, Taiwan) has recently been described as a rapid, simple system for quantitation of thin-layer chromatograms with Igor Pro v. 3.13 software (Wavemetrics, Eugene, OR) (37). Stroka and coworkers (5) recently reported a very useful modification of a computer-driven office scanner that had been modified to enable measurement of fluorescence. The light tube was replaced with a black light tube, and a special cutoff filter was installed. The modified scanner was used to determine aflatoxins at low nanogram levels. It enables monitoring of the compound in food and feed at the levels stipulated by European law (2 ng/g aflatoxin B1 and 4 ng/g of total aflatoxins).

Of course, these results do not mean that image processing analysis is better than classical densitometry in every case. They show that there are samples that can be successfully measured with video systems in a shorter time and with much easier access to information coming from the relationships between the lanes.

When deciding to buy an image-analyzing system, we have two possibilities. Due to the wide availability of digital camcorders, CCD cameras, flatbed scanners, frame grabbers, software, and other components on the market, we can build our own image-

analyzing system, or we can buy one. We are using both options for testing of qualitative, semiquantitative, and quantitative evaluation of developed TLC plates.

The contemporary progress in the field of software and CCD sensors could be the reason for the revolution in the field of quantitative evaluation of TLC plates. We are sure that in the near future image-analyzing systems will enable the gathering of spectral information. Finally, image analysis may revolutionize the learning laboratory for planar chromatography if we make a digital imaging workstation for students at universities and others working in the field of planar chromatography.

Nevertheless, it is clear that lower prices, more user-friendly systems, and a greater choice of products will make image analysis an increasingly important tool in many laboratories. Finally, image analysis as a modern approach to quantitative TLC and documentation of TLC chromatograms still offers many possibilities for further improvements of the state of the art; however, the producers should be hand-in-glove with the end users. Better cooperation between producers

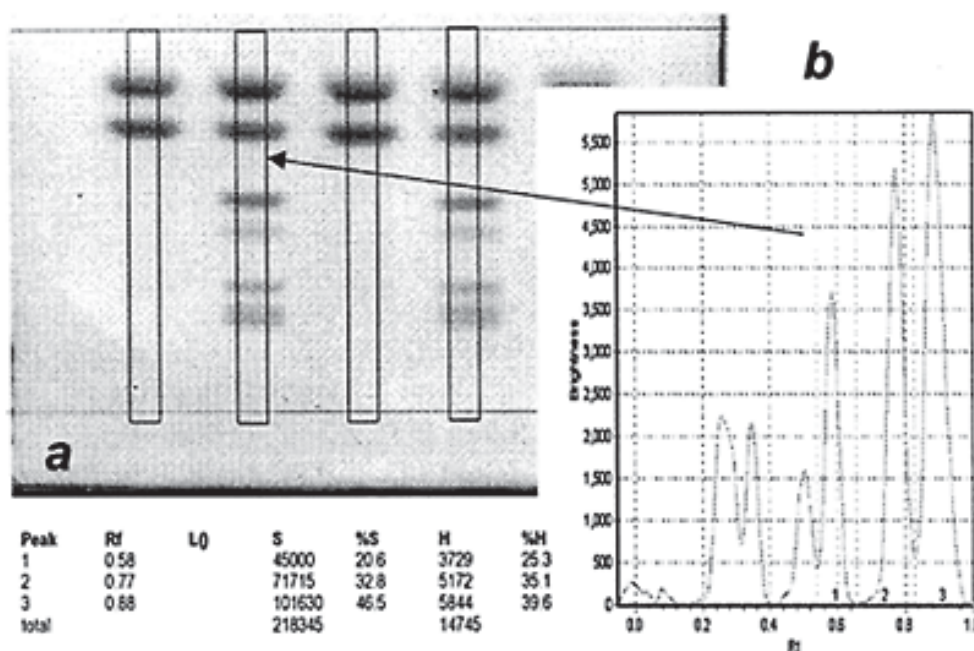


Figure 14 Semiquantitative TLC. Different sugars separated on HPTLC plates, (a) Scanned with office scanner, Hewlett Packard OfficeJet Pro 1175c. (b) Quantification performed with Sorbfil software (Krasnodar, Russia).

and end users is the basis for celebration of the rebirth of planar chromatography in this millennium.

IV. INTEGRATION IN QTLC

A. Calculation and Reporting

In densitometry and image processing the integration procedure is more complicated than the quantitative evaluation of chromatograms in other separation techniques. In TLC, components remain in the stationary phase, and the integration algorithm must find cardinal peak points and the correct baseline shape because chromatograms (densitograms) are the sum of signals from samples and plate. The integrated algorithm must find positions and concentrations of light-absorbing components located in a stationary phase that scatters and absorbs, possibly through the remaining film of the mobile phase, which also absorbs light, especially in the UV region. In the reflectance mode, which is commonly used in TLC, the detected signal represents only a small part (5–10%) of the available signals, which are mixed with information of the plate structure and the remaining mobile phase.

Twenty years ago the introduction of low-priced personal computers completely changed data processing in TLC. Many commercially available programs were prepared, and integration and quantification became relatively simple tasks. Personal computers enabled the preparation of homemade programs for integration and calculation when data acquisition and integration were complicated. In these cases we had to be familiar with programming, and in the case of regular analysis we had to validate our programs. Today, in addition to the software prepared by producers of equipment it is possible to obtain programs that have been prepared by some independent groups. These programs are often more user-friendly than factory-prepared solutions; however, it is difficult to say which type of software is better. The best solution is to use both of them in parallel. The software prepared by vendors is tailored to certain equipment and is usually the most sophisticated solution, but in some cases vendors also use software solutions to hide some hardware errors, and in this case independent software is a very useful tool for evaluating such errors.

A flow chart of the calculation and report procedures is shown in Fig. 15. Evaluation programs are prepared so that the operator has to key in certain values that are used for identification of lanes, identification of spots in lanes, and construction of baseplane and baselines. In research work we are still using our old QTLC-pack data evaluation program. It is suitable for quantitative evaluation of TLC plates with a computer-controlled scanner and digital acquisition. The advan-

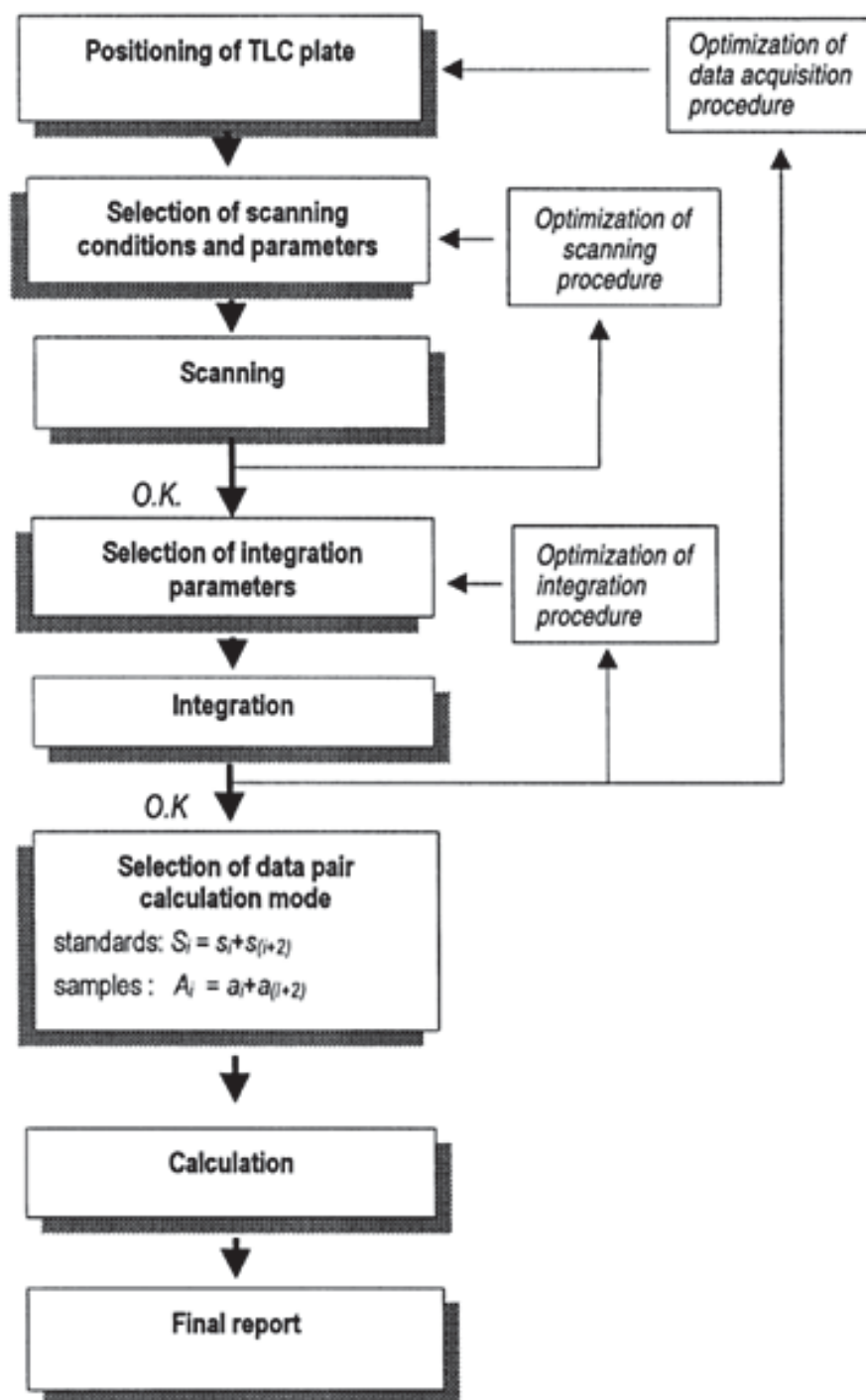


Figure 15 Scanning and calculation procedure in quantitative TLC.

tage of this software package is its very sophisticated integration algorithm, INTAL, which is the result of 20 years of nonstop development and research (38). Our ideas are used in many commercially developed TLC software packages.

Modern computer technology enables the development of a direct interface between data displayed on the CRT and the operator. The analyst can in-line construct the baseline and select positions of spots with a cursor driven by a computer mouse or special keys. Calculated R_f values, peak areas, and peak heights are usually stored in text data files. These files are later used in the identification and quantification of samples. Software such as CATS from Camag has built-in programs for different modes of calculation of concentrations. Years ago such programs were the most acceptable solution. With the development of spreadsheet programs such as Microsoft's Excel, the situation has changed. Today many analysts use Excel, because it offers them more freedom in calculation and documentation.

Integration and calculation of concentrations are also error-prone steps. Selection of integration parameters or of the calculation procedure has a great influence on the final result. Educated operators are the only solution for this problem. After the calculations, measurement uncertainty cannot be estimated from documented integration parameters, because their effect is changed from track to track and from plate to plate and documented parameters are not informative enough without documentation about why they were selected and how they were modified. The control samples and in-line assessment of a skilled operator are the best solution to minimize calculation error.

Despite the fact that numerous integration algorithms have been developed, the correct determination of multiple peaks and baseline positions remains the most demanding task in the evaluation of densitograms, especially taking into account the lower chromatographic efficiency in TLC.

V. VALIDATION IN QTLC

Today we are faced with a growing demand that analytical data used in any decision process must be technically sound and defensible. Limits of uncertainty, together with documentation, are required for each of our results. We are expected not only to standardize our methods but also to control variability in our measurements.

It is possible to find good advice in the work of J.K. Taylor published about 20 years ago. Quality assurance of a measurement process such as QTLC involves two related activities, quality control and quality assessment. Quality control develops and implements the tasks necessary to produce a measurement of requisite quality; quality assessment verifies that the quality control system is operating within acceptable limits, and thus controls the quality of the measured data (39–42).

The modern approach is not so clear and is not uniformly accepted (43, 44). To evaluate measurement uncertainty, a special calculation procedure called the "error budget model" was prepared. This approach is usually performed according to the expectations of metrologists working on physical metrology, pointing out traceability and a hierarchical chain of standards (45). Analysts in regulatory laboratories and assessors from accreditation bodies tend to accept this concept without question, without seriously testing its behavior in real life.

To see how the error budget model can be applied in TLC, we evaluated measurement uncertainty in our laboratory. Quantitative determination of monosodium glutamate in food products was taken as an example, and measurement uncertainty was calculated

according to Eurachem/ Citac guidelines (46). The TLC procedure was divided into stages, and in each stage the size of identified potential sources of uncertainty were evaluated. The cause-and-effect diagram was constructed, and identified sources of uncertainty were listed. Quantifying the measurement uncertainty, we found that the error budget method is not acceptable, because in TLC many parameters that are not described mathematically contribute much more than all the sources identified with equations (Table 3).

Thin-layer chromatography is a very reliable analytical technique, but it cannot be evaluated according to metrological expectations. The fact is that acceptable and analytical results in chem-

Table 3 Reported Variances Calculated from Validation Experiment^a

Variance	Description	Value (%)	Remarks
u_{vol}	Volume of H ₂ O	0.10	Temperature, dilution.
u_{w1}	Weight of sample	0.03	Balances.
u_{w2}	Weight of standard	0.05	Balances.
$u_{\%}$	Purity of standard	0.30	Declared value > 99%.
u_{mol}	Molar mass of L-sodium glutamate	0.002	Calculated.
v_{ex}	Extraction	2.5	Validation parameter.
v_v	Application (Linomat)	0.5	Application: 5 μ L bands, 6 mm.
v_c	Chromatography	1.2	Validation parameter.
V_d	Derivatization	0.8	Estimated (data-pair technique).
v_g	Concentration gradient	0.4	Estimated (data-pair technique).
v_m	Scanning	0.3	Scanner error calculated from 10 measurements of one band.
v_p	Positioning	—	Not important (application in form of bands).
v_i	Integration	1.0	Peak start, peak end, baseline construction depends on noise.
v_{HPTLC}	Selection of HPTLC plate	—	Not possible to estimate. If normal TLC plate is used, the total error is 4.5%.

^aFour HPTLC plates were prepared; six samples and six standards were applied according to the data-pair technique. Uncertainties are taken from Eurachem experiments.

Results:	Uncertainty contribution from described sources	0.32%
	Total error calculated from error propagation	3.15%
	Final result (combining)	3.17%

ical laboratory in routine and research work are primarily the result of the right analytical management and strategy and not metrological quality. Well-educated and well-trained analytical chemists are the guarantee for the quality of the analytical work. Measurement uncertainty cannot estimate the reliability of analytical results, because it evaluates the quality of only certain procedures. The reliability of analytical work is obtained with a constant quality throughout time, and so it can be evaluated only with a carefully planned validation procedure, accompanied with an adequate number of quality control samples and/or interlaboratory comparisons.

Although the error budget approach is not applicable in routine TLC, it can be used as a tool for planning certain steps in the validation of TLC methods. It is possible to prepare a computer program to quantify measurement uncertainty associated with potential sources of uncertainty in quantitative TLC. The analyst selects basic chromatographic parameters and the program estimates the uncertainty of the analyst's method, using relevant associated uncertainty values obtained from previous experiments. This approach is justified for TLC, which is a technique with clearly separated analytical tasks: preparation, application, development, and evaluation. It is not necessary to use a holistic approach as in HPLC or GC.

Our program was tested with a set of experimental data. TLC plates with different qualities of stationary phases (TLC, HPTLC) were spotted with different samples. Application was performed manually and also automatically with the Camag Linomat IV. Plates were scanned with the Camag TLC Scanner II and Camag TLC Scanner III and Camag Videoscanner in remission and transmission modes and fluorescence. Although the predicted values are close to the values obtained with the validation procedure, we cannot use them as substitutes for the results obtained with the validation procedure. The weakness of our program is common to all calculation models. It is not possible to prepare enough reliable information. In order to test 17 parameters with three free steps each, it is necessary to prepare more than 100 million experiments, which would take us more than 1000 years.

The result of a measurement is always subject to error. Precision is daily confused with accuracy, and the agreement of successive results from some analytical methods falsely inspires analysts with a degree of confidence that the method does not merit. This is typical for column separation techniques. Inter- and intraday precision are often very different. It is necessary to distinguish between statistical and systematic errors. In TLC the contribution of systematic errors is normally smaller than the contribution of statistical errors because TLC is an open system. It is evident that one or a few sources of error can be the major contributors to the total error, owing to the addition of the squares of variances. TLC is not strongly influenced by biases. It is possible to get a reliable "true" result by eliminating biases and reducing variances with band applications and data-pair technique. In this situation quality assessment is very important factor.

Quantitative TLC consists of five main and three optional stages. To estimate the uncertainty of the procedure as a whole, relevant uncertainty sources at each stage must be determined. The chromatographic process starts with selection and sometimes preconditioning of the stationary phase. These procedures have an indirect influence on the measured uncertainty, because further steps such as separation and scanning are influenced by this selection.

A certain number of samples and standards are applied to the TLC plate in the form of spots or bands. All applied tracks are developed simultaneously. For the inexperienced TLC user, all tracks seem equal, but in quantitative TLC, spotting positions have an important influence on the final result. If we want to minimize the measuring uncertainty, we have to carefully plan the positions of the spots.

Application can be done manually with micropipets or automatically with special application devices. A procedure for approximate determination of sample application error was proposed by Ebel and Glasser (47). It is based on the fact that accurate determination of sample application error is not possible using TLC methods alone, because the error due to chromatography will always be measured at the same time, but for substances with low R_f values the influence of chromatography is almost negligible. In the case of manual application with micropipets of 1–10 μL , the RSD of applied volume is $\pm 1.5\%$, and in the sophisticated mode of application with an autosampler it is $\pm 1.0\%$, according to vendors' specifications. If we need to apply a larger volume, 5–100 μL , the best solution is to use a Linomat. In this case the precision of applied volume is between $\pm 0.5\%$ and $\pm 2\%$.

The spotted plate is normally dried before development. The drying step can be a big, unpredictable source of error. If too much heat is used, spots can remain at the start and samples or standards can be degraded. To eliminate uncertainty, application procedures must be validated, and standard operating procedures (SOPs) with precise instructions about plate handling before, during, and after application for each TLC method should be set out.

Chromatographic separation is the main factor in reliable quantitative TLC. Development starts with the immersion of the spotted plate into the chromatographic chamber, previously filled with a selected solvent or a mix of solvents. After the plate is immersed into the developing solvent, the chromatographic process starts. Uncontrolled separation conditions produce poor reproducibility. Due to the numerous parameters influencing a composition and the existence of a gradient of the developing solvent in the vertical (depth) direction of the plate and alongside the developing path, it is not possible to mathematically predict its profile.

The drying stage is also an important source of error. During the drying process, the mobile phase evaporates from the upper part of a plate and produces secondary chromatography, which is the main reason for poor precision in TLC. With up to 10% RSD, it is by far the greatest source of uncertainty. To reduce this influence it is necessary to prepare and carefully follow an SOP for the drying stage. In addition, it is possible to reduce the influence with clever distribution of samples and standards. If samples are spotted on one side of a plate and standards on the other, an error of 5% or more is typical. If samples and standards are applied one after the other, then a 3% error can be expected; and with data-pair techniques, an error of 1–2% can be expected. The influence of inhomogeneous distribution is further reduced with an increased number of applications of the same sample or with application in the form of bands and a correctly selected scanning slit.

Sometimes separated components have no chromophore and we have to make these components visible before measuring. It is possible to do this with post-run chemical derivatization. A solution with a special reagent is prepared, and the plate is dipped into the solution for a certain time, usually some seconds. After that the plate is heated and

components on the plate and the derivatization reagent react and produce colored spots or bands. This operation is strictly empirical (dipping time, time and temperature of heating, cooling process, etc.). Its influence on the final result must be validated. Spraying has been used rather than dipping and is less reproducible.

The chromatographic step is followed with quantification of the separated components. The previously described equations for quantitative evaluations show how complicated is the relationship between the concentration of a sample and the intensity of diffusely reflected light. To obtain reliable analytical results it is necessary to prepare standards with different concentrations and, with carefully planned validation, estimate the sensitivity, working range, the limit of detection (LOD), limit of quantification (LOQ), etc. A nonlinear response can be easily overcome by the use of a limited concentration range or the selection of nonlinear calibration functions.

Data acquisition is performed with three different types of instruments: classical densitometers, video systems, and digital image scanners. Each system has some advantages and some disadvantages.

The advantage of densitometry is its very sensitive measurement in the UV and visible ranges and possible acquisition of spectra directly from a plate. The disadvantage is its slow scanning speed. The sources of uncertainty are mechanical, electronic, and optical stability of the instrument. A typical scanning time for one plate is 30 min.

The advantage of video systems is simultaneous acquisition of the whole plate and the large amount of collected raw data (pixels). In this case it is possible to construct computer programs for post-run data processing that can detect and eliminate chromatographic and scanning errors. Sensitivity is obtained with a longer scanning time, and the signal-to-noise ratio is improved by accumulation of more scans. Video systems illuminate the whole plate, and the results obtained with image processing techniques are not so sensitive to the depth concentration gradient. However, the whole power of image processing is lost if scanning is performed with inhomogeneous illumination of the TLC plate.

Plates with visible spots can also be scanned with low-priced flat digital scanners. In the case of intensive spots, reproducibility and accuracy are comparable to the results obtained from more expensive video systems and densitometers.

Data acquisition is usually not a source of random errors, because the analyst assesses the plate prior to scanning. A correctly selected scanning procedure will strongly reduce data acquisition error. The error budget approach is acceptable, because it predicts error sources, but it must be used in-line and approved by an expert. Reliable results should be obtained with a validated method, transparent documentation, and a sufficient number of good quality control samples.

Integration and the calculation of concentrations are also error-prone steps. Incorrect selection of integration parameters and of the calculation procedure greatly affects the final result. One set of parameters can be optimal from some tracks and unacceptable for others. Their values have to be changed from track to track and not just from plate to plate. Selected parameters are not informative without documentation as to why they were selected. The use of control samples and in-line assessment by a skilled operator are the best ways to minimize calculation error.

Analysts have made errors and estimated the errors of analytical measurements for years. In our opinion, it is not possible to get reliable measurement uncertainty of an

analytical procedure without consideration of quality assessment. Assessment shows how well-trained and error-prone the laboratory staff and facility are.

VI. CONCLUSION

Today we look for analytical procedures that give the greatest output of information in the shortest time. In this respect QTLC is a very promising method, and these trends must be developed. With the present hardware and software we can start a new page in the quantitative evaluation of TLC. A developed plate is a bank of information that needs to be read and processed. In our opinion, it is more suitable for data acquisition to select a multisensor device with more than 2 million pixels than to measure a plate with a large scanning slit and limited speed of movement. TLC is a planar technique and is, like photography, more reliable when it is described with a larger number of pixels. We hope that in the future QTLC will proceed in the direction of the new video-oriented data acquisition and processing systems that can be used in both research and routine QTLC.

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11

Preparative Layer Chromatography

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I. INTRODUCTION

Preparative layer (planar) chromatography (PLC) is a liquid chromatographic technique in which the solvent–solvent composition migrates through the stationary phase either by capillary action or under the influence of forced flow with the aim of separating compounds in amounts of 10–1000 mg (1). The compounds can be isolated for structure elucidation (IR, UV, MS, ^1H -NMR, ^{13}C -NMR, CD, etc.), for various analytical purposes or for determination of biological activity (2).

Depending on the mode of solvent composition migration, PLC can be classified as classical PLC (CPLC) or forced-flow planar chromatography (FFPC). In the first type of PLC, the solvent migrates by capillary action (3). The category FFPC includes all methods in which the mobile phase migrates not only by capillary action but also by forced flow. For preparative purposes, two basic FFPC methods have so far become available: forced flow can be achieved either by application of external pressure [overpressured layer chromatography (OPLC)] (4–8) or by use of centrifugal force [the various types of rotation planar chromatography (RPC)] (9–12). The enhanced efficiency of FFPC techniques as evidenced by a comparison of their analytical properties with those of classical thin-layer chromatography (TLC) and high-performance TLC (HPTLC) is well known (e.g., 2), because by use of FFPC techniques the advantage of the optimum mobile-phase velocity can be practically exploited over the entire separation distance without loss of resolution. This effect is independent of layer thickness and the type of forced flow applied.

Both types of FFPC may be used as on-line preparative techniques (13, 14), i.e., techniques in which the separated compounds are eluted from the stationary phase and isolated from the instrumentation. This enables connection of a flow detector, recording of chromatograms, and collection of separated compounds with a fraction collector (Fig. 1). FFPC methods enable not only micropreparative (OPLC) and preparative (RPC) separations but also, by using appropriate split systems, the coupling of these methods with various spectroscopic techniques, as is apparent from Fig. 1. In this way, not only isolation but also structure elucidation can be carried out in a single process.

The chromatographic processes operative in CPLC, preparative OPLC, and RPC basically resemble those of analytical TLC, OPLC, and RPC, respectively. The most important factors that may influence a PLC separation are shown in Fig. 2.

Working with PLC requires consideration of some special characteristics, such as the average particle size, the thickness of the stationary phase layer, the chamber type, the application of large amounts of sample, the location and detection of the separated compounds, and the removal of the desired compounds by elution or extraction. The type of stationary phase, the composition of the mobile phase, the separation distance, the mode of development, and the working temperature may be identical with those in analytical TLC. The procedures have been described extensively for analytical TLC (15–18) and summarized for PLC (19–21).

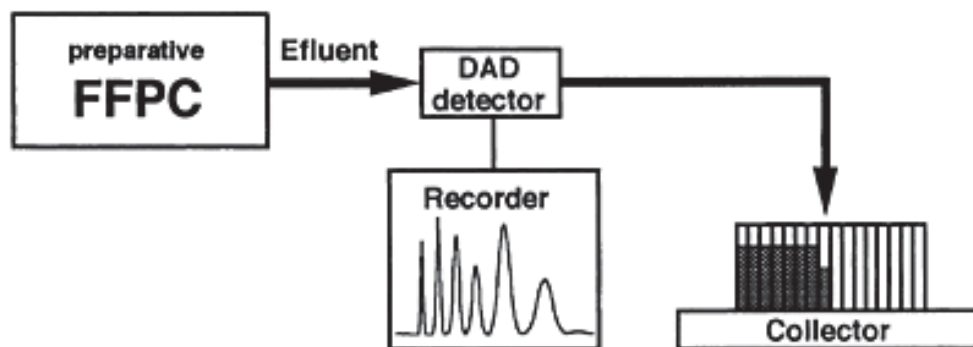


Figure 1 Schematic diagram of preparative on-line FFPC.

In the following discussion, these points are considered first for CPLC, then for the two preparative forced-flow techniques OPLC and RPC.

II. CLASSICAL PREPARATIVE LAYER CHROMATOGRAPHY

Classical preparative layer chromatographic (CPLC) separation methods require minimal financial outlay and employ the most basic equipment (2). Sound chromatographic knowledge is much more important than equipment, and the operational skills are very simple to master and easy to apply.

A. Factors of Principal Importance in Classical PLC

1. Stationary Phase

Most users prefer to use commercially available precoated preparative layers rather than produce their own. Besides saving time, precoated layers have the advantage of much higher reproducibility.

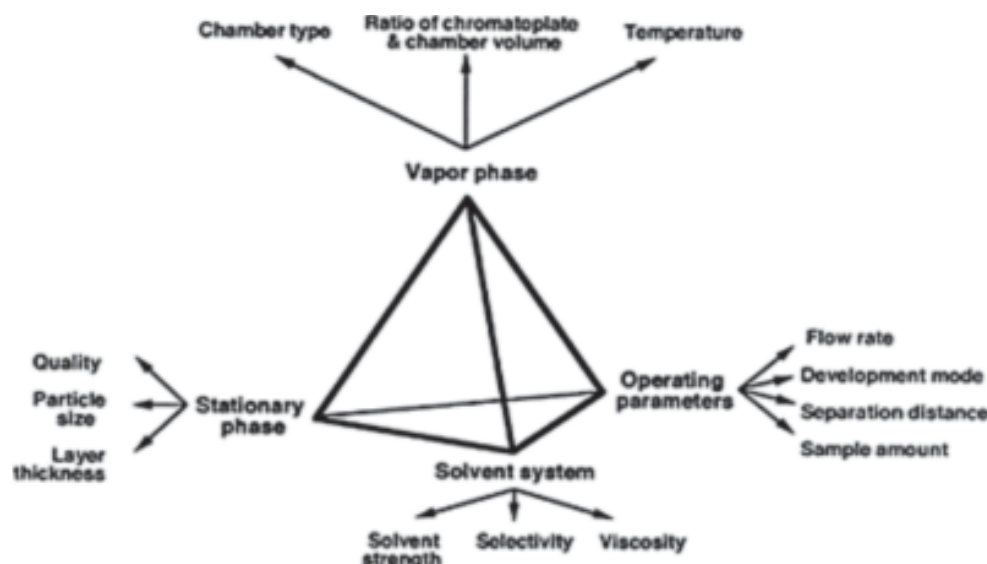


Figure 2 The principal factors affecting preparative layer chromatography.

bility than homemade layers. Three types of precoated preparative plates—silica, alumina, and RP-2—are generally commercially available in layer thicknesses of 0.5–2 mm.

It is generally accepted that higher resolution can be achieved on thin preparative layers (0.5–1 mm) (22). The resolution is much more limited on a high-capacity (1.5–2 mm) layer because of the thickness of the stationary phase. The loading capacity of a preparative layer increases with the square root of the thickness, with little if any loss of separating power. The loading capacity of a 0.5 mm layer is approximately half that of a plate with a layer thickness of 2 mm.

Commercially available plates have dimensions of 20×20 cm or 20×40 cm. The general problem with precoated plates is that the commonly used silica for preparative separations has extremely coarse particle sizes (average of ~25 μm) and their distribution is too wide (5–40 μm) (23). Figure 3 compares the quality of precoated analytical TLC and HPTLC silica as well as that of silica for TLC, whose average particle size is 15 μm . Unfortunately, at present there are no commercially available precoated preparative plates with reasonable average particle size and particle size distribution.

To increase the separation power, various precoated preparative layers with a preadsorbent zone are commercially available. The effect of the concentrating zone on resolution is illustrated schematically in Fig. 4. This part of the layer serves as a holding zone for the sample until development begins. Soluble compounds migrate with the mobile-phase front through the preadsorbent zone and are concentrated in a narrow band before entering the chromatographic layer, thus improving resolution.

Layers with favorable average particle size and particle size distribution for CPLC can be made in any laboratory with commercial “thick-layer” spreading equipment. For self-prepared preparative plates, the stationary phases most often applied are silica, alumina, cellulose, and plaster of Paris. Layers with thicknesses between 0.5 and 2 mm may be

produced from the so-called P-type sorbents. Sorbents designated P+CaSO₄ are suitable for preparing layers up to 10 mm thick. Slurrying the sorbent and drying and activating the preparative layers should be performed in compliance with the manufacturer's instructions; otherwise the layer may be damaged

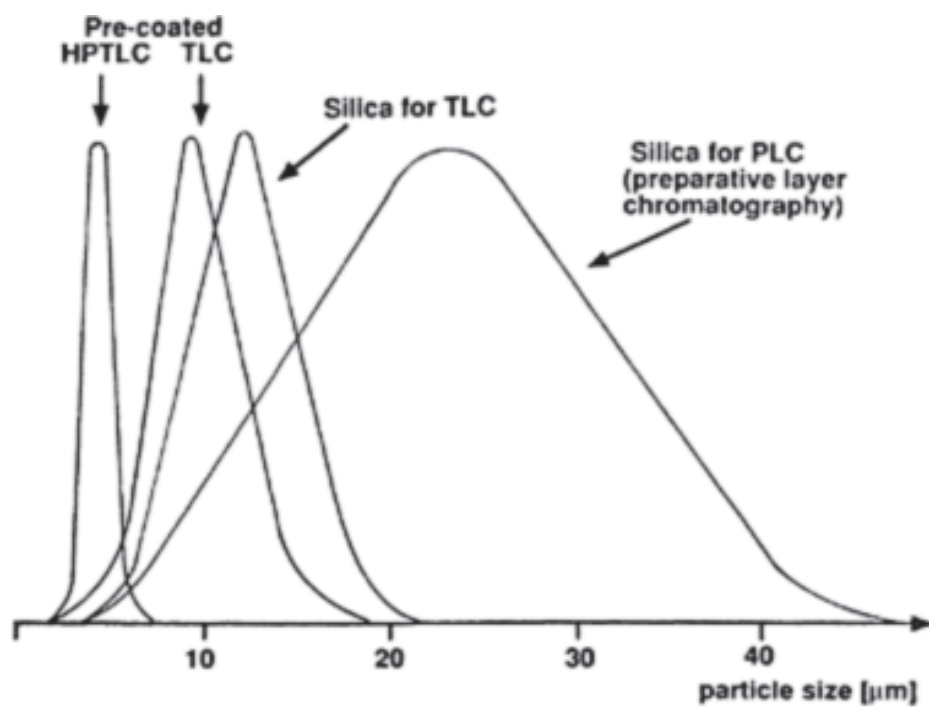


Figure 3 Particle sizes and particle size distributions of silica stationary phases for planar chromatography.

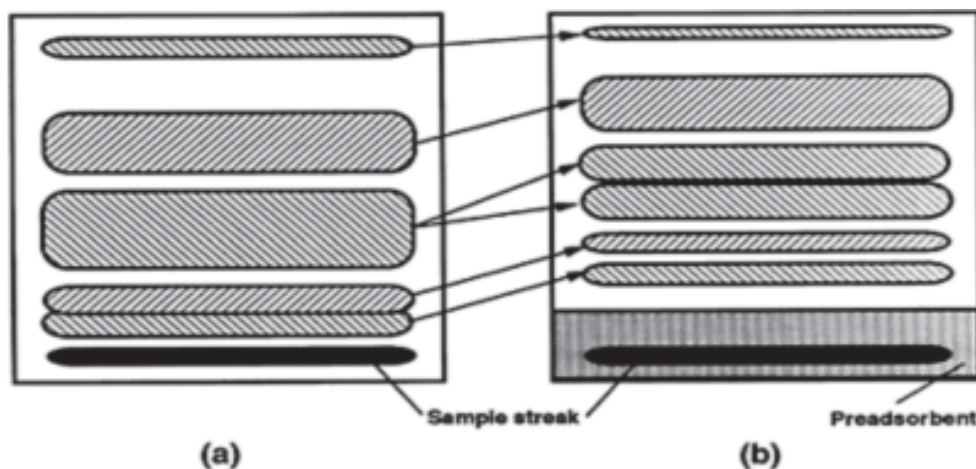


Figure 4 The effect of the concentrating zone in preparative separations, (a) Precoated layer

without concentrating zone; (b)
precoated layer with concentrating
zone.

by pitting, cracking, or flaking. The advantage of preparing one's own plates (up to 10 mm) is that any desired thickness or layer composition (incorporation of salts, buffers, etc.) becomes feasible. It is also possible to produce 20×100 cm plates for the separation of larger amounts of sample, but special equipment is required for the development of these.

2. Sample Amount and Application

Sample application is one of the most important steps of a successful preparative separation (24). The sample should be dissolved in a nonpolar, volatile solvent at such a concentration that the components of the sample are adsorbed throughout the entire thickness of the layer, not only on its surface. Local overloading may distort the applied bands because the rate of dissolution of the components in the mobile phase becomes a limiting factor.

The preferred method of placing a sample on a preparative layer is to apply it as a narrow streak across the plate (2). It is highly desirable to have the streak as straight and as narrow as possible. With practice, skill, and care, it is possible to streak a plate correctly by hand using a syringe. The use of a Teflon tip on the end of the syringe has the advantage that the layer is not mechanically disturbed. The quality of streaking is not as important for precoated preparative layers with a concentrating zone, because the sample is applied to a practically inert zone. Nevertheless, better separation can be achieved if the sample is applied carefully across the plate.

Application of a continuous streak is possible with modern instrumentation (25). Most commercially available sample applicators (e.g., those supplied by Desaga and Camag) may give a sample zone for preparative separations less than 3–4 mm wide. As is generally accepted, the streak is applied across the plate starting 2 cm from both edges. These clear areas are left free partly because of the edge effect, which may cause the motion of the mobile phase to be faster or slower at the edge than across the center of the plate. For CPLC separations of extremely large amounts, the sample may be applied to several plates, which can then be developed concurrently in a large tank.

A modern solid-phase sample application (SPSA) method was presented by Botz et al. (26) that enables regular sample application in the whole cross section of the preparative layer with the advantage of in situ sample concentration and cleanup and an extremely sharp interface leading to the chromatographic layer. With the proposed SPSA device, the sample can be applied to improve the starting situation for a preparative planar chromatographic separation, independent of whether the migration of the mobile phase is achieved by capillary action, as in conventional layer chromatography, or by forced flow, as in OPLC and RPC.

For SPSA, the sample has to be dissolved in a suitable solvent and mixed with about 5–10 times its weight of deactivated sorbent. The sorbent with the regularly adsorbed sample is carefully dried in a rotary evaporator and then introduced into a layer that has to be prepared to accept it. For preparation of the layer, the preparative plate is first fixed

in the application device. In the hard-coated alumina cover plate of the device, two 190×5 mm channels are present (Fig. 5a); one is for the application of 1 g of solid-phase sample (including the inert support) when using layers with 2 mm thickness, and the other for a 0.5 g sample for layers of 1 mm thickness. With the help of these templates, the appropriate channel can be scratched out of the stationary phase (Fig. 5b) with a thin needle, after which the stationary phase is removed from the channel (Fig. 5c).

It must be ensured that the channel in the sorbent has a regularly shaped profile, like that shown in Fig. 5c. Afterward, the prepared sorbent with the adsorbed sample is filled into the channel (Fig. 5d) and pressed evenly with a form (Fig. 5e) to ensure optimal contact between the stationary phase of the plate and the applied sample. No special care is needed in handling these layers; the pressed adsorbent will not fall out when the plates are placed vertically in a chromatographic tank. However, when using RP-18 as the support for the sample, it is advantageous to cover the channel with a suitably thin (3 mm) cover plate (190×5 mm) to eliminate the possibility that a small amount of the applied sample might fall into the mobile phase.

3. Solvent System

Because the particle sizes and size distribution of sorbents for preparative purposes are not optimal and the plates are overloaded with the compounds to be separated, an inferior separation is always achieved on preparative compared to analytical plates. This means that for a successful preparative separation, an optimized solvent system is needed. The volatility of the individual solvents must be considered during the optimization process; otherwise several problems may occur in subsequent steps (e.g., elution of the compound from the stationary phase, evaporation of the solvent). Preparative separation also precludes the use of, e.g., acetic acid as a component of the mobile phase because of the possibility of chemical degradation during concentration of the isolated compounds (27). Multicomponent solvent systems should not be used repeatedly, whereas single solvents can be used repeatedly until they become contaminated.

The solvent system can be selected by performing preliminary analytical TLC experiments. Because development of preparative plates is much slower than analytical development, the chromatographic tank will become saturated within 2 h. During the selection of the solvent system composition in the analytical preliminary assay, the atmosphere of the chromatographic tank must

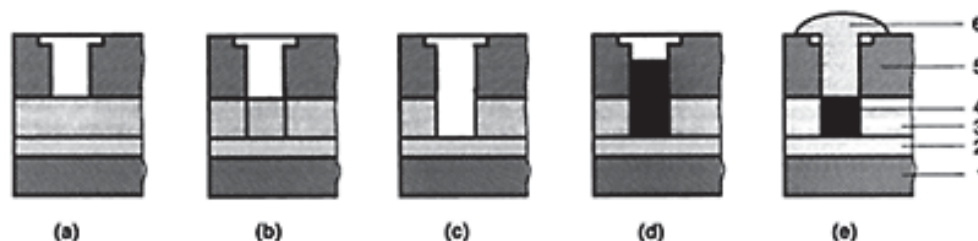


Figure 5 Principle of solid-phase sample application for preparative separation, (a) The preparative

chromatoplate is put in the SPSA device; (b) a profile is scratched out from the stationary phase; (c) the sorbent is removed from the channel; (d) the channel is filled with the prepared deactivated sorbent; (e) the solid-phase sample is pressed to ensure optimal contact between the stationary phase of the plate and the applied sample. 1, Lower part of the device; 2, glass plate; 3, stationary phase; 4, adsorbed sample; 5, upper part of the device; 6, form to press solid sample. (Reproduced from Ref. 26, with permission.)

be kept saturated with the solvent by incorporation of a sheet of filter paper that dips into the solvent. The optimized analytical solvent system may then be transferred unchanged to preparative separations using saturated chromatographic chambers.

Recently, Siouffi and Abbou (28) summarized the most important possibilities for solvent system (mobile-phase) optimization. On the basis of Snyder's system for characterization of solvents (29), the PRISMA optimization system was developed (30). PRISMA enables not only optimization of solvent strength and solvent system selectivity but also the transfer of the optimized solvent system between the different planar chromatographic techniques (31).

4. Chamber Type

One of the most important experimental variables in TLC is the vapor space, because the separation process occurs in a three-phase system of stationary, mobile, and gaseous phases, all of which interact with each other until equilibrium is reached (32, 33). Whereas many chromatographic chambers are available for analytical TLC separations (34), the rectangular glass tank, or N-chamber, with inner dimensions of 21×21×9 cm is the most frequently used for CPLC. These tanks can be used for simultaneous development of two 20×20 cm preparative plates using 50–100 mL of mobile phase. The chamber has to be lined on all four sides with thick filter paper thoroughly soaked with the mobile phase by shaking. The prepared tank should stand for 60–120 min to enable the internal atmosphere to become saturated with mobile-phase vapor. Each plate must lean against a side wall so that the plates do not touch each other. The advantages of saturated tanks are that the α front is much more regular and the separation efficiency is higher for a development distance of 18 cm (35).

5. Development Modes

The ascending mode, in which the mobile phase moves up the plate, is most frequently used for CPLC separations. The angle at which the plate is supported during development affects the rate of development as well as the shape of the spots (35). As the angle of the plate decreases toward the horizontal (horizontal development mode), the flow of the mobile phase increases but so also does spot distortion. An angle of 75° is recommended as optimum for development.

The use of descending development for preparative separations has no significant advantages with regard to resolution, and it is therefore rarely used.

The positive advantages of circular development for the analytical separation of compounds in the lower R_f range is well known (36). A comparison of circular, linear, and anticircular separation is given in Fig. 6. Remarkably, circular development has not been accepted for preparative separations because the mobile-phase velocity would be too slow. The definition of the circular development mode means, however, that the mobile phase migrates radially from the inner part

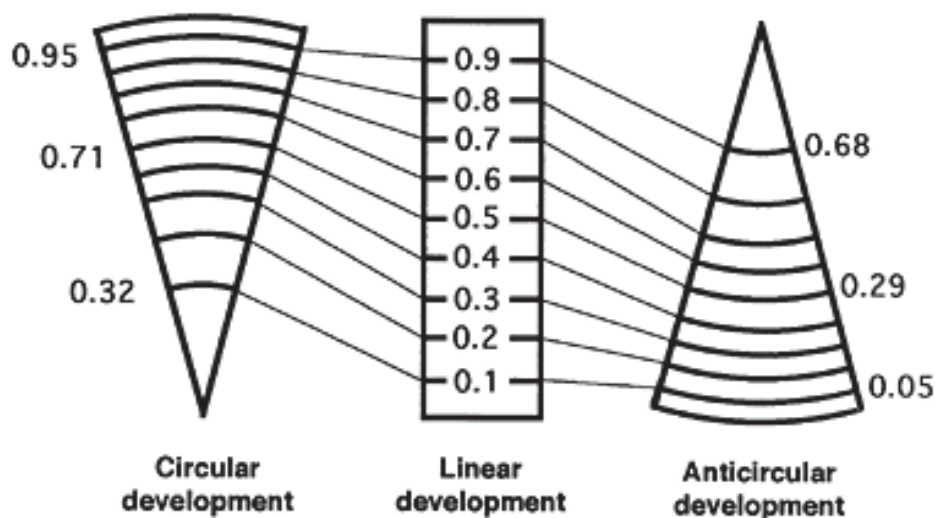


Figure 6 Comparison of circular, linear, and anticircular development.

of the plate to the outside (37). It is therefore possible to start development not directly from the center but from a 2–3 cm radius; i.e., the mobile-phase inlet is not a point but a circle. At the beginning of migration, the mobile phase moves faster in the anticircular direction toward the center than in the circular direction. Once the mobile phase reaches the center of the plate, migration will start in the circular direction with a higher velocity. Because the size of the mobile-phase inlet and the velocity of the mobile phase are related linearly, a relatively high mobile-phase velocity can be achieved over a separation distance of 7–8 cm.

A schematic drawing of a circular preparative chromatography chamber (38) is shown in Fig. 7. This device enables a suitable mobile-phase velocity to be used in the circular

development mode of classical PLC. A solvent reservoir made of steel and a rubber sealing ring are placed on the layer and fixed by a magnet located below the chromatoplate. To start the separation, adsorbent is scratched from the center of the plate, and the recess produced is filled with mobile phase. The device can be used for different types of chambers. The entry of sample and mobile phase is regular over the entire cross section of the preparative layer, regardless of whether the sample is applied in liquid or solid form. The method and device presented ensure rapid, efficient separation with all the advantages of circular development. The resolution is significantly higher than that obtained from linear development.

The 20×20 cm precoated glass plate (see Fig. 7) is placed in an aluminum holder that is adjustable in the horizontal plane by means of three legs (and can therefore be leveled). A magnet is placed into the holder. With the help of a template, the center of the stationary phase is scratched out to a diameter of 2–3 cm. A suitable elastic sealing ring is placed between the layer and a stainless steel reservoir, which is held firmly in place by the magnetic field. Depending on the chromatographic conditions selected, either an M or an N chamber can be chosen. In the case of the M chamber, the glass cover plate is placed directly on the surface of the chromatoplate.

Using a normal chamber, the cover plate is placed on a 19 cm diameter metal ring, the height of which can be varied between 0.5 and 2 cm depending on the type of chamber applied. To start development, the solvent reservoir is filled with the appropriate mobile phase, the level of which is kept constant by applying a constant hydrostatic pressure by means of a second reservoir. To stop development, the tap of the second reservoir is turned off. Using this device, sample can be applied either as liquid or in the solid phase.

Anticircular development is rarely accepted in analytical TLC for increasing resolution in the higher R_f range. For preparative separations, a special device was presented by Studer and Traitlet (39).

Although the different types of multiple development (40) are also rarely used for preparative purposes, the advantage of the method may be understood. The location of the compounds to be

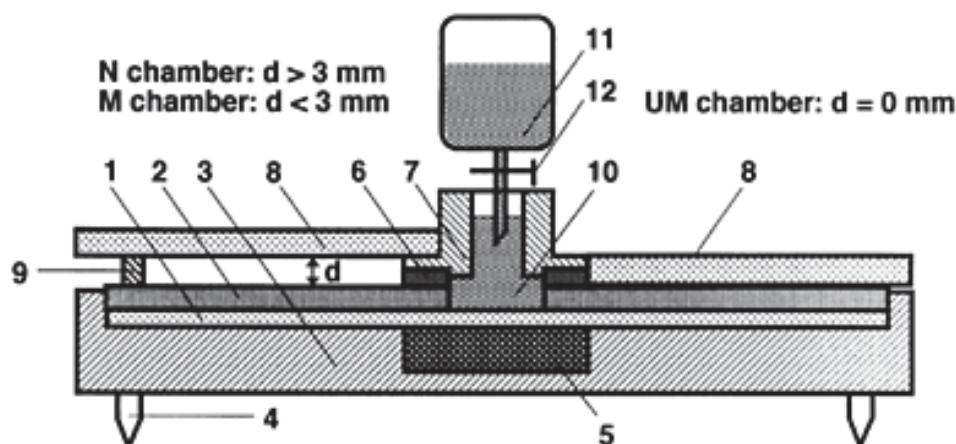


Figure 7 Schematic diagram of a circular preparative chromatographic chamber. UM=ultramicro. 1, Glass

plate; 2, stationary phase; 3, alumina holder; 4, legs; 5, magnet; 6, sealing ring; 7, stainless steel mobile phase reservoir; 8, glass cover plate; 9, metal ring; 10, mobile phase.

separated, and hence the ΔR_f values, can be influenced by the number of developments using the unidimensional multiple development (UMD) technique. UMD is the repeated development of the chromatographic layer over the same development distance with a mobile phase of constant composition. Perry et al. (41) reported that using UMD, if the R_f after the first development is R_f , then the R_f values of the multiply developed solute can be predicted by use of the equation

$$R_f^n = 1 - (1 - R_f)^n$$

where n is the number of developments. In this way, the R_f values, and thus the ΔR_f values also, can be determined for all of the compounds of interest.

Szabady et al. (42) reported that using incremental multiple development (IMD), a variation of UMD in which rechromatography is performed over increasing development distances with the same mobile-phase composition, the R_f value can also be calculated. Using IMD with linearly increasing distances, the following formula for prediction of R_{fx} values can be used:

$$R_{fx} = 1 - (1 - R_f) \frac{1 - (1 - R_f)^n}{nR_f}$$

Multiple development can also be performed in the same direction and with the same development distance using different mobile phases [gradient multiple development (GMD)]. It is also possible to develop preparative plates, especially 0.5 mm layers, with the bivariate multiple development (BMD) technique, in which development distance and mobile-phase composition are varied simultaneously during successive chromatographic runs (40).

6. Flow Rate

The mobile-phase velocity is the variable that, in principle, cannot be influenced by the chromatographer who is relying on capillary action. The only possibility of exerting any influence is to avoid solvents of high viscosity during mobile-phase optimization. Saturated chromatographic systems also have the advantage that development is much faster, which means that the mobile-phase velocity is higher. A special possibility of increasing the local mobile-phase velocity is provided either by the taper plate (see Sec. II.C.1) or by the circular preparative chromatographic chamber (see Sec. II.A.5).

7. Separation Distance

The separation distance depends on the dimensions of the plate, the development mode, and the particle size and size distribution. The last property cannot be influenced by the

user of precoated plates. Because capillary action is effective only for plates up to 20 cm in length, the maximum separation distance is 18 cm. For anticircular development, the separation distance is 9 cm; using the circular mode for special separation problems, this distance is 7–8 cm. Despite the short separation distance, the correct selection of mobile phase and development mode may give high resolution.

8. Temperature

In saturated chromatography chambers, the temperature does not exert a great influence on preparative separations. However, it is important to note the temperature if separations are to be repeated reproducibly (33).

B. Location and Removal of the Separated Compounds

After the preparative plate has been developed and the mobile phase has evaporated, the separated bands must be located and the desired compounds removed from the plate. Many methods are available for the location or detection of the separated components. If the separated compounds are colored, their position on the layer can be located under white light. If the desired compounds are fluorescent or become so after postchromatographic derivatization, their position on the layer can be determined under ultraviolet light. Conversely, a PLC plate containing a fluorescent material will indicate the separated compounds as dark zones on a bright background when examined under 254 and/or 365 nm UV light if they absorb light of these wavelengths. Precoated plates containing 254 or 365 nm fluorescent indicators should be used if possible, because they provide a mode of detection that is generally nondestructive (43).

If the compounds themselves are not visible or fluorescent, they can be detected by use of iodine vapor in a closed chamber. This technique can be used for visualization of substances from a large variety of chemical classes as dark or light brownish zones on a tan background. In most instances, the iodine can be evaporated after the compound spots have been marked, leaving the desired compounds chemically unchanged.

If destructive reagents (e.g., vanillin–sulfuric acid) are necessary for detection of the separated compounds, a vertical channel must be scraped in the layer about 0.5 cm from the edge of the streak. After covering the major portion of the layer with a suitable glass plate, the part of the layer that is not covered is sprayed and thus serves as a guide area. If heating is necessary for detection, the sprayed portion of the plate must be detached from the rest by use of a glass cutter, because heating the developed preparative plates can lead to decomposition of the compounds of interest.

After location of the desired compound, the subsequent steps are mechanical removal of the adsorbent zone, extraction of the compound from the stationary phase with a suitable solvent, separation from the residual adsorbent, and concentration of the solvent. The areas of the layer containing the compounds of interest are scraped off cleanly down to the glass with a suitable scraper or spatula.

Several commercially available inexpensive devices and individually developed methods exist for extracting the compounds from the stationary phase. Vacuum collectors are particularly recommended. This method is not very practicable for sensitive substances because the stationary phase containing the desired compounds is in constant

contact with a stream of air, and there is some risk of oxidation. In our experience, one of the best methods is to put the adsorbent with the compound to be extracted in an empty receptacle containing a sintered glass filter to retain the adsorbent, then extract the compound with a suitable solvent with the help of vacuum.

The substance should be highly soluble in the solvent or solvent mixtures used to extract a compound. The solvent used for adsorbents such as silica gel should also be as polar as possible but free from water and methanol. If water is the chosen solvent, it should be removed by lyophilization. Because silica is significantly soluble in methanol, and some of its common impurities are also soluble, the use of methanol as solvent should be avoided. Chloroform (the safer methylene chloride) is widely used for apolar substances, and ethanol or acetone for polar compounds. The mobile phase used for the separation is highly recommended for extraction also. As a rule of thumb, the volume of solvent (V_{solvent}) required when the chromatographic mobile phase is chosen for extraction is (44)

$$V_{\text{solvent}} = 10 \times (1.0 - R_f) V_{\text{scraped}}$$

It should be noted that the longer the substance is in contact with the adsorbent, the more likely it is that decomposition will occur. Once the solution of the compound to be isolated is obtained (free from adsorbent), the extract must be evaporated to dryness. The evaporation temperature should be as low as possible to avoid chemical decomposition.

C. Special Techniques

1. Gradient of Layer Thickness

A common problem in CPLC is the loss of resolution compared with analytical TLC. Two reasons for this are the wide range of particle size distribution of the stationary phase and the layer thickness. Use of the Uniplate-T™ taper plate (45) provides improved resolution; spot elongation and overlapping are greatly reduced as a result of the gradient effect of layer thickness. This plate has a wedge-shaped layer; it is thin (0.3 mm) at the bottom and thick (1.7 mm) at the top. The preadsorbent part of the layer has a thickness of 0.7 mm. A schematic drawing of this plate is shown in Fig. 8. The improved performance of the taper plate is similar to the improved resolution in the lower R_f range that results from the use of circular TLC.

The cross-sectional area traversed by the mobile-phase front increases during development. The cross-sectional flow per unit stationary phase area is therefore always highest at the bottom

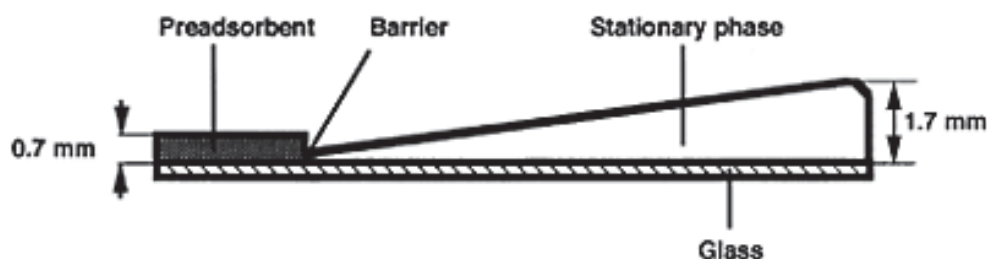


Figure 8 Schematic diagram of the Uniplate-T™ taper plate.

of the layer, decreasing toward the mobile-phase front. As a result, the lower portion of a spot moves faster than the top portion, keeping each component focused in a narrow band. Band broadening is significantly reduced, especially for compounds with higher R_f values. Compounds with lower R_f values are subject to greater mobile-phase velocity relative to higher R_f compounds than on conventional plates. This is because of the increase in solvent front size with migration distance. Because of this, the distance between bands at lower R_f values is increased, providing better separations.

A theoretical separation on a taper plate compared with a conventional precoated plate with preadsorbent is depicted in Fig. 9. The improved resolution that results from the higher local mobile-phase velocity is a clear recommendation for wider use of a layer thickness gradient.

2. Sequential Technique

The sequential technique for CPLC is a means of improving resolution and reducing separation time by supplying mobile phase to different regions of the plate at different times. The principle of the technique is based on the fact that mobile-phase velocity is much higher at the beginning of the separation than later. After a first separation the layer is dried, and the mobile-phase applicator is placed between two separated zones and used to introduce either the same or a different mobile phase. The supply of mobile phase may be stopped at any time in order to transfer it directly to the region of the plate containing the compound zones to be separated. As a result, separation always occurs at the highest initial mobile-phase velocity, which substantially shortens the analysis time. The sequential technique for preparative separations can be performed with the

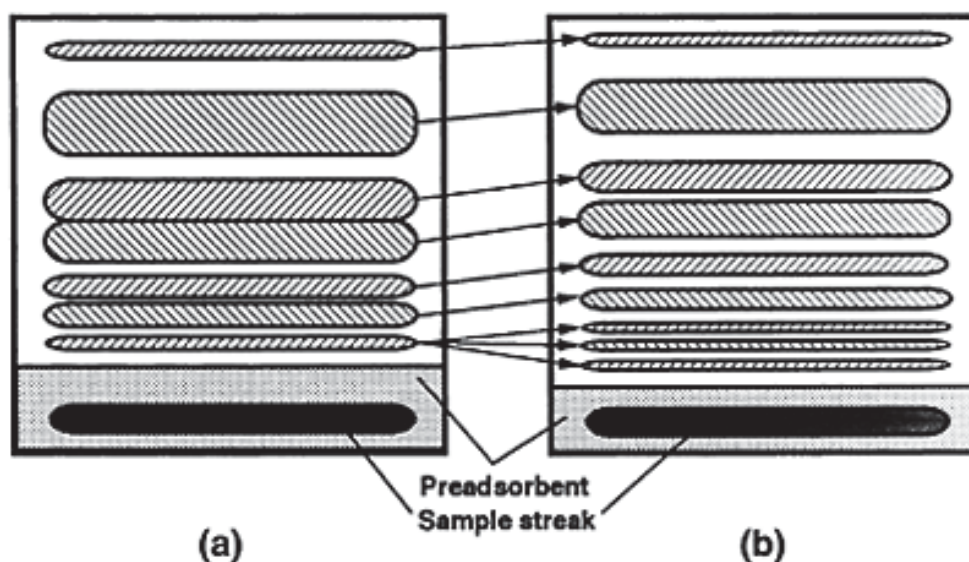


Figure 9 Comparison of separations on conventional and taper plates, (a) Preparative plate with concentrating zone; (b) Uniplate-T taper plate.

Mobil- R_f chamber developed by Buncak (46, 47). The method can be used with success for zone concentration of the applied sample, as demonstrated in Fig. 10, where X is a solvent with high solvent strength (e.g., methanol) and Y is the optimized solvent system for the separation process.

D. Applicability of CPLC

Classical preparative layer chromatography may be used for preparative separation and isolation of substances in amounts ranging between 10 and 1000 mg, depending on the separation problem and the number of compounds to be separated. Generally, it is used for the separation of two to five compounds in quantities of less than 150 mg. Especially good separations can be achieved if the ΔR_f values in the analytical TLC experiments exceed 0.1. CPLC is equally applicable to the separation of synthetic polymers (e.g., 48), for natural products from tissue cultures (e.g., 49) or from various plant materials (e.g., 50, 51), for metabolites from biological fluids (e.g., 52), or for differentiation of the chemical configuration of synthetic and natural products (e.g., 53). Although CPLC is widely used as an economical routine method for the isolation of 10–150 mg of pure compounds, few papers have been published about this technique in recent years.

III. OVERPRESSURED LAYER CHROMATOGRAPHY

Overpressured layer chromatography (OPLC) developed by Tyihak and coworkers (4–8), is a forced-flow liquid chromatographic technique that combines the advantages of classical TLC, HPTLC, and high-performance liquid chromatography (HPLC) and has

provided many new possibilities in planar chromatography. Although the many advantages of the technique would indicate that it has considerable potential for preparative separations, there have so far been few publications on the subject.

A. Principle of the Method

Overpressured layer chromatography is a planar chromatographic technique in which the vapor phase has been eliminated, the sorbent layer being completely covered with an elastic membrane under external pressure. After the mobile phase has been introduced, by means of an appropriate dispenser in addition to capillary action, it migrates through the layer (54) as a result of the "cushion system" at overpressure. Elimination of the open system enables separation to be performed in a closed system under controllable conditions. Thus, the chromatographic layer becomes a "planar column." The absence of any vapor space must be considered in the optimization of the solvent system. For more details about the method see Chapter 7.

B. Description of the Instrumentation

Earlier OPLC separations could be performed on two conventional Chrompres chambers, the Chrompres 10 and Chrompres 25 (Labor Instrument Works, Budapest, Hungary). The advantage

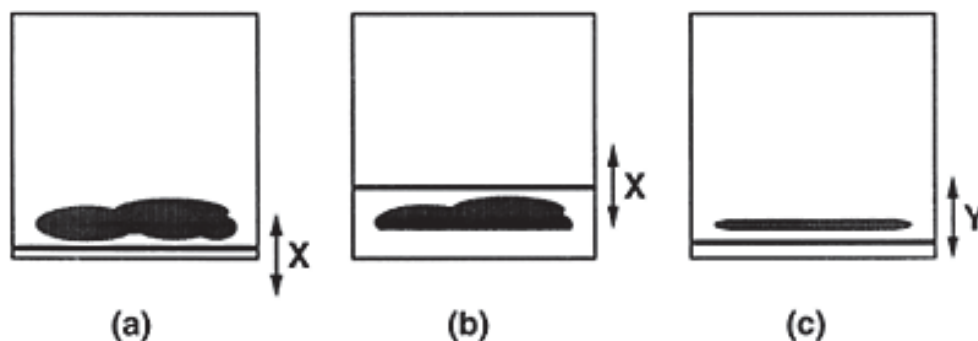


Figure 10 Concentration of the applied sample using the sequential technique.

of the first type lies in the ability to perform preparative on-line separations not only over a separation distance of 20 cm but also over 40 cm on 20×40 cm precoated plates (55). Circular separations were also possible with the Chrompres 10, but only in the off-line mode, which means that the separated compounds could be isolated only by scraping the adsorbent from the plate and subsequently extracting them. The Chrompres 25 could be used with a higher overpressure (25 bar), which enables the use of more viscous mobile phases (56).

Based on experience with conventional chambers, OPLC-NIT (Budapest, Hungary) recently developed an automated personal OPLC system working at 50 bar overpressure. With this instrument, all the conventional operating modes (off-line linear, one- and two-directional, and two-dimensional and on-line linear one-directional separations) can be

exploited by use of an appropriate cassette system (4). With this P-OPLC-50 equipment and analytical chromatoplates, semipreparative separations can be carried out. However, due to the short distance, there is no place for precoated preparative chromatoplates with a layer thickness of 1 or 2 mm. The microprocessor-controlled liquid delivery system includes a two-in-one hydraulic pump and a mobile-phase delivery pump, which enables isocratic and two- and three-step gradient developments.

On-line separations are generally performed in the linear operating mode. This requires specially prepared plates (56) with chamfered edges impregnated with a suitable polymer suspension to prevent solvent leakage at overpressure. To ensure that mobile-phase migration forms a linear front, either a channel is scratched from the layer or a channel is located in the Teflon cover plate of the cassette. A second channel at a distance of 18.3 cm (20×20 cm plate) from the inlet channel enables collection of the eluent.

C. Factors of Principal Importance in OPLC

1. Stationary Phase

It is not only the quality, particle size, and layer thickness that are important in OPLC separations. Because of the high overpressure applied (10–25 bar), the mechanical stability is also important. Zogg et al. (57) tried to prepare their own plates from TLC silica gel GF₂₅₄ (Merck, Darmstadt, FRG) of average particle size 15 μm . However, the layers were not sufficiently compact for use in OPLC separations; in particular, the layers crumbled around the channels when pressure was applied to the chamber. Although particle size could be one of the most important variables in preparative OPLC, lack of knowledge of how to prepare plates with the required mechanical stability has precluded the use of 15 μm particles.

Appropriate mechanical layer stability could not be achieved with 25 μm particles either. At present, only commercially available precoated plates can be used for OPLC separations. These have an average particle size of 25 μm and a broad particle size distribution (5–40 μm). The results show that higher resolution can always be achieved by use of a thinner layer (<1 mm). The production of preparative plates with a smaller particle size and narrow particle size distribution is necessary for full utilization of the potential of preparative OPLC.

2. Sample Amount and Application

For OPLC separations, the sample can be applied either to a dry layer (off-line sample application) or to a stationary phase already equilibrated with the mobile phase [on-line or on-plate (58) sample application].

To avoid the time-consuming and tedious procedure of streaking sample onto the plate prior to development, the influence of on-line sample application was tested (57) on plates with and without concentrating zones. For preparative plates without a concentrating zone and on-line sample application, the separation time increased as a result of an increase of approximately 0.5 cm in the separation distance. For plates with concentrating zones, the effect of the reduced separation distance (2.5–3 cm) was overcome by the efficiency of the concentrating zone. For these reasons, preparative

separations on plates with concentrating zones gave practically the same resolution in a shorter separation time.

Experience shows that the mode of sample application has no significant influence on the resolution of the compounds to be separated, irrespective of the type of plate used. It could be observed (56) that good channel preparation and plate impregnation are very important for on-line sample application.

The solid-phase sample application mode can easily be used for linear OPLC separations (26). The prepared plate is placed in the OPLC chamber horizontally, without the cover plate, and the separation can be started with a relatively high inlet pressure. Note that when using OPLC, the channel has to be completely filled, otherwise part of the mobile phase can overflow into the surface of the sample, which can distort the separation process. If the channel is filled completely, any possible lack of correct contact between the stationary phase and the sample containing the inert support has, due to the forced flow, no effect on the efficiency of the separation.

3. Mobile Phase

The optimized analytical TLC mobile phase obtained in an unsaturated chromatographic chamber can generally be transferred from analytical to preparative OPLC without modification (31, 59). To eliminate the adsorbed air and/or gas in and on the stationary phase, a prerun has to be performed after sample application and closure of the chromatographic chamber (60). The prerun must be performed with a solvent in which the substance zones to be separated do not migrate. Thus, hexane may be used for nonpolar compounds. An appropriate solvent miscible with the mobile phase must be used for polar compounds; such a solvent must be selected during mobile-phase optimization (31). After a prerun to drive all bubbles from the layer, the separation can be started with the optimized mobile phase.

In some circumstances, the solvent strength can be reduced slightly because of the larger particle size and the wide particle size distribution of the precoated preparative plates. This reduction in solvent strength leads to increased separation time. Because the drop in solvent strength also influences the resolution between consecutively eluted compounds, such a reduction must always be tested by use of analytical OPLC.

4. Chamber Type

Overpressured layer chromatography is one of the planar chromatographic methods that is devoid of any vapor space both theoretically and in practice; i.e., the OPLC chamber is completely unsaturated. This must be considered during mobile-phase optimization and also in connection with the disturbing zone (60), a specific feature of the elimination of the vapor phase. The negative effect of the latter can be eliminated by a suitable prerun, as mentioned above.

5. Development Mode

Two basic operating modes exist in preparative OPLC: linear and circular for on-line and off-line separations, respectively (61). For linear separations, impregnation of the plate is

always necessary. In addition, the separation must be started with a suitable mobile-phase inlet pressure; otherwise the front of the mobile phase cannot migrate regularly (61).

The circular separation mode, in the off-line operation mode, has the advantage that no preparation of the layer is necessary, and excellent separation can be achieved in the lower R_f range (60), as can be seen in Fig. 11a. No prerun is necessary with lower mobile-phase velocities because of the reduced effect of the disturbing zone. Especially high resolution can be achieved when the point of sample application is directly below the mobile-phase inlet, i.e., when the sample is applied in the exact center of the plate. The separation distance can be increased to 18 cm with special preparation of the plate, as is demonstrated in Fig. 11b.

Conventional anticircular separation cannot be performed because of the large perimeter (~60 cm on a 20×20 cm plate) required for introduction of the mobile phase. Regular distribution of the mobile phase by means of one or two inlet valves is impossible because of the decreasing mobile-phase inlet pressure. After suitable preparation of the plate by scraping a segment from the layer and sealing with polymer suspension, circular and anticircular off-line separations (see Figs. 11b and 11c) can be performed over a separation distance of 18 cm on a 20×20 cm preparative plate. Both development modes can be used for on-line operations.

6. Flow Rate

Whereas the aim is to work at the optimum mobile-phase velocity in OPLC, Zogg et al. (57), using preparative silica layers, found that the influence of mobile-phase velocity was not signif-

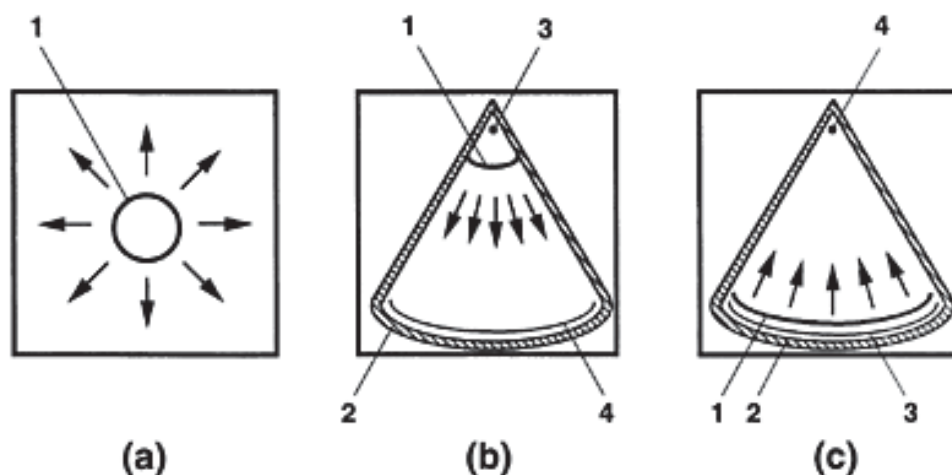


Figure 11 Preparation of chromatographic plates for off-line preparative OPLC. (a) Circular separation with 8 cm development distance; (b) circular separation with 18 cm development distance; (c)

anticircular separation with 18 cm development distance. 1, Sample; 2, polymer suspension; 3, mobile phase inlet; 4, effluent outlet.

icant within the usual working range (3–6 mL/min on 2 mm layers), but that at lower mobile-phase velocities the separation time increased dramatically. The upper limit of the applicable flow rate depends on the viscosity of the mobile phase and the overpressure applied. At higher flow rates (~10 mL/min), the counterpressure increases up to the applied overpressure, and the mobile phase can then flow over the surface of the layer. The inlet and outlet channels are often destroyed by the use of high mobile-phase velocities; this can result in a loss of resolution between the separated compounds during the collection of the eluted substances.

7. Separation Distance

With commercially available OPLC instruments and precoated preparative plates, a maximum 18 cm separation distance can be used for all three development modes. Better resolution would theoretically be expected over a longer separation distance. Working with Chrompres 10 and 25 chambers, Zogg et al. (57) showed that within the usual working range of flow rate and a 36 cm separation distance, the resolution is practically the same as at 18 cm because of the great diffusion of the substances to be separated. At low mobile-phase velocities (<3 mL/min), the separation time and diffusion increase dramatically. These operating conditions are not, therefore, of use in practice for this amount of sample (<100 mg). For full exploitation of the advantages of the linear development mode over 36 cm using 20×40 cm plates, a chamber enabling application of higher pressure would be required for highly efficient preparative separations.

On a 20×20 cm chromatoplate in the circular development mode, the maximum separation distance is 10 cm if the separation is started at the center of the plate and the sample is applied exactly at the center of the layer. With a specially prepared chromatoplate, an 18 cm separation distance can be achieved (see Figs, 11b and 11c) off-line as well as for on-line operations in the circular and anticircular development modes.

8. Temperature

As has been shown for analytical separations, the temperature used for isothermal OPLC separations has no significant influence on the separation. It is important to note the temperature if separations are to be repeated reproducibly. A dramatic change in the resolution can be achieved by using temperature gradients (62). These new possibilities have not yet been implemented in commercially available equipment.

D. Scale-Up

Analytical TLC separation of the sample under investigation can be performed on TLC plates in unsaturated chambers with the optimized mobile phase. For the scale-up

procedure, sample amounts of 2–10 mg can be tested on 20×20 cm analytical TLC plates with a layer thickness of 0.25 mm. The greatest amount of sample that gives a satisfactory analytical separation must be determined.

Because 2 mm precoated preparative plates are eight times as thick as the equivalent analytical plates, and because analytical separations are performed off-line whereas preparative separations are on-line (so all compounds have the same separation distance), a factor of 10 can be used to determine the amount of sample applicable for preparative separations. Of the various possible means of starting a preparative OPLC separation, to eliminate the negative effect of adsorbed air and gas the generally accepted method is, as in analytical OPLC, to start the separation with a hexane-equilibrated layer (59).

E. Reproducibility

Because of the great difference in quality (average particle size and particle size distribution) of precoated preparative plates from different batches, the resolution can change under the same separation conditions. Experience shows (57) that working in the fully on-line mode and at lower flow rates (<3 mL/min), five to eight separations can be performed on the same plate without loss of resolution. Between consecutive separations, the plate must be washed with a solvent of high strength and then reconditioned for 1 h with a mobile-phase velocity of approximately 3 mL/min without opening the chamber. The solvent used for reconditioning is the same as that used for the prerun. Depending on the quality of the plates, the inlet and outlet channels may be destroyed after a few hours or days. When this happens, the counterpressure will increase, and a freshly prepared plate must be used for the next separation.

F. Special Techniques

1. Mobile Phase Gradient

The advantages of a step gradient in analytical OPLC separations were demonstrated by Vajda et al. (63). The efficiency of such a step gradient for the preparative separation of secoiridoid glycosides from a plant extract is seen in Fig. 12.

2. Fully On-Line Operating Mode

Mincsovcics and Tyihak (64) summarized the possible combinations of off-line and on-line OPLC. The fastest separations can be obtained in the fully on-line mode (on-line sample application and on-line detection) using chromatoplates with a concentrating zone. This operating mode is also the simplest and most economical method of preparative OPLC, because, after cleaning and re-equilibration, the same plate can be used several times without loss of resolution. Zogg et al. (65) demonstrated that on the basis of experience gained in the HPLC separations, fully on-line OPLC separations could be carried out with the same mobile-phase composition. The results showed that practically the same resolution and peak order as in HPLC could be achieved, but over a longer separation time.

3. Micropreparative Separation

Because of the forced flow used for separation of samples up to 10 mg, excellent resolution can be achieved by using analytical HPTLC plates for OPLC. In the off-line mode, the improved separation results from the use of the optimum mobile-phase velocity. When the on-line mode is used, all the compounds also migrate over the entire separation distance (66). This results in better separation in the lower R_f range. Oroszlán et al. (67) reported the successful micropreparative separation of cannabinoids.

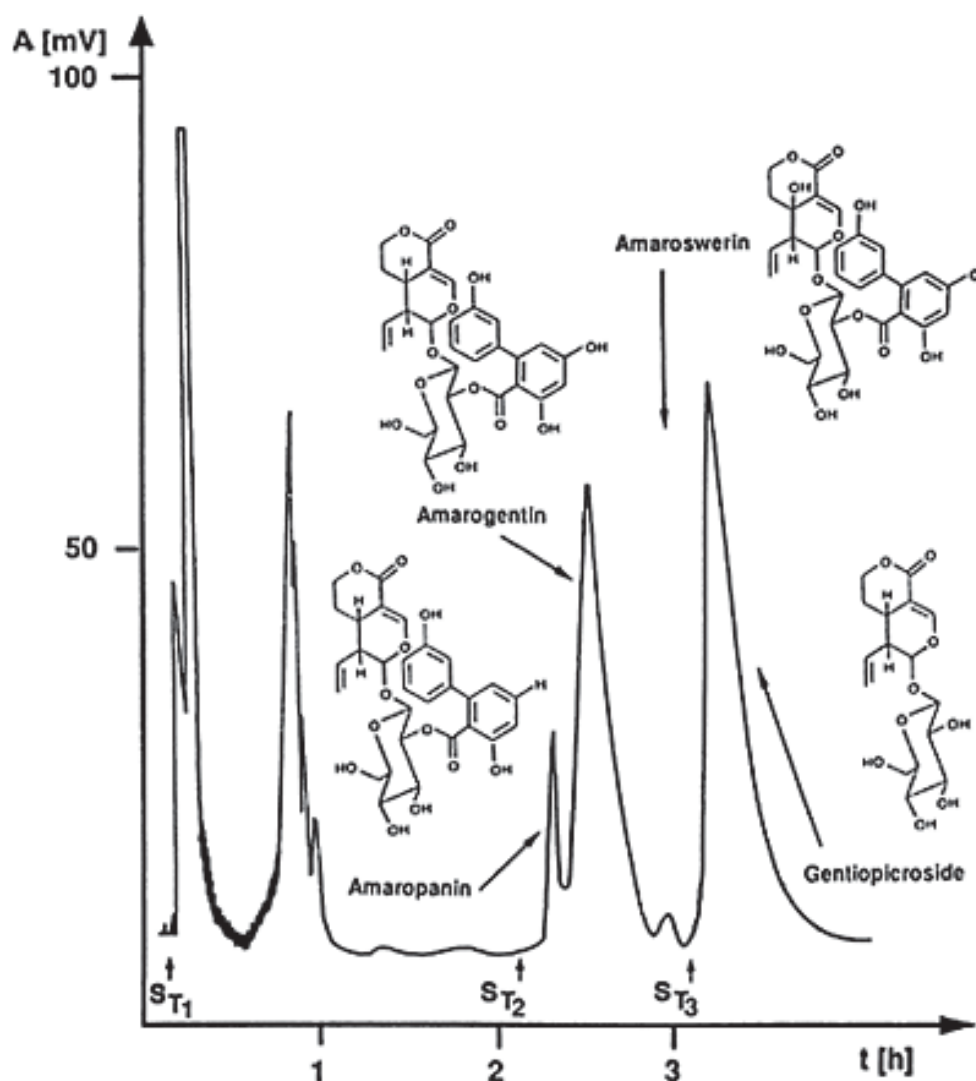


Figure 12 On-line OPLC separation of secoiridoid glycosides using a solvent strength step gradient. Conditions: Stationary phase, PSC precoated silica 60 F₂₅₄; layer thickness, 2 mm; separation distance, 36 cm; flow rate, 5.7 mL/min; cushion pressure, 10 bar;

on-line detection, UV 254 nm. Mobile
phase: Methanol–chloroform–
tetrahydrofuran–*n*-hexane
 $S_{T_1} = 1.86$ (12.2:15.1:15.5:57.2); $S_{T_2} = 2.58$ (16.9:
20.9:21.5:40.7); $S_{T_3} = 3.30$ (21.6:26.8:27.5:24.1).

4. Multilayer Separation

Mincsovcics et al. (68) found that OPLC was suitable for the development of several chromatoplates simultaneously if the plates were specially prepared. With this multilayer technique, a large sample can be separated during a single chromatographic run. In this version, using the same type of stationary phase and chromatoplates of the same size, the mobile-phase velocity is identical on all the plates. Development can therefore be performed simultaneously on several chromatoplates in the same chromatographic run. Figure 13 demonstrates the preparation of chromatoplates for off-line linear one- and two-directional, as well as circular, multilayer OPLC separations.

G. Applicability of OPLC

Experience shows that preparative on-line OPLC can be used for the separation of two to seven compounds. Major advantages of this technique are that all the separated compounds migrate over the entire length of the stationary phase and that the separation distance is longer than in CPLC, especially for compounds of lower R_f . For these substances, the resolution is significantly greater than that obtained by CPLC. Sample sizes for OPLC separation may range between 50 and 300 mg, depending on the separation problem. Especially good separations can be achieved in the linear development mode on precoated preparative plates with a concentrating zone when the ΔR_f values obtained by analytical OPLC on TLC plates are greater than 0.1.

The applicability of this method has been summarized elsewhere for various types of naturally occurring compounds (e.g., 4, 55, 56, 58, 66).

IV. ROTATION PLANAR CHROMATOGRAPHY

The first forced-flow planar liquid chromatographic technique was achieved with centrifugal force (9). The first apparatus, called the Chromatofuge, for the separation of substance groups on a 100 mg scale was introduced by Hopf (69). The main parts of this instrument were a special perforated cylinder filled with support material and a central tube down which sample and mobile phase were introduced. In this system, the radial forced-flow migration of the mobile phase was solved by rotation around the axis of the basket. Heftmann et al. (70) modified this apparatus, making it more suitable for preparative separations. The parallel development of TLC and centrifugal techniques finally led to centrifugal layer chromatography, especially after the introduction of two commercial instruments, the Chromatotron (Harrison Research, Palo Alto, CA, USA) and the centrifugal preparative chromatograph CLC-5 (Hitachi, Tokyo, Japan) (2).

The initials of centrifugal layer chromatography (CLC) have mainly been used for column liquid chromatography, so it was necessary to establish a new nomenclature in which one of the most important effects in planar chromatography, the role of the vapor space (9), is also considered. The term rotation planar chromatography (RPC) was introduced on the basis of Zink's suggestion of a rotating disk (71). Irrespective of the type and quality of the stationary phase, this term embraces not only different on-line preparative, but also analytical and off-line micropreparative, forced-flow chromatographic techniques, where the mobile phase migrates mainly by the action of centrifugal force but also by capillary action.

The following text summarizes the principles of the various preparative RPC methods and describes the features of four instruments, the Chromatotron, the CLC-5, the Rotachrom®, and the Extrachrom®.

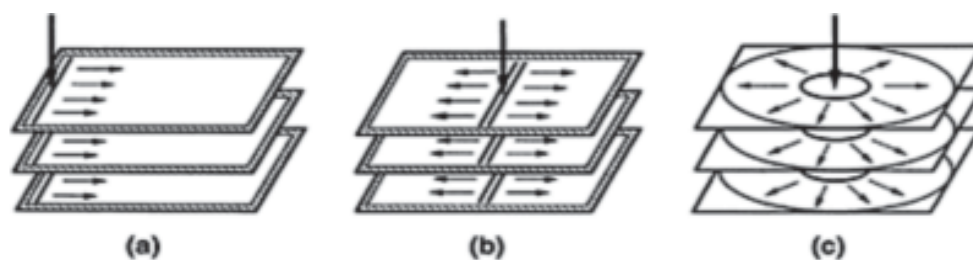


Figure 13 Preparation of chromatoplates for multilayer OPLC separations (a) Linear development; (b) linear, bidirectional development; (c) circular development.

A. Principles of the Methods

The term rotation planar separation (RPS) (9) covers a solid-liquid extraction technique, including rotation planar extraction (RPE) and five different on-line preparative methods with different chromatographic properties, as may be seen in Fig. 14. Among the first four RPC methods [normal chamber RPC (N-RPC), microchamber RPC (M-RPC), ultramicrochamber RPC (U-RPC), and column RPC (C-RPC)], the main difference lies in the size of the vapor space, which is an essential criterion in rotation planar chromatography. The circular mode of development is always used for all on-line preparative RPC methods. The sample is applied near the center of the layer, and the mobile phase is forced through the stationary phase from the center to the outside of the round chromatoplate or planar column. The separated compounds are eluted from the layer or planar column as a result of the centrifugal force and collected in a fraction collector.

1. Rotation Planar Extraction

Rotation planar extraction (RPE) is a preparative, on-line, exhaustive, relative countercurrent extraction technique in which the solid phase to be extracted is placed in a

closed circular chamber (column) and the linear flow of the extraction solvent is accelerated by centrifugal force. Owing to the geometric design of the planar column, the mass of the solid phase to be extracted is constant over the cross section. This special geometric design, which can be seen in Fig. 15a, can be described by the function (72)

$$h = \frac{K}{a + br + cr^2}$$

where h is the actual height of the planar column at radius r , r is the radius of the planar column, and a , b , c , and K are constants.

2. N-RPC, M-RPC, and U-RPC

In N-RPC (73), the layer rotates in a stationary chromatographic chamber (Fig. 15b); in M-RPC, which employs a corotating chromatographic chamber, the vapor space is reduced and variable; in U-RPC (74), the layer is placed in the corotating chamber from which the vapor space has been almost eliminated. Figure 15c shows a schematic drawing of preparative M-RPC (9) on a layer approximately 2 mm thick.

The chromatographic layer used in the ultramicrochamber (Fig. 15d) is thicker (4 mm), and the quartz glass cover plate is therefore placed directly on the layer, almost eliminating the vapor space. In N-RPC (according to classical centrifugally accelerated layer chromatography), the quartz glass cover is removed to furnish a standing chromatographic chamber and hence also a larger vapor space.

N-RPC may be used for not only on-line circular development but also off-line anticircular development. In this mode the layer rotates slowly (50–150 rpm), the sample is applied at the outside of the layer near the edge of the stationary phase, and the mobile phase migrates through the stationary phase as a result of capillary action (against centrifugal force) from the outside to

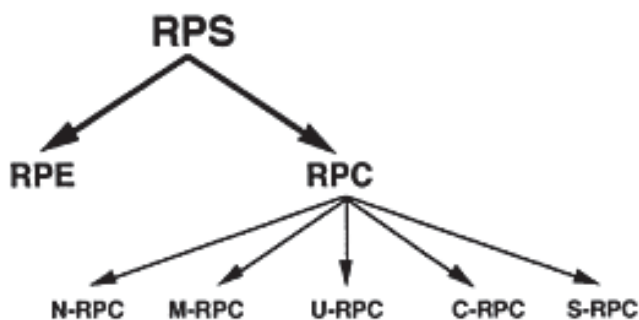


Figure 14 Classification of rotation planar separation (RPS) methods.

RPE=rotation planar extraction;

RPC=rotation planar chromatography;

for other abbreviations, see the text.

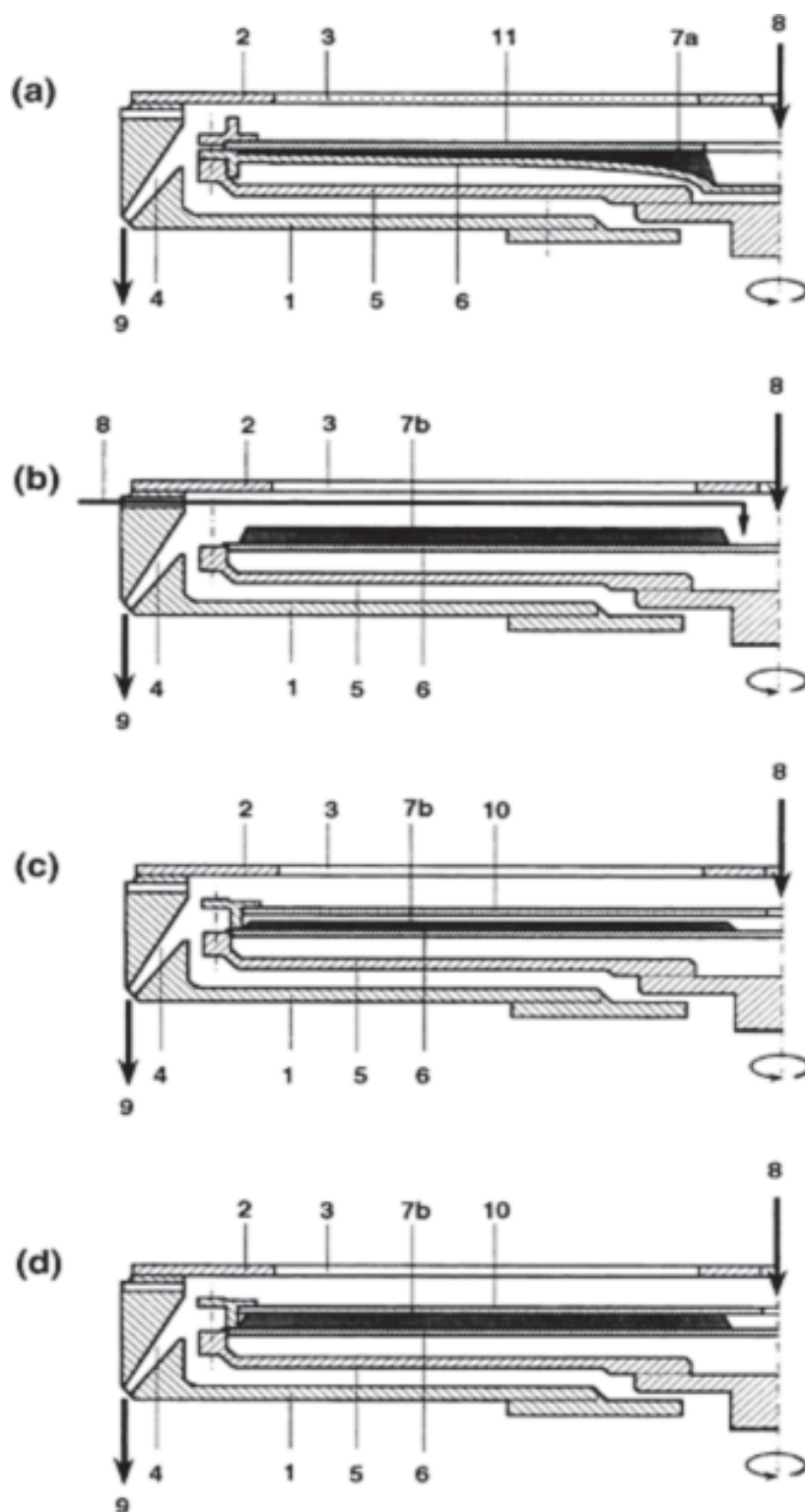


Figure 15 Schematic diagram of chambers for the various preparative RPS methods, (a) RPE and C-RPC; (b) N-RPC; (c) M-RPC; (d) U-RPC. 1,

Lower part of the stationary chamber;
 2, upper part of the stationary chamber;
 3, inspecting window; 4, collector; 5,
 motor shaft with the rotating disk; 6,
 rotor; 7*a*, material for extraction; 7*b*,
 stationary phase; 8, mobile phase inlet;
 9, eluent outlet; 10, quartz glass cover
 plate; 11, stainless steel cover plate.

the center of the round plate. The separated compounds can be removed from the plate when the separation is finished.

3. S-RPC

A special combination of circular and anticircular development modes is possible with the sequential technique (S-RPC), in which the mobile phase can be introduced onto the plate at any desired place and time (9, 55, 75) using the N-chamber configuration (Fig. 15b). In S-RPC, the solvent application system, a sequential solvent delivery device, works by centrifugal force (circular mode) and with the aid of capillary action against the reduced centrifugal force (anticircular mode). Generally, the circular mode is used for the separation and the anticircular mode for pushing the substance zones back to the center with a strong solvent (e.g., methanol). After the plate is dried under nitrogen at a high speed of rotation, the next development with another suitable mobile phase can be started. This combination of the two operating modes makes the separation pathway in S-RPC theoretically unlimited.

4. C-RPC

In column RPC (Fig. 15a), there is no vapor space (76). The stationary phase is placed in the closed circular chamber (column), and the flow is accelerated linearly by centrifugal force. The volume of stationary phase remains constant along the separation distance, hence the name "column" RPC. Because it is a closed system, there is no vapor space, and any stationary phase of fine particle size may be used with or without binder. The rotating planar column has the same special geometric design that was described for RPE. This design eliminates the extreme band broadening (77) that normally occurs in all circular development techniques and thus combines the advantages of planar and column chromatography.

The mode of operation of the CLC-5 cannot unambiguously be classified into one of the five categories mentioned. In one respect, it is a column RPC method because no vapor space exists and the device has to be filled like a column. The volume of stationary phase, on the other hand, is not constant along the separation distance as in a column but increases along the radius as in circular CPLC. The major advantage of this method is the ability to use any type of sorbent, with or without binder, in finer particle sizes.

5. Selection of RPC Methods

The choice of RPC technique and the mode of development depends on the separation problem and the result of the analytical TLC preassay.

Preparative N-RPC is chosen when the mobile phase is an azeotropic mixture or consists predominantly of one component (>80%). This condition is a necessary consequence of the large vapor space of the stationary chamber. Because extensive evaporation of the solvent as a result of rotation has certain negative effects, N-RPC can be employed only if these conditions are met.

The criterion for selection of preparative M-RPC or U-RPC is whether the TLC preassay was performed in a saturated or unsaturated chamber. If the first, preparative M-RPC should be employed; otherwise preparative U-RPC is used (2).

C-RPC is employed when stationary phases with finer particle sizes have to be used without binder. The preassays can be performed either by analytical U-RPC, starting the separation with a dry stationary phase, or by HPLC, starting the separation with an equilibrated system.

S-RPC is chosen when the separation problem cannot be solved with a single mobile phase, but optimized mobile phases are available for the separation of the different compounds. The sequential technique can also be employed for fast elution of previously separated compounds in a small volume of eluent of high solvent strength (9).

B. Features of the Instrumentation

Every apparatus for performing RPC consists of a chamber (or chambers), a device for holding the chromatographic stationary phase during rotational motion, and an arrangement for delivering the mobile phase to the stationary phase. Four instruments are or were produced commercially for preparative RPC: the Chromatotron Model 7924 (Harrison, Palo Alto, CA), the centrifugal preparative liquid chromatograph Model CLC-5 (Hitachi, Tokyo, Japan), the Rotachrom® Model P rotation planar chromatograph (Petazon, Zug, Switzerland), and the Extrachrom® rotation planar separator (RIMP, Budakalász, Hungary).

1. Chromatotron

The Chromatotron Model 7924 consists of an annular chamber inclined at an angle and fixed on a pedestal (55). A flat glass rotor covered with stationary phase is mounted within the chamber by means of a fixing screw. The motor-driven glass disk, 24 cm in diameter, rotates at a constant speed of 750 rpm. The chamber is provided with a circular channel for collection of the eluting compounds. The mobile-phase inlet is located eccentrically on a quartz lid that covers the chromatographic chamber. The solvent outlet is placed at the lowest point of the collection channel. An inlet tube is mounted on the side of the chamber for flushing with nitrogen or other inert gas. An adsorbent layer is produced on the glass rotor by casting a slurry of the adsorbent, followed by drying and then scraping to 1,2, or 4 mm with a rotating scraping tool.

2. CLC-5

The CLC-5 apparatus incorporates a rotating disk column comprising two removable disks 30 cm in diameter (71). The upper disk is made of stainless steel and tempered glass. Four screws are spaced evenly on the outer edge of this disk, and a mobile-phase reservoir is located at its exact center. The lower disk, made of stainless steel, has a stem on its base that is used to hold the disk on a rotor. A removable porous spacer is located between the two disks. On its outer edge are four holes into which the screws from the upper disk fit, thereby holding the system together. Various spacers with thicknesses of 2, 3, 5, and 10 mm are used to vary the amount of packing material that can be packed as slurry between the plates on the separation disk column. The CLC-5 instrument enables continuous variation of the rotation speed from 0 to 1000 rpm. The complete instrument consists of the rotating unit, a UV detector, and an automated fraction collector.

3. Rotachrom

The Rotachrom Model P rotation planar chromatograph consists of four main parts (2, 9): (a) the casing of the instrument with the motor, the electronics, and connections for nitrogen and the eluent outlet; (b) the lower part of the stationary chamber; (c) the collector system with the chamber types; and (d) the upper part of the stationary chamber with the solvent delivery system and integrated UV lamps.

The rotating collector is used for collection of the separated compounds in the various preparative modes and can be adapted as a chromatographic chamber for the various preparative methods. The collector is fixed in the lower part of the stationary chamber. The inside of the collector has a special ellipsoidal shape for eluent collection (55). As a result of the centrifugal force, the eluent collects in the holes at the ends of the larger shaft. From there it flows continuously under nitrogen overpressure through the two tubes inside the collector, against the centrifugal force, via the center and motor shaft to the eluent outlet unit on the right of the instrument.

For preparative N-RPC, S-RPC, M-RPC, and U-RPC separations, the glass rotor supporting the stationary phase layer is fixed at the center of the collector. In N-RPC and S-RPC, the layer rotates with the collector in the instrument; no quartz glass cover is used, so the chromatographic chamber is, in practice, the lower and upper parts of the stationary chamber.

The upper part of the stationary chamber is constructed of thick safety glass; the two UV lamps are integrated beneath the lid. The two solvent delivery devices, which are horizontally and vertically adjustable, are located symmetrically on the right and left sides on the top of the instrument. Vertical adjustment is necessary to accommodate layers of different thicknesses; horizontal adjustment is especially necessary for S-RPC. The delivery needles with Teflon tips lead from the solvent delivery systems to the center of the chamber. The upper part of the stationary chamber can be closed with screws, which are tightened and loosened with a lever.

4. Extrachrom

The Extrachrom is a multifunctional instrument in which two basic separation methods can be carried out: (a) a solid-liquid extraction method, the RPE (72), and (b) off-line analytical, micro-preparative and on-line preparative RPC methods. The construction of the instrument is based on the Rotachrom's chamber types and uses the principle of the collector system of the Chromatotron equipment.

C. Description of Operation

The most important operating steps in RPC are preparation of the chromatographic layer or planar column, application of the sample and introduction of an appropriate amount of the optimized mobile phase.

1. Preparation of the Layer

For preparation of the layer, the glass disk is mounted in a special coating arbor. Adhesive masking tape is then attached around the whole plate. The amounts of stationary phase and binder required are mixed by shaking with an appropriate volume of cold water, and the resulting slurry is poured in a continuous stream onto the glass disk to furnish a nearly uniform layer, which is left to set. After removal of the tape and the coating arbor, the plate is dried and activated in an appropriate manner. The dry plate is subsequently remounted in the coating arbor, a scraping tool is placed on the arbor axis, and the layer is scraped by turning a scraper of the desired size clockwise and applying slight pressure. Scraping is continued until a completely uniform layer is achieved.

2. Packing the Planar Column

Filling of the planar column by centrifugal force is fast and easy. The activated dry material for use as an extraction or stationary phase for chromatography is introduced through the central opening in the top part of the column, and regular packing is achieved by centrifugal force (2). If the planar column is filled with a slurry of stationary phase, the mobile phase may also be added to guarantee an equilibrated system. For both packing methods, the rotor speed must be faster than that required for the chromatographic separation.

3. Sample Application

For RPC separation, the sample can be applied either with a syringe to the glass rotor near the center of the rotating plate or via the mobile-phase system. In both cases, it is preferable to dissolve the sample in a small volume of the mobile phase. If the sample is applied to an equilibrated plate, separation is started immediately after sample application, as in HPLC. The sample may also be applied to a dry plate, in which case it is first dried, and separation is then started with the mobile phase, similar to CPLC.

D. Factors of Principal Importance in RPC

1. Stationary Phase

For N-RPC, M-RPC, U-RPC, and S-RPC separations, the preparative plates must be prepared by casting adsorbent on the plane parallel glass rotors. After the layers have dried, they are shaped by scraping with a special tool. Because these layers rotate at high speed, more binder has to be used than in conventional CPLC; insufficient binder results in layers that are very soft, loose, and powdery on the surface and have a tendency to crack. The slurry has to contain a certain amount of water to ensure regular flow; very liquid slurries will not give a homogeneous layer, whereas a thick slurry will not flow readily.

Selection of stationary phases is therefore limited for these preparative techniques. The only materials that can be used are those for which an additional amount of binder endows the necessary stability and does not impair the separation; these can be silica, kieselguhr, alumina, plaster of Paris (78), and their combinations. In general, silica gel of TLC quality (15 μm average particle size) with gypsum binder is used with 3.5% calcium sulfate hemihydrate as an additional binder. If other types of silica without binder are used, approximately 20% binder must be added. It is recommended that several layers be prepared together and stored in a safe place. They can be activated before use.

Because the planar column is a closed system, any commercially available stationary phase such as silica or modified silica (RP-18, RP-8, amino, cyano) can be used with or without binder. Compared with other preparative planar techniques, a finer particle size material (5–15 μm) can be used; this significantly improves the separation.

2. Sample Amount and Application

Depending on the separation problem and irrespective of the type of chamber used, between 50 mg and 1 g of sample can be applied to a single preparative plate. Because of the smaller volume of stationary phase in the planar column (4 mm layer), the amount of sample is limited to 500 mg.

The SPSA mode can also be used for C-RPC (26). Therefore, the selected stationary phase (e.g., silica of 15 μm average particle size) is filled into the center of the empty planar column at a rotational speed of 1500 rpm. The rotational speed is then increased stepwise to 2000 rpm. If the stationary phase is compressed and the profile at the beginning of the column is appropriate, a certain amount of carefully dried support (e.g., kieselguhr) is filled with the adsorbed sample.

3. Mobile Phase

Selection of the mobile phase depends on the RPC method used; the RPC method can also be selected after TLC preassays. We prefer the PRISMA system for TLC optimization (30, 31).

For separation of nonpolar compounds by N-RPC, the solvent strength of the mobile phase must be reduced by dilution with hexane. For polar compounds, the composition of the solvent system will depend on the volatility of the single solvents used for the mobile

phase. If analytical TLC separation was performed in a saturated chromatographic tank, the microchamber (M-RPC) must be employed for preparative separation. Because U-RPC is performed in an unsaturated chromatographic system, the mobile phase obtained by TLC preassays in unsaturated chambers can be transferred by means of analytical U-RPC to preparative U-RPC or C-RPC without modification; as with these techniques, the separation is started on a dry stationary phase. The mobile phase can also be transferred from HPLC to C-RPC or U-RPC if the separation is started with an equilibrated chromatographic system.

The guidelines for choice of the mobile phase for S-RPC are the same as those for N-RPC. The choice of mobile phase is summarized in Table 1.

4. Chamber Type

The type of chamber used in RPC depends on the analytical preassay and the separation problem. If analytical preassay was performed in an unsaturated chromatographic tank, the ultramicrochamber (U-RPC) should be used; if preassay was performed in a saturated tank, RPC should be performed in the microchamber (M-RPC). Planar column RPC can be used if the analytical separation was performed by HPLC; if the HPLC mobile phase is used, equilibrated planar column chromatography (C-RPC) has the same chromatographic properties as HPLC. Normal chamber RPC (N-RPC) is acceptable for the separation of nonpolar compounds, but it is difficult to use for the separation of polar substances.

As a guideline, it can be stated that the simplest instruments to use are the ultramicrochamber and the planar column, but if the presence of the vapor phase is important for the separation of substance classes, then the microchamber has to be used.

Table 1 Selection of the Mobile Phase in RPC

System	Nonpolar compounds	Polar compounds	RPC method
Unsaturated TLC	Without modification		U-RPC, C-RPC
	Dilution	Change of composition	N-RPC, S-RPC
Saturated TLC	Without modification		M-RPC
HPLC	Without modification		C-RPC, U-RPC

5. Development Mode

The circular development mode is used in preparative N-RPC, M-RPC, and U-RPC. In S-RPC, the circular and anticircular development modes can be combined as often as is required by the separation problem. Although C-RPC appears to be a circular development mode, it is, in effect, a linear development mode because the volume of the stationary phase is constant along the radius.

6. Flow Rate

In RPC, the mobile-phase velocity is influenced primarily by the centrifugal force or speed of rotation; the faster the rotation, the faster the migration of the α front (79). At high speeds of rotation, the function approaches a straight line but will never reach it in the circular development mode. In the anticircular mode, the mobile-phase velocity will increase along the radius by an amount depending on the reduction in surface area. In C-RPC, the mobile-phase velocity is always linear. Note that the flow rate cannot be greater than the amount of mobile phase the layer can absorb; otherwise solvent will flow over the surface of the layer (14).

Because the Chromatotron works at a constant speed of rotation, the flow rate of the mobile phase cannot be influenced in this way. With the Rotachrom or Extrachrom instruments, mobile-phase velocity can be varied by adjusting the speed of rotation; as is apparent from Fig. 16a, the greater the speed of rotation, the faster the migration of the mobile phase (9). The other way to increase the mobile-phase velocity is to increase the diameter of the hole in the center of the stationary phase at a constant speed of rotation, as can be seen in Fig. 16b.

The optimum speed of rotation depends on the separation problem and on what mobile phase is used. The flow rate is limited by the amount of solvent that can be accommodated by the layer without flooding the surface. The greater the amount of solvent applied, the higher the rotation speed must be to keep the mobile phase within the layer.

According to the results of Stahl and Müller (80), the optimum speed of rotation is generally 700 rpm for preparative separation using silica with an average particle size of $25\ \mu\text{m}$. Using the

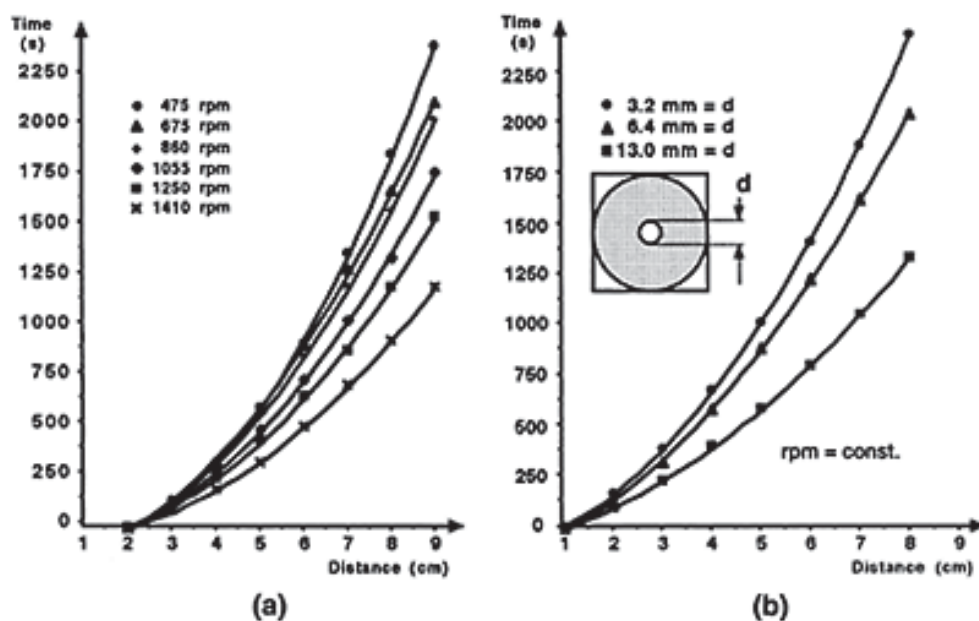


Figure 16 Characteristics of RPC separation, (a) Relationship between

rotational speed and the migration of the α front on a preparative plate, (b) Relationship between the hole in the center of the stationary phase and the migration of the α front on a preparative plate.

planar column for C-RPC, the maximum speed of rotation can be increased, because a smaller ($3\ \mu\text{m}$) average particle size can be used.

7. Separation Distance

For N-RPC, M-RPC, and U-RPC, the separation distance is 8 cm working with the Rotachrom equipment and 10 cm with the Extrachrom instrument. For S-RPC, the separation distance is theoretically unlimited because of the combination of the circular and anticircular development modes. With this technique, the separated compounds are eluted; the unseparated compounds can be pushed back to the center of the plate, and, after drying, a fresh development can be started with another mobile phase. The separation distance using C-RPC is 7.5 cm with the Rotachrom and 10 cm with the Extrachrom instrument (9).

8. Temperature

The temperature of the chromatographic chamber cannot be regulated with the RPC instruments. The chamber temperature of the instruments may be held constant during the separation by thermostatic control. Because of the heat generated by the motor, the chamber must usually be cooled to keep the temperature constant at 20°C .

E. Scale-Up

Because M-RPC and U-RPC can be used not only for on-line preparative separations but also for analytical purposes, direct scale-up is possible for both analytical methods. From TLC separations using unsaturated or saturated chromatographic tanks, the mobile phase can be transferred via analytical U-RPC and M-RPC to preparative U-RPC and M-RPC, respectively (2), if the solvent strength and selectivity are kept constant. For scale-up, the sample can be applied in a circle on a 20×20 cm analytical TLC plate, and the amount of sample is increased stepwise in subsequent separations.

The resulting plates are scanned (off-line) to find the limit at which resolution becomes unsatisfactory. From these experiments, the maximum amount of sample for on-line preparative separation can be predicted, taking the particle size and the volume of the stationary phase into account. In analytical U-RPC and M-RPC, the separation distance is 8 cm and the average particle size $11\ \mu\text{m}$; in preparative U-RPC and M-RPC, the separation distance is increased by 30%, but the particle size is approximately 30% larger. These adverse effects practically cancel each other, so only the layer thickness has to be considered in the scale-up procedure. In our experience, therefore, a factor of 20 is

generally appropriate (74). The flow rate of the mobile phase has to be adapted to preparative separations so that the migration of the *a* front is as fast as in the analytical separation. (See Fig. 17.)

F. Reproducibility

Reproducibility in RPC depends upon the preparation of the layer (planar column), the vapor space, and sample application. If the same amount of stationary phase is used, layer preparation does not exert a significant effect on reproducibility. Production of a highly reproducible planar column requires the use of exactly the same amount of stationary phase under the same conditions of compression.

The second effect that influences reproducibility is the vapor space; the best reproducibility can be achieved using C-RPC and U-RPC. To ensure sufficient reproducibility when working with the M- or N-chambers, the temperature must be kept constant or evaporation of the mobile phase will no longer be under control.

The samples must always be applied in the same amount of the appropriate solvent (preferably the mobile phase) as a fine stream during the rotation of the rotor. The resolution obtained from a thin streak is usually superior to that from a thick streak.

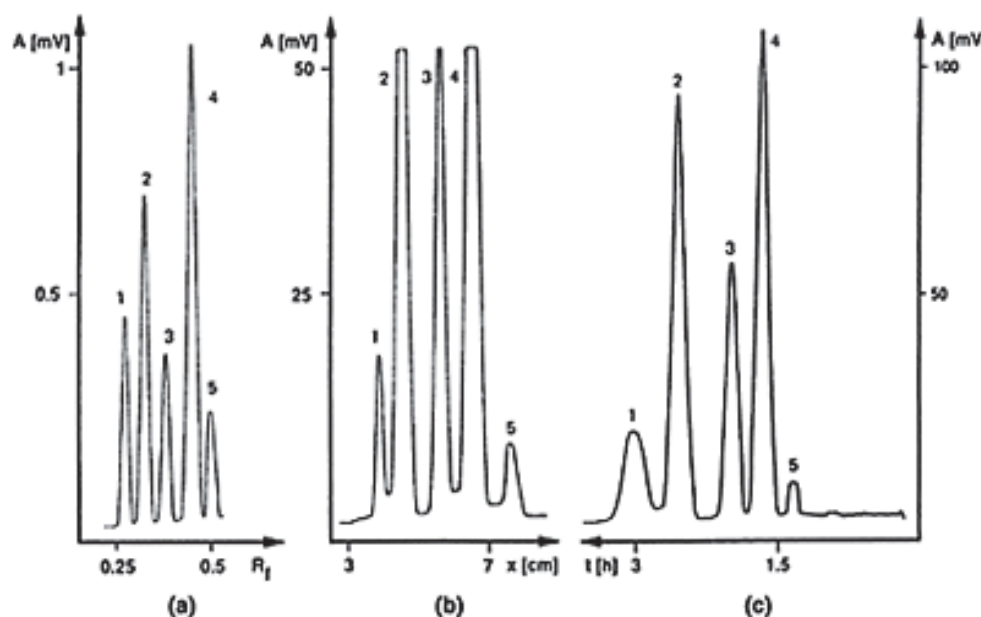


Figure 17 Scale-up steps and on-line U-RPC separation of furocoumarin isomers. (a) Off-line separation of 50 μ g of extract on an HPTLC plate; (b) off-line separation of 8 mg of extract on a TLC plate; (c) on-line separation of 160 mg of extract on a preparative layer. 1, Sphondin; 2, isopimpinellin;

3, bergapten; 4, pimpinellin; 5, isobergapten. Conditions: Stationary phase, TLC and HPTLC precoated silica 60 F₂₅₄; TLC silica 60 GF₂₅₄ for preparative separation; layer thickness for preparative separation, 4 mm; separation distance, 8 cm for analytical and 10 cm for preparative separations; flow rate, 0.17 mL/min for analytical and 3.5 mL/min for preparative separations. Detection: UV 313 nm (off-line) and UV 279 nm (on-line). Mobile phase: *n*-hexane–dichloromethane–chloroform–tetrahydrofuran (72.8:10.8:8.3:8.1).

G. Special Techniques

1. Mobile-Phase Gradient

Gradients similar to those used in OPLC separations can be used with all the commercially available RPC instruments. Rapid change of mobile phase is easily achieved with the Rotachrom and Extrachrom instruments thanks to the two solvent delivery devices.

2. Fully On-Line Operating Mode

The fastest separations can be obtained in the fully on-line mode (on-line sample application on an equilibrated stationary phase and on-line detection), especially when the C-RPC technique is used. This operating mode is also the simplest and most economical method of RPC, because after cleaning and reequilibration, the same stationary phase can be used several times without loss of resolution.

3. Mixed Stationary Phases

A special possibility is the use of mixed sorbents, which is possible with the CLC-5 equipment and C-RPC techniques. The simplest method is to fill most of the planar column with stationary phase A and then the last 1 cm with stationary phase B as a preadsorbent zone (e.g., 14). It is also possible to fill the same column with more than two successive stationary phases in order of increasing or decreasing polarity.

4. Indirect Detection

If the compounds to be separated are not visible or UV-active and the S-RPC technique has to be used for a certain separation problem, an indirect method can be used to detect the substances in situ on the layer (14). A segment is cut from an aluminum-backed analytical TLC plate and immediately pressed for a few seconds against the wetted preparative layer to make a copy of the separation. After drying, the analytical plate can be sprayed with a suitable reagent. With the help of this print, the separated compounds can be located on the preparative plate. An example of an S-RPC separation is presented in Fig. 18; the location of the separated compounds was detected indirectly after each of the 12 operating steps.

H. Applicability of RPC

The application of RPC covers a wide range of substance classes and polarities from synthetic compounds (e.g., 81) to various natural products (e.g., 82). Its use for the latter is summarized by Hostettmann et al. (21, 83), who listed not only the types of naturally occurring compounds

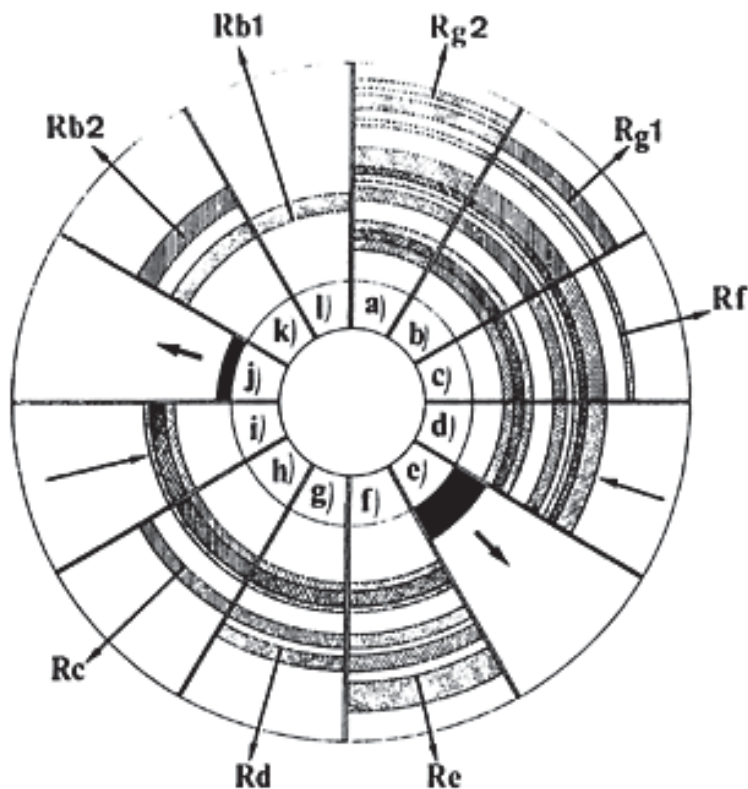


Figure 18 S-RPC separation of ginsenosides with indirect detection, (a) The outer three ginsenosides (Rg2, Rg1, and Rf) could be baseline

separated with eluent A. Using eluent C, the three separated compounds were eluted sequentially, first Rg2, (b) second Rg1, and in step (c), Rf. (d) The remaining ginsenosides were pushed back to the center with eluent C with the help of capillary action at a low rotational speed of 150 rpm. (e) After drying the plate with nitrogen, the separation was continued with eluent A. (f) Using eluent C, Re was eluted from the chromatographic plate, and then, in step (g), Rd and, in step (h), Rc. (i) The two remaining compounds (Rb1 and Rb2) were pushed back to the center of the plate with eluent C. (j) After drying of the plate, compounds Rb1 and Rb2 were separated with eluent B. (k) Both separated compounds were eluted from the plate with eluent C—first Rb2, then Rb1. Conditions: Stationary phase, self-prepared TLC silica GF₂₅₄; layer thickness, 4 mm; speed of rotation, 700 rpm; temperature, 21.8°C; flow rate, 3 mL/min; mobile phase A, water–isopropanol–acetonitrile (5:21:74); mobile phase B, water–isopropanol–acetonitrile–acetic acid (8:20:72:2.5); mobile phase C, ethanol–water (9:1). (Reproduced from Ref. 14 with permission.)

but also the types of sorbents (with layer thickness), the sample sizes, and the mobile phases used. The normal chamber was used in more than 95% of the separations, although efforts are being made to introduce separations by the CLC-5 technique (71). Although the new types of chambers (micro, ultramicro, and planar column) were introduced relatively recently, their efficiency has been demonstrated for various classes of substances (9, 82–84).

V. COMPARISON OF VARIOUS PREPARATIVE LAYER CHROMATOGRAPHIC TECHNIQUES

The principal differences between CPLC, OPLC, and RPC are summarized in Table 2, which lists the generally accepted characteristics of the methods. As is apparent, the major difference among the three methods of PLC is the nature of mobile-phase migration. Better resolution can always be achieved by use of forced-flow techniques (OPLC, RPC) because of the mobile-phase velocity and the use of on-line separation, which eliminates the need to scrape the separated compounds from the plate and in which all the compounds migrate over the entire separation distance. These techniques require more sophisticated instrumentation.

The greatest flexibility with regard to choice of stationary phase (a variety of stationary phases with smaller particle sizes and particle size distributions), layer thickness, and chamber type is provided by RPC. The greatest separation distance (up to 36 cm) is possible with micropreparative long-distance OPLC (LD-OPLC) (85, 86). Unfortunately, the particle size and size distribution of precoated preparative plates are inadequate for this OPLC technique.

Because of the availability of suitable stationary phases and development modes, RPC offers the greatest separating power in terms of both the amount of sample and the number of compounds to be separated.

VI. TRENDS IN PREPARATIVE LAYER CHROMATOGRAPHY

The trends in PLC can be summarized under four headings:

1. Development and/or a combination of different precoated preparative plates with smaller average particle size
2. Development of various types of chambers
3. Introduction of different development modes
4. Combination of PLC and various methods of preparative CLC (column liquid chromatography)

Table 2 Comparison of PLC Methods at Their Present State of Development

Property	Classical PLC	OPLC	RPC
Migration of mobile phase	Capillary action	Pressure	Centrifugal force/capillary action
Layer/column	Precoated	Precoated	Self-prepared/filled
Stationary phase	Mainly silica	Silica	All available
Particle size of stationary phase	5–40 μm	5–40 μm	3–15 μm
Layer thickness	0.5–2 mm	0.5–2 mm	1–4 mm

Volume of stationary phase	Constant	Constant	Increasing/constant
Vapor space	Normal tank	None	Variable
Separation distance	18 cm	18 (36) cm	10 (9) cm
Separation mode	Linear (circular)	Linear/circular	Circular/linear
Isolation	Off-line	On-line	On-line
Typical amount of sample	50–150 mg	50–300 mg	50–500 mg
Number of compounds	2–5	2–7	2–10

The introduction of precoated preparative plates with smaller particle size distributions and various types of stationary phases (e.g., RP-18, Sephadex) is essential. Hostettmann et al. (83) tried to prepare preparative octadecyl layers, but the technique still has to be perfected. A real novelty to appear in recent years has been the taper plate; this dramatically increases resolution, especially in the lower R_f range.

Of the various possibilities of hyphenated ML-OPLC techniques (85, 86) illustrated in Fig. 19, a combination of parallel and serially connected chromatoplates can be envisaged. Such an arrangement is especially suitable for micropreparative separations on HPTLC plates. With the arrangement shown in Fig. 19, the middle three plates are connected in parallel and the bottom and upper plates in series. The type of sorbent material is also varied, the different stationary phases being indicated by various shades of gray in Fig. 19. Note that with such an arrangement, the local mobile-phase velocity is different for the parallel and serially connected plates (87). A number of significant changes in the practice of multidimensional planar chromatography may be expected in the next years.

The development of different types of chambers (e.g., M- and U-chambers) enables variation of one of the most important experimental conditions in PLC, the vapor space. The last decade has seen considerable developments, especially the various forced-flow methods. In both theory and practice, OPLC and C-RPC have no vapor space, whereas in U-RPC a certain amount of vapor space is present in theory but can be neglected in practice. The M-chamber is suitable for the separation of substances for which the vapor space has a pronounced influence on the separation, e.g., ammonia in the separation of alkaloids.

The N-chamber can be chosen when the mobile phase is an azeotropic mixture or consists predominantly (>80%) of one component (9). This condition is necessary because of the evaporation that occurs in the large vapor space of the stationary chromatographic chamber. Because the extensive evaporation of the mobile phase that results from rotation has specific negative effects (73), N-RPC can be employed only if this condition is met. Only preliminary studies have so far been performed on the correct choice of chamber type; this subject should be studied in greater depth in the near future. Because it employs a closed chromatographic chamber, OPLC would be an ideal separation technique if a higher (up to 100 bar) external pressure could be used and if better quality precoated plates were commercially available.

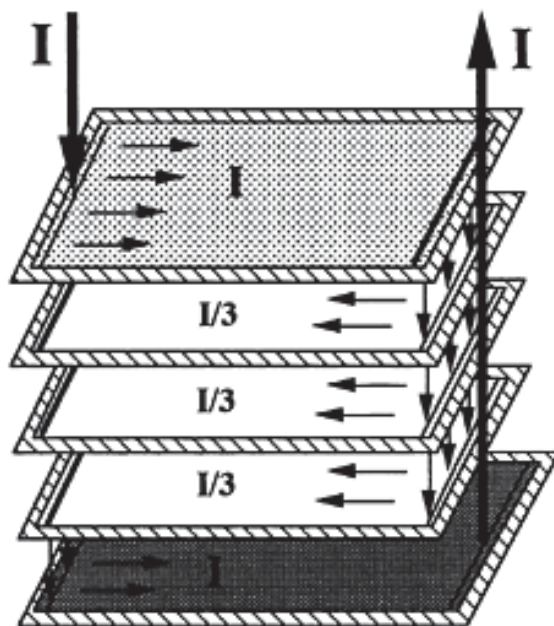


Figure 19 Schematic diagram of combined parallel and serially connected multilayer OPLC for fully on-line preparative separation, as a type of multidimensional forced-flow planar chromatography.

Whereas the importance of the various modes of development is well known for analytical TLC, linear development is the only method widely used for preparative CPLC. Remarkably, this is also the only development mode reported for preparative OPLC. In contrast, so far all RPC separations have been performed in the circular development mode. A new possibility in RPC is the planar column (76), where, in contrast to the use of centrifugal force, the flow is accelerated linearly to give linear development. As already mentioned, anticircular and circular development are also possible for on-line OPLC separations using a separation distance of 20 cm (2, 9).

The advantages of the different multiple development techniques for preparative separations were summarized recently (40). However, they are rarely used for classical PLC. Appropriate combination of PLC and preparative CLC for the isolation of compounds from complex matrices is very important. PLC is a highly suitable complementary method in the isolation process for the final separation of two to five substances from mixtures containing 50–500 (or 50–1000) mg of material.

It is expected that as a result of new developments, especially in modern forced-flow planar chromatographic techniques and multiple development modes, PLC will not only maintain its importance (88) but expand further as a successful method for the isolation and purification of synthetic and natural products.

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12

Thin-Layer Radiochromatography

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I. INTRODUCTION

Thin-layer chromatography (TLC) as it is applied today was developed in the 1950s. Standardization of sorbents and layer preparation led to wide application of TLC in analytical laboratories (1, 2). The main advantages of this separation method are its simplicity, low cost, and flexibility, and therefore it is now a general laboratory tool similar to titration, crystallization, and so on. Instrumentation for TLC resulted in automated sample application and development and thus good reproducibility. Modern scanning and video instruments can achieve accurate and precise recording of the separated constituents and quantification of chromatograms.

With the advent of high-performance liquid chromatography (HPLC) in the 1970s, however, a competitive method became available that attained higher resolution and sensitivity. As a result, a decline in the use of TLC was experienced. This is also true for radiochromatography, because HPLC with radiodetectors provides a convenient quantification method. In the past, separation of radiolabeled compounds was quite often achieved with good resolution by TLC; nevertheless, separation without loss in resolution was feasible only with film autoradiography. Quantification was performed then by zonal analysis, which is a time-consuming and tedious procedure. So, in most cases, on-line radio-HPLC systems proved to be superior to the radio-TLC procedure.

Over the last few years, the technologies for detection of radioactivity in TLC have been greatly improved. Today, separated mixtures can be detected without a significant loss in resolution, enabling in situ quantification of chromatograms. Moreover, these new radioimaging detectors provide higher sensitivity than that of HPLC detectors. A number of advantageous characteristics of TLC can be utilized; namely, in each experiment fresh sorbent is used, and it is suitable for fast method development and serial analysis. In addition, during TLC no sample loss due to irreversible absorption on the column can take place; therefore, the entire sample is detected (with the exception of infrequent cases when volatile constituents are present). As a result of this, a combination of planar

chromatography or overpressured layer chromatography (TLC/OPLC) with radioactivity detection can be successfully used as an independent method for the analysis of radiolabeled mixtures or can serve as an excellent complementary method to radio-HPLC.

A number of recent publications have reviewed radiochromatography, including detection in TLC (3–11). This chapter reviews the development and current status of planar radiochromatography. The advantages and disadvantages of the various detection methods are outlined, plate characteristics and handling of the layers are discussed briefly, and a few applications of TLC using radioactivity detection are illustrated.

Hyphenated techniques of planar radiochromatography and other radiochromatographic techniques are also reviewed, including a multiaspect comparison of techniques for different detection possibilities from soft beta- to gamma-emitting isotopes (6, 11, 12).

II. METHODS OF RADIOACTIVITY DETECTION IN PLANAR CHROMATOGRAPHY

The principal method used to detect and quantify radioactivity on TLC plates is autoradiography. It is a method used to detect the distribution and measure the quantity of a radioisotope that is deposited on a surface. A more formal definition of autoradiography might be “a method for locating and quantifying radioactive substances by placing the sample under investigation in close contact with a position-sensitive detection system.”

Conventional autoradiography utilizes direct contact of the radiolabeled sample with a photographic (X-ray) film or emulsion. Films and emulsions have been the traditional tools of autoradiography since 1867, when Niepce de St Victor first observed the blackening of silver chloride and silver iodide emulsions by uranium nitrate and uranium tartrate (13). Traditionally, in film autoradiography, the first step was the location of the spots or bands of interest, and then quantification was achieved by removing the zone of interest for subsequent liquid scintillation analysis (zonal analysis). For data capture of the image formed on the film, various instruments are commercially available today (light densitometers, laser densitometers, flatbed scanners, and video and CCD camera systems). Then, with suitable software, quantification of the optical density of the exposed film can easily be carried out (see below).

Methods for detecting radioactivity in situ (i.e., directly on TLC plates) improved dramatically during recent decades. After TLC separation, electronic autoradiography (i.e., linear analyzers and multiwire proportional chambers), as well as bioimaging/phosphor imaging analyzers, are employed for detection and quantification of radiolabeled compounds. The basic functioning of the above-mentioned new instrumentation is outlined below. Descriptions of outdated detectors (e.g., radioscaners) can be found in the literature (6, 14).

The method of choice for data evaluation depends on the available instrumentation, the type of experiment, and the information required.

Table 1 summarizes the most frequently used radioactive isotopes in the various planar radiochromatographic techniques, including detailed physical parameters of these radionuclides (15–17).

A. Film Autoradiography

Film autoradiography is a method of detecting β -particles that is based on the conversion of silver ions to reduced silver atoms within a film emulsion. TLC plates are subjected to exposure (ex-

Table 1 The Most Frequently Used Radioactive Isotopes in Planar Radiochromatographic Techniques

Element	Radionuclide (${}^A_Z\text{X}$)	Decay mode (radiation emitted)	Physical half-life (time)
Hydrogen (tritium)	${}^3_1\text{H}$	β^-	12.35 yr
Carbon	${}^{14}_6\text{C}$	β^-	5730 yr
Phosphorus	${}^{32}_{15}\text{P}$	β^-	14.28 days
	${}^{33}_{15}\text{P}$	β^-	25.3 days
Sulfur	${}^{35}_{16}\text{S}$	β^-	87.4 days
Technetium	${}^{99m}_{43}\text{Tc}$	IT, γ	6.0 h
Iodine	${}^{125}_{53}\text{I}$	EC, γ , e^-	60.25 days
	${}^{131}_{53}\text{I}$	β^- , γ , e^-	8.04 days

A, atomic number; Z, mass number; X, radionuclide; β^- , negative beta particle (negatron) emission; EC, electron capture; IT, isomeric transition; γ , gamma radiation; e^- , electron emission.

Source: From Refs. 15–17.

posure time depends on the amount of radioactivity applied to the plate), and the image is then revealed by subsequent processing of the film.

Various types of films for use in autoradiography are available on the market. The largest manufacturers of film for radionuclide analysis are Kodak (Rochester, NY), Fuji (Tokyo, Japan), Dupont (Wilmington, DE), Ilford (Essex, England), and Agfa Gevaert (Brussels, Belgium). Among the films used for detection of radionuclides, the most recently developed is the new Kodak Biomax MR film (18). According to the manufacturer, Kodak's patented "T-grain" emulsion technology enables BioMax MR film to provide a twofold to fourfold increase in sensitivity compared to other films in the detection of ${}^{35}\text{S}$ -, ${}^{33}\text{P}$ -, and ${}^{14}\text{C}$ -labeled samples while offering maximum resolution that results in improved detection of low-intensity bands.

For maximum sensitivity, the film emulsion must be efficiently penetrated and should interact with the radioactive emission. Autoradiography is best suited (in terms of sensitivity) for ${}^{35}\text{S}$ and ${}^{14}\text{C}$ emitting β -particles at a medium energy level. The most difficult task is the detection of tritium-labeled substances because of the low energy of the emission and the high probability of self-absorption during autoradiography. To obtain higher sensitivity to ${}^3\text{H}$ -labeled compounds, special films have been developed that do not contain a protective layer on the film surface (applied to most of the other films), making them more sensitive to the low-energy emissions of this nuclide.

Film autoradiographic technology can be divided into three steps: (a) exposure of the chromatographic plates, (b) film development, and (c) evaluation of chromatograms.

1. Exposure of Chromatographic Films

The method of film exposure is dependent on the type of experiment and the type of information required. The three principal exposure methods used are direct exposure (autoradiography), direct exposure with an intensifying screen, and fluorographic exposure (fluorography) (see Fig. 1). The best exposure procedure is generally determined experimentally, and numerous investigations and examples have been described in the literature (e.g., 19, 20).

The length of exposure can vary over a wide time period. It depends on the type of isotope and the amount of radioactivity applied onto the chromatoplate. Exposure conditions for a particular autoradiographic procedure are determined by exposing the film to plates containing calibrated amounts of radioactivity. When properly exposed, the autoradiographic resolution is comparable to that of the original chromatogram. Overexposure of the film will cause a more diffuse and larger image of the spots and result in poorer resolution of closely eluted spots. Quantification of the radiographic images produced requires comparison of the measured variations in optical densities to a radiation response curve (characteristic curve) generated with radioactive standards on the same piece of film. Standards can be purchased as radiolabeled plastic polymers (21) or prepared from dilute solutions of known amounts of radioactive material. It should be borne in mind that concentration vs. optical density curves are linear over only a limited range. Above a certain exposure level, the film will not darken further, and therefore quantification of the chromatogram by image analysis is not possible. On the other hand, radiolabeled compounds on the plate can be located and further quantification can be carried out by zonal analysis. Because film emulsion can also be darkened by the presence of organic solvents, the plates must be free of mobile-phase components before exposure.

The simplest method for detection consists of direct exposure produced by intimate contact of the developed plate with a photographic or X-ray film (autoradiography). Direct exposure is useful for all of the beta emitters, although to various extents. The choice of the most appropriate film is crucial.

To improve the detection efficiency for gamma-emitting (e.g., ^{125}I) and high-energy beta-emitting isotopes (e.g., ^{32}P), the plates are exposed with intensifying screens placed behind the film (22). These screens are used to reduce the exposure time or increase the sensitivity in the detection of radiolabeled samples. However, they diminish the resolution of an image compared to a direct (no intensifying screen) exposure. The decrease in resolution is due to the increased distance between the origin (sample) and the emulsion, where the image is formed. To get the best resolution, a sensitive single-emulsion film (such as the BioMax MR film mentioned above) (18) should be used.

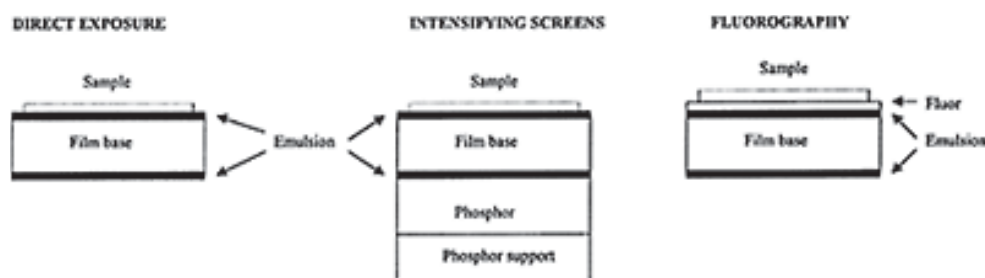


Figure 1 Comparison of exposition methods for film autoradiography.

Intensifying screens work by generating photons through the interaction of the energy of the radiolabeled particles and the phosphor in the intensifying screen. The energy from the β -particles interacts with the phosphor to generate a large number of photons (optimal sensitivity in such a system is reached when the photons generated by the screen match the peak spectral response wavelength of the film). Keep in mind that the photons are less energetic than the β -particles used to create them.

All intensifying screens perform optimally at -60 to -80°C . The reason for this simplicity is that the activation energy required to form a stable latent image on the film is lowered (chemicals are required to create a permanent image). At room temperature, greater activation energy is required to form the stable latent image. Therefore, more energy is required to achieve an image at room temperature when using an intensifying screen than when using a screen at -60°C . Activation energy needs to be reduced because the photons (though more numerous) are less energetic than the radioisotope particles.

Conventional intensifying screens work by placing a radiolabeled sample on a sheet of autoradiographic film with the intensifying screen lying under the film (i.e., the film is sandwiched between the screen and the sample). To make use of the intensifying screen, the radioisotope particles must have enough energy to pass through the film. ^{32}P and ^{125}I have sufficient energies to penetrate the film. Radioisotopes such as ^3H , ^{14}C , ^{35}S , and ^{33}P lack sufficient energy to penetrate the solid matrix of the emulsion coated onto the film. Therefore, intensifying screens used in this way offer no benefit to weak and medium energy radioisotopes such as ^3H , ^{33}P , ^{35}S , and ^{14}C . Recently, Scientific Imaging Systems (Kodak) introduced an innovative intensifying screen system called the BioMax TranScreen system (23). This system solves the problem of the film attenuating the β -particle before it reaches the intensifying screen. It is designed to be used with medium- and low-energy beta isotopes, i.e., ^{35}S , ^{33}P , ^{14}C , ^{45}Ca , ^{59}Fe , and ^3H . The manufacturer claims that BioMax TranScreen LE can achieve a medium image 5–35 times faster than direct autoradiography for detection of low-energy radioisotopes. This means that an exposure (data capture) period 5 to 35 times shorter is required.

The detection of lower energy isotopes (e.g., ^3H) adsorbed on thin layers may be enhanced by the use of an organic scintillator such as 2,5-diphenyloxazole (PPO). This technique is termed "fluorography." Fluorography involves the overcoating or impregnation of a scintillator into the TLC plate followed by direct exposure of the treated plate to an X-ray film. The scintillant, being in direct contact with the isotope, emits light when activated by the emitted β -particles and exposes the film photographically. For efficient detection, the spectral sensitivity of the film should be

matched to the wavelengths of light emitted by the scintillator. The scintillants can be incorporated by mixing the scintillator with the adsorbent during preparation of the TLC plate or applied after development. Fluorographic reagents can be added by spraying or dipping the plates. Solutions and spray reagents are commercially available and allow for simple and even application of the reagent (24).

2. Film Development

Film development requires a darkroom for processing (developing, fixing, and drying) the film. There are two methods for manually processing autoradiographs. The difference lies primarily in the volume of chemicals used and the method of transferring the films between solutions. The recommended processing chemistry is the same for both methods. The deep tank method usually includes developer tanks (with a volume of a few liters) and fixer tanks sitting in a water bath. The water bath controls the temperature of the developer and fixer. The film is moved from tank to tank by hand, using metal film hangers. The tray method includes at least three trays that are 2 cm or more larger than the sheet of film to be processed plus an adequate amount of running water for washing. The film is moved from tray to tray by hand using print tongs.

Maintaining fresh processing chemicals is critical to achieving high quality autoradiographs. Old developer and fixer will adversely affect the image quality of processed film even if they are used infrequently. It is highly advisable to change the developer and fixer chemicals frequently (e.g., every month) to ensure optimal processing conditions. They should also be replenished during processing. There are five steps in film processing: development, rinsing, fixing, washing, and drying. Today, automated processing units are also available, combining performance with convenience. Detailed descriptions of the developing process as well as the photofinishing chemicals can be found on the web sites of suppliers (25).

3. Evaluation of Chromatograms

After exposure and film development, the radioactivity is located as darkened spots or bands. Their optical density is related to the amount of radioactivity.

Because the autoradiographic film is an analog representation of the chromatogram obtained on the plate, qualitative evaluation of films is carried out by visual inspection. Because human intelligence is excellent in evaluation of patterns, visual inspection is a perfect method for qualitative assessment of chromatograms.

In contrast, quantitative evaluation of the chromatoplates by visual inspection is inaccurate. There are two principal methods for quantification after autoradiography: zonal analysis and computer evaluation after image capture digitization of films.

4. Zonal Analysis

Zonal analysis is a traditional procedure that involves removing sectioned areas (separated spots and/or bands) of chromatographic adsorbent from a TLC plate, followed by liquid scintillation counting of radioactivity (i.e., performing an off-line measurement of radioactivity). The zones are removed either by scraping the adsorbent from the plate

(plate scraping) or by cutting pieces from plates with a flexible backing and transferring the segments into counting vials. In an alternative procedure that allows isolation of the radiolabeled sample, the plates are segmented and the radioactive components are eluted from the adsorbent with solvents and counted.

A prerequisite of an accurate determination is good chromatographic separation. In addition, the plate should be carefully segmented to obtain zones corresponding to the compounds separated. In the course of the chromatographic process, irreversible binding of small amounts of material at the site of application and onto the adsorbent might be a potential source of error. This is particularly common with tritiated compounds that possess high specific activity and when very low sample masses are chromatographed. The problem can usually be corrected by deactivating the adsorbent at the application site (e.g., by spotting the radioactive sample directly over a previously spotted sample that contains the identical unlabeled compound).

Measurement of radioactivity is generally accomplished by using a liquid scintillation counter (LSC) for weak beta emitters, whereas for gamma emitters the sectioned zones are counted without further sample preparation by an appropriate gamma counter. It should be remembered that chromatographic agents, solvents, and samples frequently influence the liquid scintillation counting by reducing the counting efficiency. This effect (known as quenching), however, is taken into account by modern LSC instruments. In addition to quenching, other interfering processes such as chemiluminescence, phosphorescence, and efficiency losses due to self-absorption of labeled compounds in the heterogeneous system (i.e., on the sorbent surface) can affect quantitative measurement (26). Samples with low levels of radioactivity can be counted for longer periods to obtain a statistically suitable number of counts. For samples with low levels of radioactivity, a background correction should be performed. The number of background counts can be determined by counting a section of adsorbent equivalent in size to the sampled sections. This section should be taken from a closely adjacent portion of the plate free from radioactivity and chemical contamination.

Plate scraping can be done either manually or with an automated plate scraper. Manual scraping is done with a sharp, flat spatula or with one of the commercially available adsorbent scrapers. When a lane of the TLC plate is segmented by hand, good results are easily obtained if large areas of adsorbent are removed. However, when greater resolution is required, a high number of small, reproducible zones must be removed from the plate. In this case, a number of difficulties (incomplete removal of the lane, loss of adsorbents, etc.) are encountered. For this situation, the measurement of a zone cut from the plate with a flexible backing is a better choice.

Good results can also be obtained using plastic- or aluminum-backed TLC plates followed by elution from individual sections with spots or zones that have been cut from the plates and transferred into scintillation vials. Samples that require recovery can be eluted from the adsorbent with one or more appropriate solvents and then dissolved in a counting cocktail. The elution can be achieved in three ways: (a) by removal of the adsorbent followed by elution with solvent, (b) by washing the spot or zone with solvent, and (c) by immersing the spot or zone in solvent. It should be kept in mind that before using one of the latter two methods, the recovery of radioactivity should be checked to ensure that good recovery is obtained.

The zonal analysis technique is relatively sensitive and provides quantitative detection even for samples with low levels of radioactivity. Single peaks containing only 100 dpm (disintegrations per minute) can yet be detected (27). However, in serial studies aimed at quantification (e.g., determination of the mass balance of metabolites), a higher activity of the separated compounds (at least 500 dpm) is required to achieve reliable results.

For data presentation, the number of counts measured for each spot or zone is used to determine the distribution of the radioactivity of the measured zones. Then the counting data obtained are plotted to give a histogram profile of the radioactivity along the entire lane of the TLC plate.

5. Image Analysis

In recent years, image analysis has been developed very intensively. Image analysis is a broad concept that includes image capture, image processing, image evaluation, image handling, and image representation (28). Newly developed methods for the image capture of separated radio-nuclides (i.e., electronic autoradiography and phosphor image technology) are discussed below. Devices for electronic autoradiography and phosphor image analysis are supplied with dedicated software for data capture and data processing. Image analysis, however, has been introduced for film autoradiography, too. A number of devices are available now [charge-coupled device (CCD) cameras and various scanners] that can be used for image capture of the optical density of a film. Image capture of TLC separations by an inexpensive flatbed scanner has been reported (e.g., 29), particularly for autoradiographic films (30). Image capture is often called “digitization” because the generated image is a digital representation of the image. This digital image can be evaluated further (particularly for quantification) by suitable computer software.

The optical density of the film is determined not only by the activity of the sample under investigation but depends also on the film type, exposure period, and film development procedure. Consequently, to achieve quantitative results, a set of standards (a calibration curve) should be applied during each exposure. By using a calibration curve, the under- and overexposure of the film is also determined.

Quantification of radionuclides by the use of image evaluation in TLC separations involves (a) using a set of standards to construct a calibration curve (to get a correlation coefficient of at least 0.95), (b) integration of separated spots or bands of interest, and (c) calculation of the unknown amount by use of the calibration curve.

The software packages used for analysis of images are briefly discussed in Section II.D.7.

B. Electronic Autoradiography

No energy storage medium is used during electronic autoradiography, and, unlike other autoradiographic techniques (film, phosphor storage screen), the radioactivity is measured directly on the chromatoplates.

The introduction of linear analyzers represented a great improvement in the detection of radioactivity in TLC. These detectors were based on imaging detectors developed in the late 1960s, and the principles of function and use were reviewed by Clark and Klein

(6). However, owing to the development of the position-sensitive multiwire proportional chamber (MWPC), the application of linear analyzers has been reduced.

1. Principle and Technology of the MWPC

With the introduction of multiwire proportional chambers by Georges Charpak, it became possible to localize charged particles, X-ray photons, and thermal neutrons with submillimeter accuracy (31). For this invention, Charpak was awarded a Nobel prize for physics in 1992. Charpak's concept includes a gridded proportional counter chamber in which positional information is obtained by establishing which anode wires in an X-Y grid are in closest proximity to the secondary avalanche produced by the passage of a β -particle through a counting gas. By this technique, compounds labeled with ^3H , ^{125}I , ^{14}C , ^{32}P , $^{99\text{m}}\text{Tc}$, etc. can be detected with extremely high sensitivity. Two instruments based on this principle (Digital Autoradiograph of Bethold and Instant-imager of Canberra Packard) gained popularity for evaluation of chromatoplates.

a. Digital Autoradiograph. The digital autoradiograph (DAR) is a two-dimensional detector that quantitatively measures the position and intensity of two-dimensional distributions of ionizing radiation from a radioisotope on a 20×20 cm surface (3, 32). The developed TLC plate is placed on a measuring table and then automatically loaded into the detector. The detector consists of three parallel wire planes, with only a few millimeters of space between the planes and between the wires. The central plane is maintained at a positive potential of 1800 V, and the counting chamber is filled with P-10 counting gas (90% argon, 10% methane). The middle plane generates a charge signal from the ionizing radiation entering the chamber. The two orthogonally crossed wire planes below and above the middle plane pick up the signal and thereby determine the position of the radioactivity on the surface measured. The signals from the three wire planes (600 wires) are transmitted via preamplifiers, pulse shapers, discriminators, and logic circuits to analog-to-digital converters and then captured by a suitable data acquisition system.

Signal analysis is achieved by measuring $5 \times 360,000$ elemental detector cells per second. DAR measurement time (run time) must be optimized on the basis of the amount of radioactivity applied to the plate (11, 32). This is accomplished by inspection of the real-time display during data acquisition.

b. MicroChannel Array Detector. The microchannel array detector (MICAD) Instantimager of Packard Bioscience (33) consists of two sections, the microchannel array plate and a multiwire chamber. The microchannel array plate is 3 mm thick and has a sensitive area of 20×24 cm. It has a laminated surface where conductive (brass) and nonconductive (fiberglass) materials alternate. A voltage step gradient is applied to the successive conductive layers to create a high electric field of approximately 600 V/mm in the microchannels. Above the microchannel array plate is a multiwire chamber similar to that described for the Digital Autoradiograph (anode plane of 200 gold wires approximately $20 \mu\text{m}$ in thickness; two cathode planes formed by metallic cathode tracks).

The entire MICAD detector is filled with a continuous flow ($25 \text{ cm}^3/\text{min}$) of counting gas (96.5% argon, 2.5% carbon dioxide, 1.0% isobutane). When a β -particle is emitted from a radioactive source, the counting gas is ionized in one of the microchannels. The

electrons produced are accelerated by the high electric field in the microchannel to further ionize the gas to produce a cloud of electrons. In this way, the microchannels serve as both collimators and preamplifiers. The cloud of electrons migrates up an electric field gradient into the multiwire chamber.

c. β -Imager of Biospace Measures. According to Charpak's original concept, positional information is obtained by establishing which anode wires in an X-Y grid are in the closest proximity to the secondary electron avalanche produced by a β -particle in the counting gas. An alternative approach is the application of a cooled CCD to detect the light emitted by β -particle interactions in a scintillator (34, 35). The use of CCD detection makes it possible to enhance the resolution of the image.

This technique is utilized in the β -Imager 1200 (36). Each β -particle that enters the gaseous detector generates an avalanche of electrons and a spot of light. The CCD camera records each event, which is analyzed and stored in a computer system. The detector is similar to that described above. Namely, it consists of two amplification gaps separated by a transfer gap. These gaps are defined by metallic grids and filled with a counting gas mixture (helium, argon, dimethyl ether). When a β -particle enters the detector, it creates a great quantity of electrons by an avalanche process. Two amplification stages result in multiplication of the number of charges from one β -particle at the entrance to 10^5 at the output. The intermediate transfer gap prevents any feedback from the output to the input.

The associated electric field induces a controlled local spark that emits visible light, which is read by the CCD camera. A calculation is performed to optimize the location of the entering particle, and the two-dimensional image that is formed is stored in a computer system. This detector enables the detection of 2.0 dpm/min on the chromatogram, whereas a quantitative measurement in the range of 5–50 dpm is possible for ^{14}C -labeled compounds.

In the improved version of this device (β -Imager 2000), the counting gas was replaced by a mixture of argon containing triethylamine (37). The maximum field of view is 20×25 cm, thus enabling the imaging of an entire standard TLC plate. The full field-of-view resolution for ^3H is $200\text{ }\mu\text{m}$, whereas for ^{14}C , ^{35}S , and ^{33}P it is $350\text{ }\mu\text{m}$. This value is $500\text{ }\mu\text{m}$ for ^{32}R (In maximum zoom position, where the field of view is 25×33 mm, resolution values are considerably reduced, e.g., $50\text{ }\mu\text{m}$ for ^3H .) These data demonstrate that by using this instrument, resolution comparable to that of a film or phosphor imager can be obtained. According to the manufacturer, the detection threshold for ^3H amounts to 0.007 cpm/mm^2 (cpm=count per minute), whereas it is 0.01 cpm/mm^2 for ^{35}S , ^{14}C , and ^{33}P and 0.1 cpm/mm^2 for ^{32}P . Counting response is linear over a range of 10^4 .

C. Storage Phosphor Screen Imaging

1. Principle and Technology

Storage phosphor screen imaging technology was introduced by Fuji Photo Film Company in the 1980s, and now these types of instruments are also supplied by other companies (e.g., Molecular Dynamics, Biorad, and Packard). From a practical point of view, the procedure carried out with the phosphor image technique is very similar to that of film autoradiography, and it is sometimes termed "filmless autoradiography." Screens

are sensitive to any source of ionizing radiation; therefore, commonly used isotopes such as ^{14}C , ^3H , ^{35}S , ^{125}I , ^{32}P , and ^{33}P can be detected.

The phosphor screen is the crucial part of this technology. It is a flexible image sensor in which bunches of crystals (the grain size is about $5\ \mu\text{m}$) of a photostimulable phosphor of barium fluorobromide are uniformly coated on a support film. The BaFX:Eu^{2+} ($\text{X}=\text{Cl}$, Br , or I) crystal is an ionic crystal having a tetragonal structure, and Ba is replaced with the Eu^{2+} ion to create a solid solution. This crystal, when irradiated, stores the energy in the crystal vacancies. The luminescence mechanism of this photostimulable phosphor is interpreted as follows. When the exposed Eu^{2+} ions become Eu^{3+} ions through primary excitation by X-rays, for example, electrons are released into the conduction band. These electrons are trapped in the Br vacancies, which are inherently present in the crystal, and color centers of the metastable state are formed. During reading, as a result of the use of red laser light (at $633\ \text{nm}$), trapped electrons are liberated again into the conduction band of the crystals. In this manner, Eu^{3+} ions are converted back to Eu^{2+} ions while releasing photons of blue light at $390\ \text{nm}$ (38). The process is schematically shown in Fig. 2. It should be noted that another formulation of the phosphor screen has also been developed

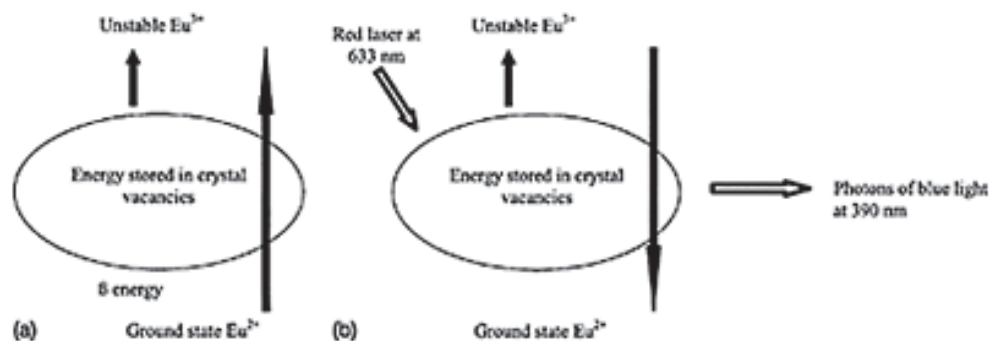


Figure 2 A schematic representation of the phosphor image mechanism, (a) Exposure, (b) scanning.

that uses different chemistry optimized for detection of luminescence (39). Today, instruments that use both techniques are available, thus enabling radioactivity and fluorescence detection in a single system.

Because phosphor screens (also referred to as “image plates”) are reusable, an additional step is also included in evaluation of chromatograms. Prior to repeated use, the screens should be erased by exposing them to visible light. This step cannot be omitted, because during the reading process the stored information is released, but not in a quantitative manner. Incorrect results can be obtained when the screen has not been erased properly, because a “ghost” image can interfere with the current results. Erasing can be performed by using bright visible light. Dedicated devices for this purpose (light boxes) are also available. The manipulation involves five steps: (a) TLC separation, (b) exposure, (c) data capture (i.e., reading or scanning), (d) image evaluation, and (e) erasing the screen.

The Fuji and Packard systems use similar formulations, whereas Molecular Dynamics and Biorad systems use phosphor screens manufactured by Kodak. Screens are available

in different sizes to suit the reading device used, and most of them are capable of capturing an image from a standard (20×20 cm) TLC plate. Because of the Packard instrument's physical design, the screen is only 12.5 cm wide. However, a standard plate can be scanned in two parts, and the image can be rebuilt by the instrument's software. Various screens, depending upon the proposed application, are available (for general purpose, for highest resolution, etc.). To detect the weak energy of tritium, signal image plates constructed without a protective layer should be used.

Storage phosphor screen technology is considered a technique that can be performed with normal lighting. However, this is true only with certain limitations. When there is bright fluorescent lighting in the room, most of the signal on the screen can be erased in a couple of minutes. In a recent study, signal loss from two types of imaging plates (phosphor screens), BAS-III and BAS-MS, was investigated in (a) normal laboratory lighting, (b) safe light, and (c) total darkness (40). The authors concluded that image plates should ideally be handled in the dark or under safe light conditions, and normal room lighting should be avoided.

There are two types of scanning mechanisms used by reading devices. One option is that the phosphor screen is kept on a flat plane while the laser beam sweeps across the screen (used by Fuji and Molecular Dynamics). The other design (Packard's Cyclone) contains a helical scanning mechanism and flexible phosphor screens loaded onto a cylindrical carousel that spins at 360 rpm. The main benefits of this system are its compactness and low cost.

2. Evaluation of Chromatograms

Each company provides its own software package for evaluation of images obtained with their phosphor image technology. The approaches (i.e., one- and two-dimensional evaluation, data handling, communication with other software packages, etc.), however, do not differ significantly.

By the phosphor image technique, 1–2 dpm mm⁻² h⁻¹ of ¹⁴C and ³⁵S and approximately 0.2 dpm mm⁻² h⁻¹ of ³²P and ¹²⁵I can be detected (41). This means that for the various nucleotides, the sensitivity is 10–100 times higher than that of film autoradiography. Due to the higher sensitivity, the use of a storage phosphor system instead of film provides either faster results or detection of samples exhibiting lower radioactivity.

It is evident that the signal intensity on a phosphor screen increases with the duration of exposition. At room temperature, the net signal (the signal of the sample minus the background signal) of a ¹⁴C sample increases at a constant rate over a seven-day period and then exhibits a plateau, but when the cassette is cooled under controlled conditions (<8°C), the signal continues to increase (42). Manufacturers, however, do not suggest low-temperature exposition, because it may cause condensation of air humidity, which affects the phosphor screen material (43). Consequently, this procedure should be performed very carefully, and it is mandatory to keep a certain temperature adaptation period for the phosphor screen.

With a longer exposure period, lead shielding reduces the background value (as a result of cosmic background radiation) and thus greatly improves the signal-to-background ratio. Dedicated shield boxes are also available for this purpose.

Resolution of phosphor image technology is somewhat like that of films and definitely superior to that of a linear analyzer (44). Figure 3 shows TLC chromatograms that were evaluated

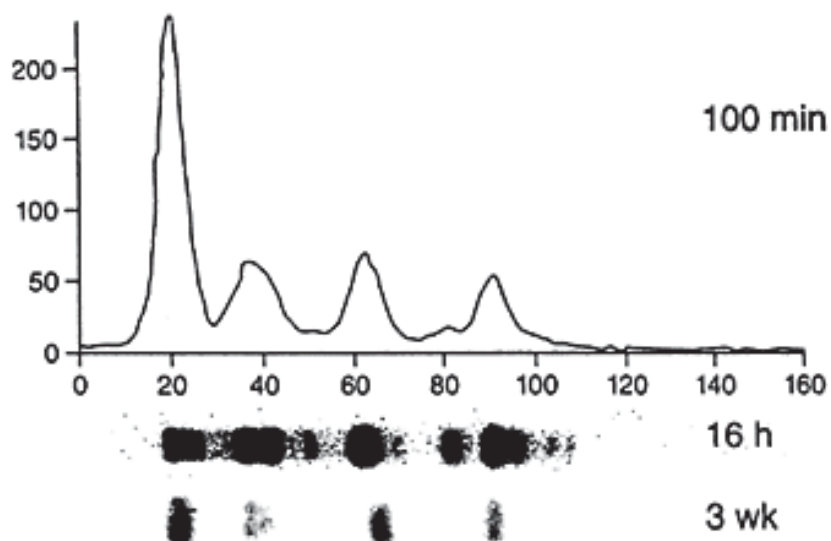


Figure 3 Resolution of various detection methods. TLC chromatograms of an extract of urine from a plant metabolism study obtained by linear analyzer (top), phosphor image analyzer (middle), and film autoradiography (bottom). (From Ref. 44.)

by the above techniques. The similarities and differences among the three methods are clearly seen.

3. Quantitative Analysis

A storage phosphor system provides results faster than film (or lower radioactivity can be detected), but the real advantage is the wide linear dynamic range of the image plates. Linear dynamic range is the range over which the instrument yields a linear response and is therefore useful for accurate quantification. It has been documented by several authors that the linear dynamic range of a storage phosphor system has a magnitude of 5 (e.g., 44, 45). This range for TLC purposes is far more than adequate.

Because phosphor screens (or image plates) are not identical, a calibration curve (similar to that mentioned for film autoradiography) should always be included when quantitative analysis is carried out. By doing this, the effect of phosphor screen type and exposition period is excluded.

Some factors can affect the results of quantification. First is the above-mentioned signal loss (termed “signal fade”) that occurs gradually after the sample is removed from the phosphor screen. Signal fade is uniform across a given phosphor screen, so it does not significantly affect the accuracy of analysis. On the other hand, it has a definite influence on the limit of detection (LOD) as well as on the limit of quantification (LOQ). Second is an artifact termed “laser bleed” or “flare.” Bleed is caused by stray laser light hitting high-intensity signals on the storage phosphor screen around the pixel being excited. It generates a real signal and will interfere with accurate quantification. This is especially a problem when weak bands are within the bleed area of intense bands. Scientists from Packard report that their Cyclon system eliminates this effect (46). Finally, although under- and overexposure of the screen are rare, they cannot be ruled out. By using a calibration process, this problem is eliminated.

D. Comparison of Detection Methods and Techniques in Thin-Layer Radiochromatography

There are three principal techniques for the detection of radiolabeled compounds in thin-layer chromatography: film autoradiography (combined either with zonal analysis of separated components or with image analysis), the phosphor image technique, and electronic autoradiography. The technique of choice depends on various parameters. Because TLC is a separation technique, the most important features to be considered are sensitivity, resolution, and the method of quantification (sample preservation included). In addition, the availability of data handling methods and data storage and conformity with good laboratory practice (GLP) are to be taken into account. Depending upon the requirements of a particular laboratory, other factors such as cost, speed, and sample throughput should also be carefully considered. The various methods are schematically summarized in Fig. 4.

The most important detailed technical parameters of the various autoradiographic techniques and their detection of beta-emitting isotopes are summarized in Tables 2 and 3, respectively.

1. Sensitivity and Speed of Detection

Sensitivity can be described as the minimum detectable level of radioactivity. Sensitivity is closely connected with the time interval of detection and should therefore be discussed in terms of the speed of the detection process. The minimum detectable levels of activity, for example, are low for film autoradiography; the time period necessary to achieve these levels (i.e., exposure time), however, is very long. The explanation for this fact is simple: Although the film has great sensitivity to photon emissions, it lacks efficiency in detecting ionizing radiation. From a practical point of view, it can be stated that the use of film, even with intensifying screens, is less sensitive than the other techniques.

2. Resolution

It is difficult to give an exact comparison of resolution power among various detection methods. Even instruments that use identical techniques provide somewhat different

degrees of resolution. From the practical point of view, however, the resolution of the detection methods can be arranged in the following order: film autoradiography > phosphor image technique > electronic autoradiography. Spatial resolution provided by film autoradiography and the phosphor image technique, as well as by the β -Imager (less than 60 μm in the latter case), completely meets the requirements of thin-layer chromatography. The limiting factor is the chromatographic resolution of the separated components.

3. Method of Quantification, Linear Dynamic Range

As discussed above, the method of quantification available for modern techniques is definitely superior to those of film autoradiography. The linear dynamic ranges of the phosphor image

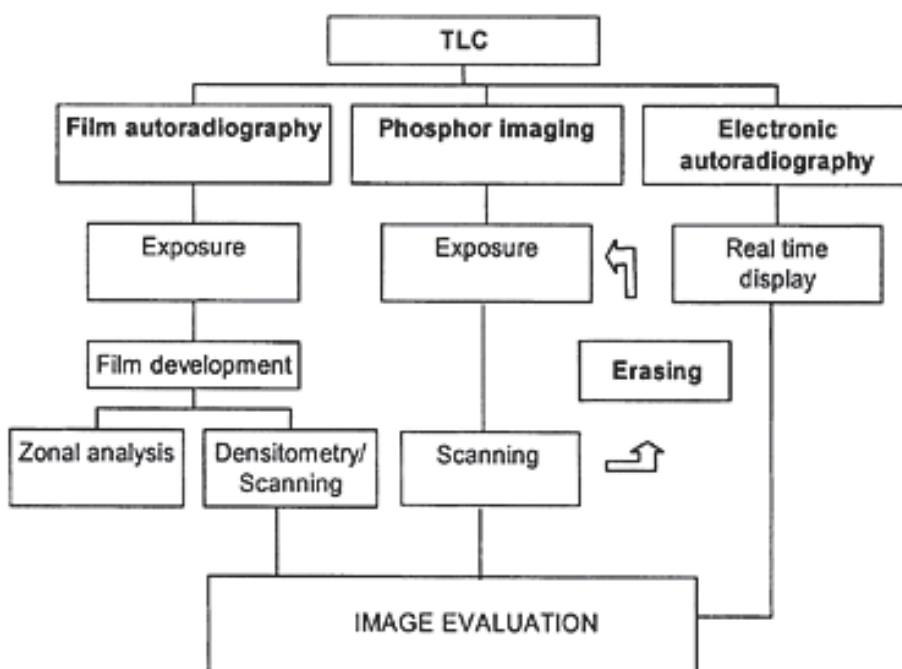


Figure 4 Comparison of methods for radioactivity detection in TLC.

Table 2 Summary of the Most Important Technical Parameters of Popular Autoradiographic Techniques

Parameter	Methods for detection of radioactive isotopes			
	Traditional contact film autoradiography	Digital autoradiography (DAR; MWPC)	Charged-coupled devices (CCD)	Phosphor imaging techniques
Linear detector response	Nonlinear	Good—linear	Good—linear	Good—linear
Dynamic range	Limited: max 10^2	Unlimited ^a ; up to 10^{5b}	Up to 10^4	Up to 10^5
Quantitative determination	Limited	Yes	Yes	Yes
Background noise level	High: 10^5 events/mm ²	Good: 300 events/mm ²	N.D.	Good: 300 events/mm ²
Sensitive area	20×20 cm	20×20 cm ^a 20×24 cm ^b	20×25 cm	620 cm ²
Spatial resolution (different from emitters)	Emulsions: 0.5–10 μ m Film: 2.8–56 μ m	(50)–500 μ m	15 μ m	100–400 μ m
Detection time reduction	1	1/1000	N.D.	1/100
Real-time detection technique	No	Yes	Yes	No
Most important suppliers	Types of film: Agfa, Kodak, Fuji, Amersham, Dupont, Ilford	EG&G Berthold, Camberra Packard, Raytest, Biospace	Biospace	Fuji-Raytest, Molecular Dynamics, Biorad, Packard
References	18–21, 25	3, 6, 11, 32, 33, 47, 48	36, 37, 47	40, 44, 46, 47

N.D.=no data.

^aEG&G Berthold.^bCamberra Packard, Raytest.**Table 3** Comparison of Various Techniques for Detection of Beta-Emitting Isotopes³

Aspect	Traditional contact film autoradiography	Digital autoradiography DAR (MWPC)	Phosphor imaging techniques

Detection of different radioisotopes	++	++++	+++
Simplicity	++	++++	+++
Speed of process	+	++++	+++
Sensitivity	++	++++	+++
Resolution	++++	++	++++
Linear range	+	++++	+++
Sample amount required	+	+++	+++
Quantitative evaluation	+	++++	++++
Cost of instrumentation	++++	++	+++
Cost of operation	++	++++	++++
GLP/GCP conformity	++	++++	++++
Overall applicability	++	++++	++++

^a Rating system—the second term in each case refers to the two cost aspects.

+ Low/expensive.

++ Good/fair.

+++ High/acceptable.

++++ Excellent/inexpensive.

Source: Refs. 11, 49, 50.

technique and electronic autoradiography are wider (by at least 10^5) than is needed for detection in TLC. In the case of film autoradiography, both the narrow linear dynamic range of the method (when using image analysis) and the lack of sample preservation (in zonal analysis) constitute a definite disadvantage.

4. Optimization of Detection Conditions

Detection conditions for film autoradiography and the phosphor image technique are optimized by estimating the exposure period. Sometimes underexposure or overexposure may take place, and the experiment has to be repeated. The unquestionable advantage of electronic autoradiography is that the image is displayed on the screen during data acquisition, making the optimization procedure easy.

5. Speed of Detection and Sample Throughput

Speed of detection is low in film autoradiography; it is higher for the phosphor image technique and the highest in the case of electronic autoradiography. The sample throughput, however, is lowest for electronic autoradiography because the detection is performed directly on the chromatoplate and the instrument measures a single plate at a

time. Visualization of the image (film development or scanning of the phosphor image plate) is a fast process, and simultaneous exposure of many chromatoplates can be carried out. This fact is especially advantageous when samples of low activity should be detected. With electronic detection, these samples may require several hours of instrument use, and in a multiuser environment (in which there are many chromatograms to be evaluated), the instrument can be in constant use. Although data capture (i.e., the exposure period) may take a few days or even weeks using film autoradiography or the phosphor image technique, the visualization process is fast enough, enabling high throughput detection of chromatograms.

6. Simplicity and Cost of Operation

Overall, radioactivity detection in TLC is not a complicated process. Film autoradiography and the phosphor image technique require very little experience, and becoming familiar with an electronic detection device is also not a long process. The cost of the various processes, however, varies significantly. The cost of equipment is lowest for film autoradiography and highest for electronic autoradiography. Once the technology is introduced, the cost of operation is reduced for the phosphor image technique (phosphor screens are reusable) and for electronic autoradiography (for direct radionuclide detection, only reactant gas is needed). It is higher, however, for film autoradiography, because both film and photochemicals must be supplied.

7. Data Evaluation

The primary source of data in a TLC separation is the chromatoplate itself. When radioactivity detection is carried out, the chromatoplates can be stored for a certain period of time (depending on the physical half-life of the radionuclide) unless zonal analysis for quantification is performed.

The radioactivity is spread along the surface of the chromatoplate to form an invisible image. This latent image should first be visualized and digitized. The visualized image, therefore, is “translated data.” Evaluation of images provides concentration plots, graphs, and chromatographic reports as results of the analysis. These considerations are of special importance with respect to the data handling, data storage, etc. according to GLP guidelines.

The process of visualization differs for the various methods of radioactivity detection in TLC. For electronic autoradiography, this procedure is carried out directly on the chromatoplate by realtime acquisition of the particles emitted from samples, and the data acquired are automatically digitized and stored in a computer. Film autoradiography and the phosphor image technique, on the other hand, use a substrate filled with grains that are sensitive to ionizing radiation, and then the “imprint” of the latent image is visualized. Exposed grains of an X-ray film are then developed by a chemical procedure to get an image perceptible to the human eye. The image then can be digitized and stored in a computer as mentioned in Section II.A.5. The grains of a storage phosphor (image plate) are illuminated with a laser beam to visualize the image. The digital signal obtained is then automatically stored in a computer.

A number of software packages for image analysis that are suitable for evaluation of autoradiograms can be purchased today. Concentration profiles of selected lanes can also be displayed and analyzed by these programs. Quantification can be performed either by a two-dimensional method (i.e., by computing the area and mean gray value of a selected spot or band on the image) or by a one-dimensional approach (peaks present in concentration profiles are subjected to quantification). Chromatographic properties such as R_f , spot area, and resolution can also be calculated.

Unlike visualization of the latent image and the digitization process, image evaluation, image handling, and image representation are the same for all methods of radioactivity detection in TLC. Electronic images are widely used in many fields of science (51). The application of image analysis for the evaluation of thin-layer chromatograms has recently been reviewed (52).

The data analysis of images obtained can then be evaluated by a number of suitable software packages. Among them are a couple of freeware programs (e.g., 53,54), both of which were developed at the National Institutes of Health to be used with IBM PCs or Macintosh computers.

The program packages allow display, editing, enhancement, and analysis of digitized images. For evaluation of TLC images, the software packages use both one- and two-dimensional approaches (i.e., selected lanes are evaluated to give concentration profiles of the lane, or in the region of interest the intensity of each spot or band is determined on a gray-scale basis). The method of data presentation is more or less standardized. Images and results (i.e., concentration profiles, result tables, etc.) are displayed directly on the screen and printed out by a high-resolution color or black-and-white printer. The exporting and importing of data as well as communication with other popular software packages (e.g., Microsoft Office) are essential requirements; therefore, popular picture files (such as BMP, JPEG, and TIFF) should be flexibly compatible.

It is highly desirable that the software package record the steps of evaluation to provide traceability. Because images produce large files, data archiving is very important, and by using a CD writer or a magnetic tape recorder, this task can be carried out in a cost-effective way.

In laboratories supervised by authorities (GLP and accredited laboratories), the use of validated computer systems is essential. Furthermore, limited access to unauthorized persons is of paramount importance to secure the data safety. Accordingly, the necessary arrangements should be made to provide standard operating procedures that comply with up-to-date requirements (e.g., Local Area Network, password, regulating supervisor, etc.).

III. PLATE CHARACTERISTICS AND HANDLING OF LAYERS

A. Sample Handling and Application onto the Layer

Radiation from radionuclides produces ionization when it passes through a material. In living tissues, this ionization (if sufficiently intense) could result in short-term damage; therefore, the importance of safety in radiation cannot be overemphasized. Radiation cannot be sensed by sight, smell, hearing, taste, or touch, and a person can easily

experience exposure to radiation without being aware of it at the time. However, the most widely used isotopes in TLC experiments (^3H , ^{14}C) present little hazard because their β -radiation only weakly penetrates tissue. Irradiation is local, usually to the hands. The eye lens is also vulnerable to β -radiation, so it is advisable to use safety glasses when working with radioactively labeled substances. ^{32}P is more hazardous than other β -emitters, because it emits higher energy β -particles.

Handling of radioactive material (during both sample preparation and chromatography) should generally be carried out in a fume hood or at least in a well-ventilated area. To minimize the risk of hazard, duration of exposure should be minimized. Because radiation dose decreases with the square of the distance from the source, a very effective yet simple protective measure is to maximize the distance between the worker and the radiation source. It is imperative to use shielding to dissipate the radiation energy for "hard" beta-emitters such as ^{32}P and ^{125}I . ^3H and ^{14}C require no shielding other than that afforded by gloves and protective clothing.

Procedures for handling chromatoplates and chromatoshets do not require special measures other than those mentioned so far. The sample can be applied by hand using a microsyringe, micropipet, or glass capillary. An automatic applicator can also be used, with which more reproducible application can be achieved. Care must be taken to avoid contamination of the plate during the application step and subsequent handling of the chromatoplate. After development, drying of the plate should be carried out in a fume hood or in an intensively ventilated area. It is advisable to store the plates in sealed containers to prevent contamination of laboratory areas.

B. Plate Characteristics

Application of TLC with radioactivity detection is mainly carried out with normal-phase layers. Silica gel 60 is by far the most frequently used adsorbent for separations. Properties of precoated layers (considering surface homogeneity, separation performance, and reproducibility) are superior to those of self-prepared layers, and therefore, ready-made layers are now used almost exclusively.

Conventional TLC is widely used owing to its low operating costs and simplicity and because it does not require instrumentation. Conventional layers are coated with $20\text{ }\mu\text{m}$ particle sorbents on various supports (glass, aluminum foil, plastic foil). Aluminum- and plastic foil-backed chromatoshets are preferred in most laboratories because the separated spots or bands can be removed by simply cutting them out for subsequent liquid scintillation counting (i.e., zonal analysis). Most applications use one-dimensional ascending development (the migration of the mobile phase is based on the phenomenon of capillary forces). In many cases, precoated layers with a concentration zone are used, because large volumes can be applied onto the layer, which is advantageous when diluted samples are to be used.

High-performance thin-layer chromatography (HPTLC; layers coated with smaller $3\text{--}10\text{ }\mu\text{m}$ particles), which provides smaller plate heights during separation, has also been used for planar radiochromatography (e.g., 44, 45).

Further decrease of plate height can be achieved by forced-flow migration of the eluent [overpressured layer chromatography (OPLC)], which is practically a planar HPLC technique. More details of this technique can be found in Chapter 7 of this

Handbook. An example of the combination of OPLC with radioactivity detection is discussed later in this chapter.

IV. APPLICATION OF TLC/OPLC WITH RADIOACTIVITY DETECTION

A. Radiochemical Purity and Stability Assessment

Radioactivity detection in TLC is widely used in the course of radiosynthesis, where it is applied for (a) evaluation of the success of radiosynthesis, (b) analysis of intermediate products and column chromatographic fractions, and (c) purification and isolation of the product. Because open-column chromatographic separation for preparative purposes is a standard method in radiosynthesis, the effluent fractions should often be analyzed simultaneously with column chromatography to determine the composition of the effluent. For this purpose, TLC with radioactivity detection is the best choice, because it can provide results very quickly if a fast detection process (electronic radioactivity detection) is applied.

The radiochemical purity of the starting material in investigations with radiolabeled compounds should always be determined. For this purpose, TLC can be successfully put to use. In this respect, TLC is very advantageous because the entire sample is detected after separation, whereas when using column chromatography, irreversible adsorption on the column of certain constituents cannot be ruled out. This phenomenon can lead to improper results. The above is true for the assessment of the stability of radiolabeled compounds as well as that of solutions and biological samples containing radiolabeled material.

Degradation of a radiochemical material may also occur during TLC separation. When this is suspected, the stability of the compound(s) should be checked in the eluent used for TLC separation. This assessment is performed by using a widely practiced method in TLC. Namely, the compound under scrutiny is applied on one edge of the chromatoplate. Then a two-dimensional separation is carried out using the same eluent in both directions for identical distances. If none of the components have degraded, a diagonal straight line containing all the components will be observed.

B. Metabolic Studies

Administration of a radiolabeled drug followed by separation of the radiolabeled compounds (i.e., metabolites) formed is a very convenient tool in *in vitro* and *in vivo* metabolic studies. TLC with radioactivity detection is widely applied in these studies because of its simplicity and low cost and the amount of information it provides. TLC is an excellent tool to determine the pattern of metabolites (metabolic profile) and the quantitative distribution of metabolites (i.e., to establish the metabolite balance). When using modern radioactivity detectors possessing high sensitivity, it is quite possible to analyze samples without any sample cleanup or preconcentration step. Nonetheless, a suitable sample preparation step in a metabolic study cannot usually be avoided. During

development of a sample cleanup procedure, TLC is usually the method of choice to characterize the fractions.

Simple visual inspection of metabolic profiles obtained by TLC very often provides important information regarding the metabolism of the compound studied. In Fig. 5, a chromatogram of rat urine samples after administration of a drug candidate is shown (56). It is apparent in the figure that two metabolites (M1 and M2) are conjugates, because they do not appear on the chromatograms after enzymatic digestion.

Metabolic profiles are usually determined in urine, feces, bile, and sera or plasma samples. They can be obtained from various organs of experimental animals, and even a discrete region of a whole-body section of the experimental animal can be the subject of TLC when sensitive radioactivity detection is applied (57). D'Argy and Sundwall have shown the differences in the patterns of metabolites in liver, kidney, lung, and blood of a cynomolgus monkey after administration of a radiolabeled test compound.

In recent years, there has been an increase in the use of *in vitro* systems for determination of xenobiotic metabolism. This is mainly because of the need for rapid screening for pharmacologically active compounds. In these studies, TLC with radioactivity detection is often the method of choice (e.g., 58–60) because of its high throughput.

In metabolic studies, another important application of TLC with radioactivity detection is to confirm structures of unknown constituents by comparing their chromatographic characteristics to those of authentic standards (provided the latter are available). It is generally accepted that compounds are identical if they possess identical characteristics as determined by two independent analytical methods. If a metabolite under study and a standard presumed to be the same compound have identical retention behavior by HPLC (usually performed by reversed-phase separation) and by TLC (usually performed by normal-phase separation), then it is highly probable that these compounds are identical. If, in addition, mass spectral characteristics provide further evidence for the similarity of the structure of the metabolite studied and that of the authentic compound, one can state that the two compounds are identical (see, for example, Ref. 60).

Preparative TLC with radioactivity detection is also used in metabolic studies to isolate metabolites for identification purposes. It is important that the plate be prewashed (usually with methanol) to remove contaminants that may be present in the layer. After separation, zones exhibiting radioactivity are removed from the plate (as described in Section II.A.4), and metabolites are eluted by a suitable solvent. Prior to structure elucidation, metabolites obtained in this way are subjected to further cleanup, mainly by preparative HPLC. With the advent of the HPLC-MS technique, however, the use of this isolation procedure for structure identification purposes has become less common. Nonetheless, this procedure is very useful when metabolites suspected of being conjugates are isolated and subsequently subjected to enzymatic hydrolysis. Repeated planar chromatography could help elucidate the identity of the compound(s) from which the conjugate formed.

OPLC-DAR, a new hyphenated technique, has numerous advantages over TLC-DAR in metabolic research (11, 61, 62). After optimization, normal- and reversed-phase HPTLC plates are equally suitable for OPLC separation and DAR detection of minor and major radioactive metabolites of different polarities. The main usefulness of this

combination of OPLC-DAR with a stepwise gradient lies in the separation and purification of weakly radioactive minor metabolites (11, 61).

The novel on-line OPLC-RD (radioactive detection) technique combined with HPLC-RD and OPLC-DAR is a new ideal, rapid, economic, and effective tool that can be applied advantageously to multicomponent metabolite research (62, 63).

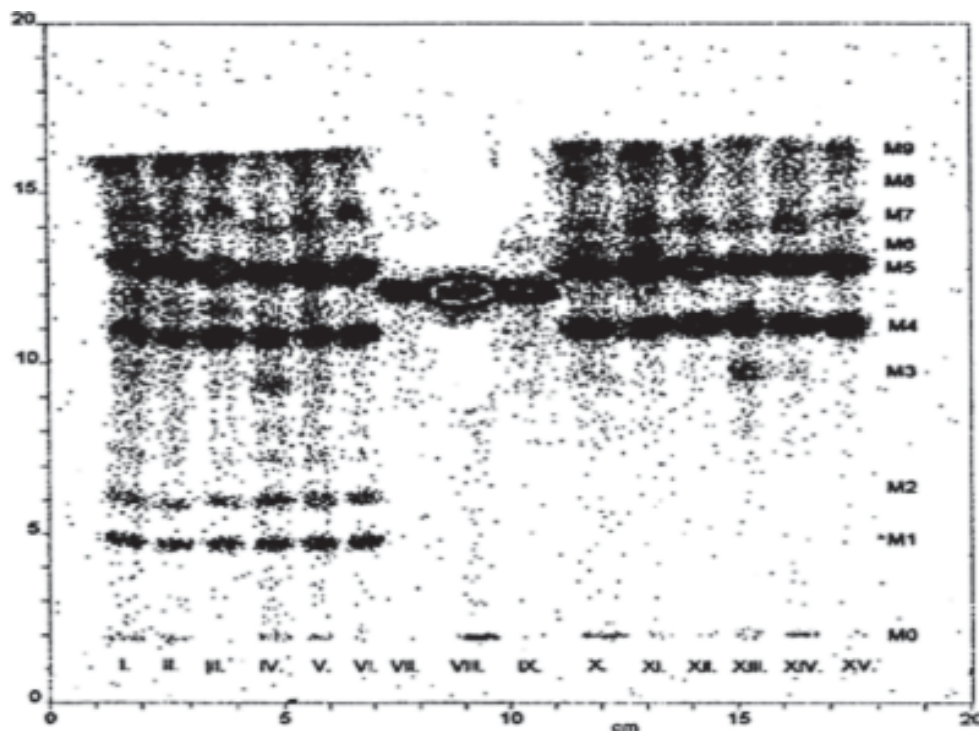


Figure 5 TLC-DAR chromatogram of metabolite profiles in rat urine after oral administration of ^3H -labeled drug candidate. Sample preparation was by SPE on Samplex C_{18} column.

Metabolite compounds were separated on a $20\text{ cm} \times 20\text{ cm} \times 0.2\text{ mm}$ silica gel 60F_{254} layer with 1-butanol–acetic acid–water (4:1:1) as the mobile phase. The DAR run time was 20 min. The tracks on the left were obtained from rat urine sampled 0–12, 12–24, and 24–48 h after oral and intravenous administration, respectively, without enzymatic hydrolysis. The tracks on

the right were obtained from rat urine sampled 0–12, 12–24, and 24–48 h after oral and intravenous administration, respectively, after enzymatic hydrolysis (β -glucuronidase–arylsulfatase). The center tracks were ^3H -labeled standards at different concentrations. (From Ref. 56.)

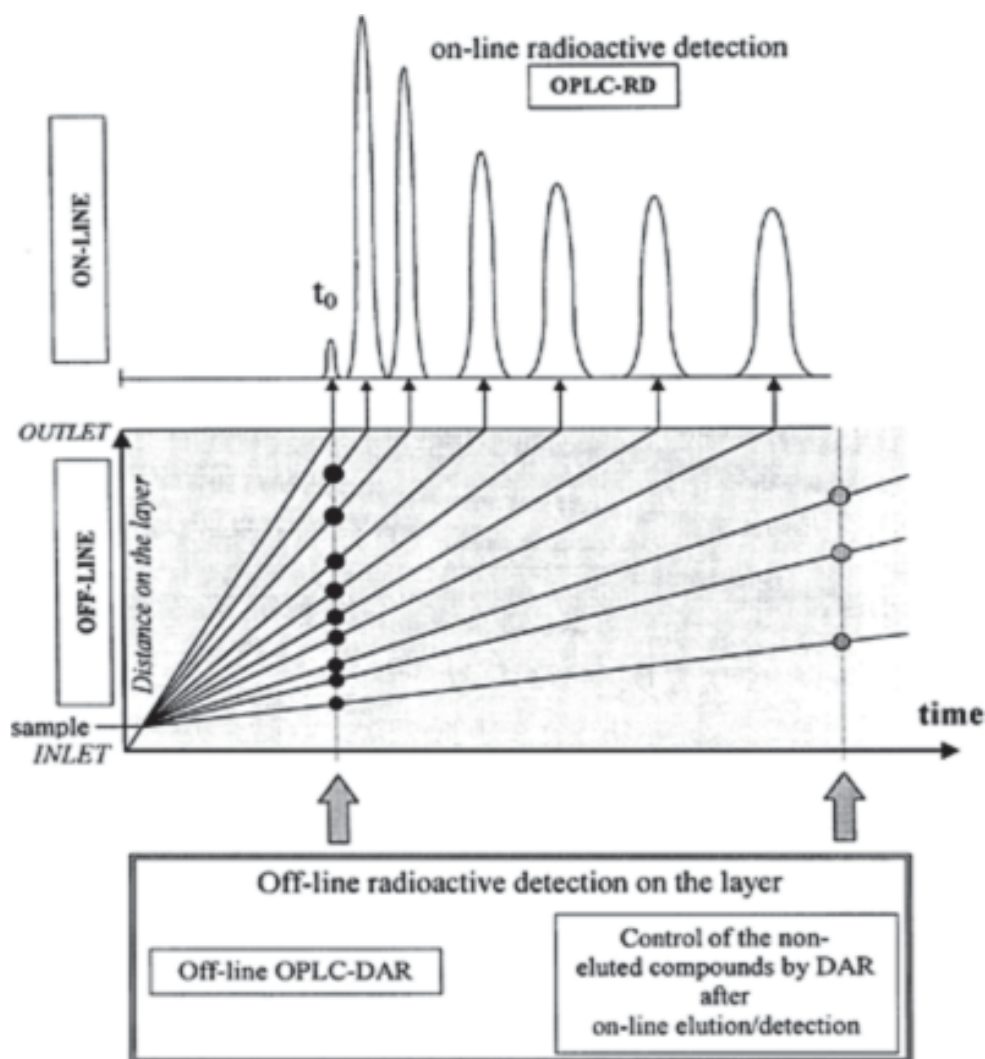


Figure 6 Scheme of OPLC separation using off-line and on-line radioactive detection. (From Ref. 62.)

The scheme of OPLC separation using off-line and on-line radioactive detection is summarized in Fig. 6. This complex procedure for metabolite separation, isolation, and identification using multidimensional chromatography combined with various spectroscopic methods proved to be useful in metabolic research (62).

C. Biochemical Investigations

Thin-layer chromatography combined with various radioactivity detection methods has been applied successfully in many fields of biochemistry. Using TLC, a simultaneous assay of several samples can be carried out in a short period of time. Both normal-phase and reversed-phase chromatography may be applied for this purpose (e.g., 64, 65). A further application is the ion-exchange TLC separation of ^{32}P -postlabeled DNA adducts (66).

V. COMBINATION OF PLANAR CHROMATOGRAPHY USING RADIOACTIVITY DETECTION WITH OTHER SEPARATION METHODS

Because there is a continuous increase in the demand for methods of analysis of complex mixtures, the coupling of two (or several) analytical techniques is a common practice. For example, TLC is often combined with another separation method such as gas chromatography or HPLC. Compound characterization (UV, IR, mass, and NMR spectrometry) techniques both in situ and after

Table 4 Summary of Important Aspects of Combined CFA, TLC-DAR, OPLC-DAR, TLC/OPLC-PIT, HPLC-RD, and GC-RD Radiochromatographic Techniques

Aspect	Technique used for separation and detection of radioactive compound						
	CFA ^a	TLC-DAR ^a	OPLC-DAR ^{a,b}	OPLC-RD ^c	TLC/OPLC-PIT ^a	HPLC-RD ^c	GC-RD ^c
Detection of different radioisotopes	++	++++	++++	+++	+++	+++	+
Simplicity	++++	++++	+++	++	+++	++	++
Speed of process	+	+	++++	+++	+++	+++	+++
Sensitivity	+	+++	++++	++	+++	++	++++
Resolution	++++	++	+++	++++	+++	++++	++++
Reproducibility	++	++	++++	++++	++	++++	++++
Linearity range of detection	+	++++	++++	+++	+++	+++	+++

Separation mode							
Two-dimensional	++++	++	++++	—	++++	—	—
Preparative	+++	++	+++	+++	+++	++++	—
On-line sample collection	—	—	++++	++++	—	++++	—
Cost of operation	++	+++	++++	++++	++++	++	++
Cost of instrumentation	++++	++++	+++	+++	++	+	+
GLP/GCP conformity	++	++	++++	++++	++++	++++	++++
Applicability in pharmaco-kinetic research	+	+	+	++	+	++++	++++
Applicability in metabolism research	+++	+++	++++	++++	++++	++++	+
Overall applicability	+	++	++++	++++	+++	+++	+++

+Low/expensive; ++good/fair; +++high/acceptable; ++++excellent/inexpensive.

^a Off-line operation.

^b With HPTLC layer.

^c On-line operation.

CFA, contact film autoradiography; PIT, phosphor imaging techniques; OPLC, overpressured layer chromatography; DAR, digital autoradiography; RD, radioactive detector.

Source: Refs. 11, 62, 63, 68.

isolation of the compound of interest are also widely applied in modern laboratories. Various analytical methods can be combined in situ utilizing the unique feature of TLC, the fact that detection is performed after chromatographic separation. Coupling of radioactivity detection and UV densitometry is a widely applied approach (e.g., 67).

Table 4 summarizes the various aspects of combined multidimensional radiochromatographic techniques. The aim of this approach is to provide a handy comparative analysis of all existing radioanalytical methods from a user perspective for everyday use.

VI. THE FUTURE OF TLC WITH RADIOACTIVITY DETECTION

The development of new detectors improved both the sensitivity and resolution of detection. It can be said that TLC provides the most sensitive detection in radiochromatography, and because of the high throughput as well as the simplicity of the procedure, TLC with radioactivity detection cannot be avoided in many fields of analysis. In addition, TLC can serve as an independent, complementary method to HPLC.

In the future the entire process will probably be automated, starting with sample application and finishing with data evaluation. The progress in the latter field is so

intense that sometime in the near future results of TLC separation will most likely be automatically incorporated into laboratory databases by means of laboratory information management systems (LIMSs). Nonetheless, this will not undermine the need for intelligent and skilled analysts during this work.

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13

Applications of Flame Ionization Detectors in Thin-Layer Chromatography

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I. INTRODUCTION

Conventional methods of quantification of fractions resolved by thin-layer chromatography (TLC) using techniques such as in situ spectrophotometry or photodensitometry are of limited utility for substances that contain weak or no chromophoric groups (1). Such fractions can be conveniently detected and quantified by sensitive vapor-phase detectors that are commonly used in gas chromatography (2, 3). Several systems for quantitative TLC using vapor-phase detectors have become known in recent years.

In one type of system, the substances fractionated on the adsorbent layer are vaporized, fraction by fraction, by successive application of heat, and the products formed are driven to a thermal conductivity detector (TCD) or a flame ionization detector (FID). In an early device working on this principle (4, 5), narrow quartz plates coated with an adsorbent such as silica gel are used for fractionation by TLC. After removal of the developing solvent, the chromatogram is encased in a rectangular quartz chamber and driven through a furnace, while nitrogen carrier gas flows through the chamber to an FID. Thereby, the fractions on the chromatoplate are vaporized consecutively by pyrolysis and/or evaporation, and the gaseous products from the various fractions are recorded as separate peaks.

In a recent modification of such a system, the substances separated on a TLC plate are consecutively vaporized by laser pyrolysis, and the resulting products are transported by a suitable carrier gas mixture to an FID or an electron capture detector for quantification (5a).

In my laboratory, the chromatoplate encased in a quartz chamber has been replaced by a chromatotube, i.e., a quartz tube whose inner surface is coated with a layer of silica gel or some other inorganic adsorbent (6). Fractionation on such chromatotubes is carried out as in conventional TLC and is followed by removal of the developing solvent by heating in a stream of an inert gas. Thereafter, the chromatotubes are scanned by driving them through a narrow furnace (800°C) while nitrogen, the carrier gas, flows through the

tube to an FID. During scanning, the individual fractions are vaporized consecutively and monitored by the FID. The technique of TLC using chromatotubes, also termed tubular TLC (2, 3, 7), was later modified by using different principles of vaporization of the fractions, i.e., combustion in situ on an adsorbent containing cupric oxide and detection of the carbon dioxide formed in a TCD with the aid of helium as carrier gas (8, 9). The techniques of pyrolysis and evaporation on an adsorbent such as silica gel and combustion on an adsorbent containing cupric oxide were subsequently integrated into a single instrumental system using the more sensitive vapor-phase detector, i.e., FID (10–12).

Tubular TLC-FID systems have been used so far mainly for the analysis of lipids and related substances. In this context, it should be of interest to note that tubular TLC systems have also been coupled with vapor-phase radiation detectors (2, 9) and with a mass spectrometer (13, 14).

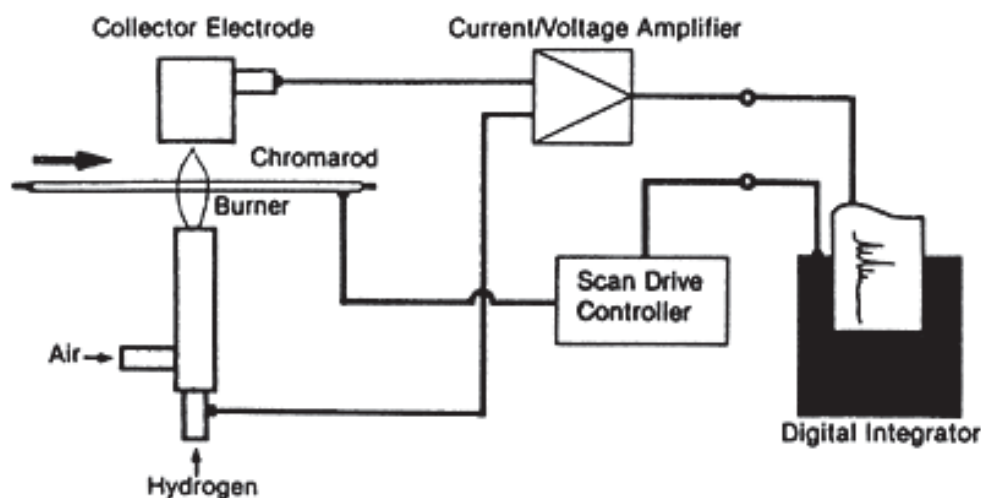


Figure 1 Scheme showing the working principle of a coated rod TLC-FID scanner, Iatroscan MK-5 series. (Reproduced with permission of Iatron Laboratories, Inc.)

In contrast to the above techniques, in which the fractions separated by TLC are vaporized from the adsorbent and transported by a carrier gas to a vapor-phase detector, another set of methods have become known in which TLC is carried out on adsorbent-coated quartz rods (14a) or quartz strips (14b). These chromatorods or chromatostrips are then driven through the flame jet of an FID to detect and quantify the separated fractions, which are recorded as peaks.

The following discussion is devoted to a description of the techniques of coated rod TLC in conjunction with an FID and the application of these techniques.

II. COATED ROD TLC-FID SYSTEMS

A. Chromatography

Thin quartz rods coated with an adsorbent such as silica gel or aluminum oxide embedded in porous sintered glass can be prepared by coating the rods with a suspension of the adsorbent and glass powder and baking at 800–1000°C (15). Such adsorbent-coated rods are commercially available. Chromarods S and S II, which are coated with silica gel having particle sizes of about 10 and 5 μm , respectively, have been recently replaced by Chromarod S III, which has better reproducibility. Chromarod A is coated with aluminium oxide with a particle size of about 10 μm .*

Before the coated rods are used for analysis, they must be meticulously cleaned free of any organic matter that produces signals in the FID. This is done conveniently, as in tubular TLC, by driving the rods through the flame jet of the FID in the TLC scanner (Fig. 1).

Sets of up to 10 coated rods are mounted on rod holders that are used for both chromatography and subsequent scanning. The samples to be analyzed are dissolved in a solvent and applied near one end of the coated rods, which are then developed with one or more suitable solvents as in conventional TLC. After that, the developing solvent is removed by heating, and finally the coated rod is scanned in the device described below.

Considerations concerning the choice of the developing solvents and the precautions to be taken to prevent contamination by foreign organic matter in coated rod TLC are similar to those

* Chromarods S III and A are supplied by Iatron Laboratories, Inc., 11–4, Higashi-Kanda 1 Chome, Chiyoda-ku, Tokyo 101, Japan, as well as its agencies, such as SES GmbH, Friedhofstr. 7–9, D-55234 Bechenheim, Germany.

Table 1 Applications of Coated Rod TLC-FID Systems

Substances analyzed	Chromarod	Solvent system (by vol.)	Ref.
Alkaloids and purine bases		Benzene–chloroform–diethylamine (36:8:1)	15
	S	Chloroform–diethylamine (30:1)	15
Alkaloids opium	S II	1. Benzene–ethanol (9.5:0.5) 2. Benzene–ethanol (9:1) ^a	33
Amino acids	S	Water– <i>n</i> -propanol (20:80)	15
Antibiotics			
Polyether carboxylic (abierixin, nigericin, grisorixin)	S II	Chloroform–methanol–formic acid (97:4:0.6)	33a
Anti-HIV agents			

<i>N</i> -Acyl aminonaphthalenesulfonic acid derivatives	S III	Methanol	34
Antioxidants and food preservatives	S	Hexane–acetic acid (125:1)	35
		Diethyl ether–hexane (2.5:97.5)	35
Fats, oils, and related products		1. Petroleum ether–benzene–formic acid (92:17:1)	36
Acylglycerols, fatty acids, and other lipid classes		2. Petroleum ether–diethyl ether–formic acid (97:4:1) ^a	
	S	Chloroform–benzene–formic acid (60:40:2)	37
	S II	Benzene–acetic acid–ethyl acetate–water (97:0.8:2:0.2)	38
	S III	Benzene–chloroform–acetic acid (70:30:2)	38a
	S IIb	Hexane–diethyl ether–acetic acid (70:30:0.1)	27
	S III	Hexane–diethyl ether–formic acid (96:31:1)	38b
	S III	Hexane–chloroform–isopropanol–formic acid (80:14:10:1)	38c
	S III	Hexane–ethyl acetate–diethyl ether–formic acid (92:4.5:3:1)	38d
	S II	Hexane–diethyl ether–acetic acid (95:5:0.3)	38e
	S III	1. Hexane–diethyl ether–formic acid (50:20:0.3)	38f
		2. Chloroform–methanol–ammonium hydroxide (58:10:2.5), twice ^{a,c}	
	S III	Hexane–acetic acid (100:1)	38g
	S III	1. Hexane–diethyl ether–formic acid (53:17:0.3), 10cm	38h
		2. Chloroform–hexane–methanol–acetone (55:5:3:7), 10 cm ^{a,c}	
	S III ^b	1. Chloroform	38i
		2. Chloroform–methanol–ammonium hydroxide (70:0.04:0.01) ^a	
Monoacylglycerol isomers	S II ^b	Chloroform–acetone (96:4)	39
	S II ^b	Chloroform–acetone–acetic acid	39

		(100:1:1)	
Surface waxes of grains	S II	Hexane–diethyl ether–acetic acid (98:2:1)	39a
Triacylglycerol subclasses	S ^d	Petroleum ether–diethyl ether–acetic acid (90:10:1)	40
	S ^d	Benzene–chloroform–acetic acid (90:8:2)	41, 42
	S	Chloroform–petroleum ether–acetic acid–methanol (25:25:1.5:0.15–0.40)	43
		Petroleum ether–diethyl ether–acetic acid (90:10:1)	44
Substances analyzed	Chromarod	Solvent system (by vol.)	Ref.
Methyl esters of isomeric fatty acids	S ^d	Hexane–benzene (1:1)	41, 45
	S ^d	Benzene	42, 46
Oxidation products of fats	S	1. Petroleum ether–benzene–formic acid (92:17:1) 2. Petroleum ether–diethyl ether–formic acid (97:4:1) ^a	47
	S II	Hexane–diethyl ether–acetic acid (97:3:1)	48
	S III	Light petroleum (b.p. 60–70°C)–diethyl ether–acetic acid (90:10:2)	48a
	S III	Hexane–diethyl ether–acetic acid (98:2:1)	48b
	S	Chloroform–methanol–acetic acid–water (60:30:9:2) ^a	49
Phospholipids of oils and oilseeds	S III	Chloroform–methanol–acetic acid–water (80:14:14:3)	49a
	S III	Petroleum ether–diethyl ether–acetic acid (90:10:2)	49b
Sucrose polyesters	S III	Petroleum ether–diethyl ether–acetic acid (90:10:2)	49c
	S	Benzene–methanol (100:1)	35
Flavoring agents			
Herbicides			
Metolachlor	A	<i>n</i> -Pentane–diethyl ether (70:30)	49d
Lipid reference mixtures			
Less polar lipid classes	S	Hexane–diethyl ether (90:10)	50
			51

Phospholipids and glycolipids		(85:15)	52
		Petroleum ether–diethyl ether–formic acid (96:3:1)	53
		(85:15:0.1)	28
		1. Diethyl ether–ethanol (75:25), 2 cm	54
		2. Petroleum ether–diethyl ether–acetic acid (90:10:1), 10cm	
		3. Methanol, 4 cm, twice ^a	
	S II	Hexane–diethyl ether–formic acid (52:8:0.1)	54a
	S III	Hexane–diethyl ether–formic acid (80:20:0.04)	54b
	S III	1. Hexane–diethyl ether–formic acid (70:30:0.2)	54c
		2. Chloroform–acetone–formic acid (99:1:0.2) ^{a,c}	
		Chloroform–methanol–water (74.1:23:1:2.8)	29
		(70.0:26.2:3.8)	54
		(80:35:5)	52
		(60:30:3.5)	50, 55
	S II	Chloroform–methanol–water (46:26:2.5)	21
	S III	1. Chloroform–acetone (30:20)	55a
		2. Acetone–formic acid (49:1) ^{a,c}	
	S III	Chloroform–methanol–water (40:20:1)	55b
	S II	1. Chloroform–methanol–15 N ammonium hydroxide (60:10:1), 6 cm	56
		2. Hexane–diethyl ether (50:2), 10 cm ^a	
Substances analyzed	Chromarod	Solvent system (by vol.)	Ref.
Lipids of biological and biomedical interest			
Animal tissue (phospholipids)	S IF	1. Hexane–diethyl ether–formic acid (70:30:1) ^c	56a
		2. Chloroform–methanol–acetic acid–formic acid–water (80:35:2:1:3), twice ^{a,c}	
		3. Chloroform–methanol–30% aqueous ammonium hydroxide (60:35:0.9) ^{a,c}	
	S III	1. Benzene–chloroform–formic acid (50:20:1.5), 30 min	56b
		2. Chloroform–methanol–29.3% ammonium hydroxide (50:50:5), 15 min ^a	
	S III	Hexane–diethyl ether–acetic acid (60:70:0.2)	56c

S III	Chloroform–methanol–water (80:35:5)	56d
S III	1. Benzene–chloroform–formic acid (50:20:1.5) 2. Chloroform–methanol–ammonium hydroxide (50:50:5) ^a	56e
S III	1. Hexane–benzene (70:30) 2. Benzene–chloroform–formic acid (70:25:2) 3. Chloroform–methanol–water (70:25:3) ^a	56f
S III	1. Hexane–diethyl ether–formic acid (98:2:0.1) 2. Hexane–diethyl ether–formic acid (80:20:1) 3. Chloroform–methanol–ammonium hydroxide (50:50:5) ^a	56g
S	Hexane–diethyl ether–formic acid (90:10:2)	56h
S	1. Chloroform–methanol–acetic acid–water (67:28:2:3) 2. Hexane–diethyl ether–formic acid (90:10:2) ^a	56h
S III	1. Hexane–chloroform–isopropanol–formic acid (80:14:1:0.2) 2. Acetone 3. Chloroform–methanol–water (70:30:3) ^{a,c}	56i, 56j
S III	Hexane–diethyl ether–formic acid (98:2:0.5)	56k
S III	Hexane–diethyl ether (87:13)	56k
S III	Chloroform–methanol–water (57:47:1.4)	56k
S III	Hexane–diethyl ether–formic acid (82:18:0.1)	56l
S III	Hexane–diethyl ether–acetic acid (87:13:0.05)	56m
S III	Hexane–diethyl ether–formic acid (99:1:0.5)	56n
S III	Acetone	56n

	S III	Chloroform-methanol-water (50:40:10)	56n
Substances analyzed	Chromarod	Solvent system (by vol.)	Ref.
	S III	1. Hexane-diethyl ether-formic acid (66:17:0.2) 2. Chloroform-methanol-water (50:40:10) ^{a,c}	56o
	S III	Hexane-diethyl ether-formic acid (97.3:2.1:0.6)	56p
	S III	Hexane-diethyl ether (87.1:12.9)	56p
	S III	Diethyl ether-acetone (57.1:42.9)	56p
	S III	Chloroform-methanol-water (56.3:42.3:1.4)	56p
	S III	1. Chloroform-methanol-water (57:12:0.6), 2.5 cm 2. 1,2-Dichloromethane-chloroform-acetic acid (46:6:0.05), 9 cm, twice 3. Hexane-diethyl ether-acetic acid (98:1:1), 11.5 cm ^a	56q
	S III	1. Hexane-diethyl ether-formic acid (98:2:0.1) 2. Hexane-diethyl ether-formic acid (80:20:0.1) 3. Acetone 4. Chloroform-methanol-ammonium hydroxide (50:50:5) ^a	56r
	S II	Dichloroethane-chloroform-acetic acid-isopropanol (92.8:0.1:0.03)	56s
	S III	1. Hexane-diethyl ether-acetic acid (55:15:0.15) 2. Chloroform-methanol-water (40:28:2) ^{a,c}	56t
Blood platelet	S	Chloroform-methanol-water (60:30:3.5)	50
Heart mitochondria	S	1. Petroleum ether-diethyl ether (85:15) 2. Chloroform-methanol-water (80:35:3) ^c	57
Thoracic aorta	S II	1. 1,2-Dichloroethane-chloroform-acetic acid (46:6:0.5), 8 cm, twice 2. <i>n</i> -Hexane-diethyl ether-acetic acid (98:1:1), 11 cm ^a	57a
Lymphocytes	S	1. Hexane-diethyl ether (9:1) 2. Chloroform-methanol-water (60:30:3.5) ^c	55
Plasmalogens in synaptosomal membrane of brain	S	1 Petroleum ether-diethyl ether (85:15), exposure to HCl 2. Chloroform-methanol-water (80:35:3) ^c	58
Heart lipids		1 1,2-Dichloroethane-chloroform-acetic acid	59

		(92:8:0.1), 11 cm 2. Chloroform–methanol–water (68.5:29:2.5), 10 cm ^c	
	S II	1. 1,2-Dichloroethane–chloroform–acetic acid (46:8:0.05), 9 cm, developed twice 2. Hexane–diethyl ether–acetic acid (98:2:1), 11 cm ^a	60
Amniotic fluid	S	Chloroform–methanol–water (80:25:3)	29
Marine organisms	S II	1. Hexane–diethyl ether–formic acid (98:2:0.2) 2. Hexane–diethyl ether–formic acid (80:20:0.2) ^a	61, 62
Substances analyzed	Chromarod	Solvent system (by vol.)	Ref.
	S II	1. Hexane–diethyl ether–formic acid (80:20:2) 2. Chloroform–methanol–water (80:35:3) ^c	63
	S II	Hexane–diethyl ether–formic acid (97:3:1)	64
	S	Hexane–diethyl ether–acetic acid (93:7:0.3)	65
	S II	Dichloroethane–chloroform–acetic acid (92:8:0.1)	66
	S II	1. Dichloromethane ^c 2. Dichloromethane–methanol–70% aqueous ethylamine (70:20:10), developed twice	67
	S III	Hexane–diethyl ether–formic acid (97:3:1)	67a
	S III	Hexane–diethyl ether–acetic acid (67:70:0.2)	67b
Serum	S III	1. Pentane–ethyl acetate (50:50) 2. Pentane–ethyl acetate (90:10) ^a	68
Phosphorylated acylglycerols	S III	1. Dichloromethane–benzene–ethanol (96%, v/v)–formic acid (70:20:8:3), 3 cm 2. Hexane–dichloromethane–benzene–ethanol (96%, v/v)–formic acid (75:15:5:5:3), 5.5 cm 3. Hexane–diethyl ether–formic acid (70:20:0.1), 9.5 cm ^a	69
Pesticides and growth regulators	S	Hexane	15
	S II	Hexane–dichloromethane–methanol (25:25:0.25)	70
	S II	Methanol–2 N hydrochloric acid (2:3)	71
Petroleum and coal products Heavy oils and synthetic fuels	S	Pentane–isopropanol (95:5)	72

	S II	1. Pentane–isopropanol (95:5) 2. Benzene–isopropanol (80:20) ^a	73
	A	1. Hexane, 9 cm 2. Benzene, 5 cm 3. Dichloromethane–methanol (60:40), 2.5 cm ^a	74
Diesel exhaust particulates	S II	Hexane	75
Liquid coal products	S III	1. <i>n</i> -Pentane–isopropanol (95:5), 8 cm 2. Benzene–isopropanol (80:20), 13 cm ^a	76
Polymers			
Dimerized fatty acids	S II	Dichloromethane–diethyl ether–acetic acid (60:1:1)	77
	S II	1. Hexane–diethyl ether–formic acid (94:3:3), 10 cm 2. Methylene chloride–methanol–acetic acid (98:0.5:1.5), 2.5 cm ^a	78
	S II	Dichloromethane–diethyl ether–acetic acid (60:1:1)	78a
Polybutadienes	S	Carbon tetrachloride–tetrahydrofuran (100:1)	79
Styrene–cellulose copolymers	S	Benzene	80
Substances analyzed	Chromarod	Solvent system (by vol.)	Ref.
Psychopharmaceuticals	S	Cyclohexane–diethyl ether–acetic acid–methanol (65:25:9:1)	81
Saccharides			
Glucitol derivatives	S II	Benzene–ethyl acetate (9:1)	82, 83
Xylitol derivatives	S II	Toluene–acetone (9:1)	83
Steroidal compounds			
Bile acids	S	1. Upper phase of toluene–acetic acid– water (50:45:5) 2. Acetic acid–water–methanol–chloroform (10:5:20:65), 5.5 cm ^a	84
Sex hormones	S	Acetone–chloroform (20:80)	15
	S	Benzene–methanol (90:10)	15
Cardiotonic steroids	S	Chloroform–methanol (90:10)	15
Suprarenal hormones	S	Chloroform–methanol (95:5) or (90:10)	15
Sulfonamides	S	<i>n</i> -Butanol–ethanol–0.1 N acetic acid (60:20:20)	15

Surfactants and detergents			
<i>n</i> -Alkylbenzenesulfonates	S	Chloroform–methanol (80:20)	85
Fatty acid esters of pentaerythritol	A	1. Acetone–petroleum ether (15:85), 12 cm 2. Diethyl ether–methanol–petroleum ether (10:12:80), 8 cm 3. Chloroform–diethyl ether–formic acid–toluene (40:10:4:60), 4 cm 4. Chloroform–methanol–toluene (40:20:40), 2 cm ^a	86
Ethylene oligomers	S II	1. Benzene–ethyl acetate (6:4), 10 cm 2. Ethyl acetate–acetic acid–water (8:1:1), 9 cm ^a	87
Tetrodotoxin	S II	Butanol–acetic acid–water (60:15:30)	88
Vitamins (hydrophilic)	S	Acetone–water (90:10)	15

^aSuccessive developments.

^bImpregnated with boric acid.

^cScanning after each development.

^dImpregnated with silver nitrate.

^eImpregnated with oxalic acid.

of tubular TLC. Reusability of the chromatorods is excellent provided proper care is exerted in their handling and storage as indicated in the supplier's manual and in a monograph (16).

B. Principle, Instrumentation, and Operation

The working principle of a commercially available instrument, the Iatroscan TH-10, designed to scan the adsorbent-coated chromatorods is depicted in Fig. 1. The rod holder carrying the developed chromatorods is driven at a chosen speed from one end to the other through the flame jet of the FID. Thereby, the fractions resolved on each of the rods are successively vaporized or pyrolyzed, and the ionizable carbon is converted to ions that are detected in the collector electrode. The FID signals from each fraction are amplified and recorded as separate peaks. The Iatroscan TH-10 MK 5 instrument is supplied by Iatron Laboratories, Inc. or by its agencies.

Proper choice of the operating variables, in both chromatography and scanning, is crucial for satisfactory sensitivity of detection and reproducibility in quantitative analysis by coated rod TLC-FID techniques (3, 16–25). Thus, the sample size (18, 19, 23, 24), the flow rate of hydrogen fed to the FID (24, 26), and the speed of scanning (23–26) have considerable effect on the linearity of response of the FID, the baseline stability of the FID signal, and the reproducibility of the response factors, respectively. Various aspects of quantification in coated rod TLC-FID techniques are discussed in several recent reviews (26a–26e).

C. Quantitative Analysis

In the coated rod TLC-FID systems, the components of various chromatographic fractions are vaporized in the flame jet partly by physical evaporation and partly by pyrolysis, i.e., breakdown of the parent molecule. Consequently, the FID response may not correlate with the amount of ionizable carbon theoretically present in the compound. Therefore, reliable quantitative results can be obtained by coated rod TLC-FID techniques only if the observed peak area is corrected by using proper calibration factors, which should be routinely determined. The use of suitable internal standards (19, 27–29) and empirical calibration with mixtures of known composition (16, 17) are the methods of choice for reliable quantitative analysis. The standard deviations reported for the major components of mixtures are of the order of 4–6% (26), 6–13% (18, 30), and 2–10% (24).

III. APPLICATIONS OF COATED ROD TLC-FID SYSTEMS

The coated rod TLC-FID system using Chromarods and the Iatroscan TH-10 instrument has found numerous applications for a wide variety of substances. Table 1 lists some of the applications of this coated rod TLC-FID system from readily accessible literature. Further applications are detailed in earlier reviews (16, 17, 17a, 26a–26e) and the brochures provided by Iatron Laboratories, Inc. Some recent developments that involve the use of novel vapor-phase detectors should be able to widen the range of possible applications of the coated rod TLC-FID systems. Examples are the flame thermionic ionization detector (FTID), which responds to compounds containing nitrogen and halogen atoms, the flame emission photometric detector (31, 31a), which detects substances containing sulfur and/or phosphorus, and the chemiluminescent nitrogen detector, coupled on-line with FID (32).

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14

Amino Acids and Their Derivatives

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I. INTRODUCTION

Thin-layer chromatography (TLC) can separate amino acids and their derivatives with high resolution and with many other advantages over other methods. This chapter emphasizes procedures that have been used successfully in this laboratory, but contributions from other laboratories are also mentioned. Thus, this is not an exhaustive review of the field; however, references of such reviews are cited. The methods described in this chapter can serve as starting points for particular applications.

II. SEPARATION OF AMINO ACIDS

A. Introduction

There are about 20 amino acids, which constitute an alphabet for all proteins and differ only in the structure of the side chain R. The amino acids exist as zwitterions at their isoelectric points (pI). The structures, names, abbreviations, and pK_a and pI values for the 20 common amino acids are summarized in Fig. 1. Amino acids are generally soluble in water, but some are less soluble than others. Alcoholic 0.5 M or 0.1% HCl should be used to prepare solutions of amino acids that are only sparingly soluble in water.

B. Preparation of Test Materials

The analysis of amino acids required either for the determination of the composition of proteins or for the investigation of certain fluids or extracts derived from animals or plants requires the removal of materials such as peptides, proteins, carbohydrates, urea,

salts, and lipids from them by specific operations, and proteins and peptides need to be hydrolyzed.

1. Removal of Macromolecules

Various kinds of precipitating agents are used to remove macromolecules. A comparison of deproteinizing methods (1) has shown that in certain cases a considerable loss of amino acids must be taken into account.

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Structure	Name	Abbreviation ^a	pKa ₁ (α-Carboxyl)	pKa ₂ (α-Amino)	pKa ₃ (side chain)	P ¹
Nonpolar side chain						
	Glycine	Gly(G)	2.3	9.6		6.0
	Alanine	Ala (A)	2.3	9.7		6.0
	Valine	Val (V)	2.3	9.6		6.0
	Leucine	Leu (L)	2.4	9.6		6.0
	Isoleucine	Ile(I)	2.3	9.7		6.1
	Methionine	Met (M)	2.3	9.2		5.8
	Phenylalanine	Phe(F)	1.8	9.1		5.5
	Proline	Pro(P)	2.0	10.6		6.3
Neutral polar side chain						
	Serine	Ser (S)	2.2	9.2		5.7
	Threonine	Thr (T)	2.6	10.4		6.5

Figure 1 Structures, pK_a values, and pI values of the 20 common amino acids.

2. Removal of Urea and Salts

The addition of a trace amount of urease (2) provides the best results for urine samples, whereas salts can be conveniently removed by passing the sample (3) through a cation-exchange resin column.

3. Enrichment of Amino Acids in Urine

Aliquots of urine (10 mL) are lyophilized and then extracted with methanol–1 M HCl (4:1) (1 mL) and centrifuged. Then 20 μ L of supernatant liquid is applied to the thin layer (4).

Structure	Name	Abbreviation ^a	pK_{a1} (α - carboxyl)	pK_{a2} (α - Amino)	pK_{a3} (side chain)	pI
	Cystine	Cys (C)	1.7	10.8	8.3 (Sulphydryl)	5.0
	Asparagine	Asn (N)	2.0	8.8		5.4
	Glutamine	Gln (a)	2.2	9.1		5.7
	Tyrosine	Tyr (Y)	2.2	9.1	10.1 (Phenolic hydroxyl)	5.7
	Tryptophan	Trp (W)	2.4	9.4		5.9
Charged polar side chain						
	Aspartate	Asp (D)	2.1	9.8	3.9 (β - Carboxyl)	3.0
	Glutamate	Glu (E)	2.2	9.7	4.3 (γ - Carboxyl)	3.2
	Histidine	His (H)	1.8	9.2	6.0 (Imidazole)	7.6

$\begin{array}{c} \text{H} \\ \\ \text{DOC}-\text{C}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}_3^+ \\ \\ \text{NH}_3^+ \end{array}$	Lysine	Lys (K)	2.2	9.0	10.5	9.8
					(ε-Amino)	
$\begin{array}{c} \text{H} \\ \\ \text{DOC}-\text{C}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-\text{C}-\text{NH}_2 \\ \qquad \qquad \qquad \\ \text{NH}_3^+ \qquad \qquad \text{NH}_2^+ \end{array}$	Arginine	Arg (R)	2.2	9.0	12.5	10.8
					(Guanidino)	

4. Enrichment of *N*-Methylated Amino Acids from Biological Fluids and Protein Hydrolysates

An aliquot of the fluid or hydrolysate is adjusted to 0.1 M in HCl, treated with an equal volume of aqueous Reinecke's salt (2%), and left in the refrigerator overnight. Then the precipitate is filtered off and dissolved in acetone. The solution is centrifuged, and the supernatant is mixed with an equal volume of water and extracted several times with ether. The lower layer containing water, acetone, and ether is evaporated to dryness, and the residue is dissolved in aqueous 10% propan-2-ol solution for use in TLC (4).

5. Hydrolysis of Proteins

Proteins are hydrolyzed to amino acids by treatment with acid, alkali, or enzymes. Each method has certain disadvantages as shown in Table 1. The most commonly used methods for total hydrolysis are described below.

Method for acid hydrolysis. A sample (50–100 mg) of air-dried or lyophilized protein is weighed into a tube, and 6 M HCl (1 mL for 5 mg of protein) is added. The tube is evacuated using a vacuum desiccator (8), sealed, and placed in a circulating air oven at 110°C with good temperature control (7). After hydrolysis for the appropriate period of time (24, 48, or 71 h), it is centrifuged gently. Then the tubes are cracked open and the HCl is removed as quickly as possible using a stream of N₂. The HCl can alternatively be neutralized by adding solid Ba(OH)₂ (up to pH 7) and removing white BaSO₄ by filtration or centrifugation. The clear hydrolysate may be frozen in an acetone–solid CO₂ bath, placed in a vacuum desiccator over NaOH or KOH, and lyophilized. However, clear hydrolysates can also be stored in the refrigerator for several days.

For more detailed discussion on hydrolysis of proteins for amino acid analysis one may consult Light and Smith (9), Moore and Stein (7), Savoy et al. (10), or Perham (11).

Method for sulfur-containing amino acids. Moore (12) determined cysteine and cystine as cysteic acid by performic acid oxidation. However, methionine can also be determined as methionine *S,S*-dioxide.

Performic acid is prepared by adding H₂O₂ (1 mL, 30%) to formic acid (9 mL, 88%) and allowing the mixture to stand at room temperature for 1 h. It is then cooled to 0°C. Performic acid (2 mL) is added to the protein (containing about 0.1 mg cystine) in a Pyrex tube, which is then allowed to stand at 0°C for 4 h for soluble proteins or overnight for proteins that do not dissolve. Then HBr (0.30 mL, 48%) is added with swirling, the mixture is evaporated to dryness at 40°C using a rotary evaporator, and the protein is hydrolyzed in vacuo with HCl (3 mL, 6 M) at 110°C for 18 h. The hydrolysate is treated as mentioned above, before analysis. A rapid method of protein hydrolysis by microwave

irradiation has been described (12a). That article describes a design for a reusable Teflon-Pyrex tube for fast inert gas flushing under microwave irradiation. Results have been compared with those of conventional heating methods in terms of destruction or degradation of certain labile amino acids and their recoveries depending upon hydrolysis time by microwave irradiation.

Table 1 Disadvantages of Methods of Hydrolysis of Proteins

Method of hydrolysis	Disadvantages
1. Acid 8 N H ₂ SO ₄ at 110°C for 18 h	1. Tryptophan is destroyed; Ser and Thr are partially destroyed. 2. Presence of carbohydrates leads to formation of a black material, humin.
6 M HCl at 110°C for 18 h	1. Tip, Asn, Gln destroyed; Ser, Thr, Tyr partially lost. 2. Cys and Met are either partially destroyed or oxidized to cysteic acid and Met-S,S-dioxide, respectively.
2. Alkali	
Ba(OH) ₂	1. Partial or complete destruction of Arg, Cys, Ser, Thr.
NaOH (5) or LiOH (6)	2. Causes racemization and some deamination. LiOH is reported to be best (6) for tryptophan determination.
3. Enzymes pepsin, trypsin, papain, chymotrypsin	1. Each enzyme is generally specific for a particular peptide bond. 2. May produce hydrolysis of enzymes, which would interfere with the amino acid analysis.

C. Chromatographic Techniques

1. Adsorbents and Thin Layers

A variety of adsorbents such as silica gel, alumina, polyamide, and cellulose are available commercially for use in TLC work. Alumina and silica gel are used with or without suitable binder such as gypsum or starch. Mixtures of two adsorbents or adsorbents impregnated with certain reagents such as 8-hydroxyquinoline and various metal ions have also been used successfully to improve resolution. By far the most thin-layer work has been done on layers prepared from water-based slurries of the adsorbents. Even with the same amount and type of binder, the amount of water that is used for a given slurry varies among the different brands of adsorbents. For example, in the case of cellulose the amount of powder to be mixed with water varies depending on the supplier; Serva, Camag, and Whatman have recommended the use of 60–80 mL, 65 mL, and 25 mL of water, respectively, for 10 g of their cellulose powders. These slurries may be prepared by shaking a stoppered flask or by homogenizing for a few seconds with a mechanical mixer. On the other hand, for the preparation of an aluminum oxide slurry (acid, basic, or

neutral) it is recommended that one use 35 g of aluminum oxide with 30 mL of water with spreading equipment, and 6 g of adsorbent in 15 mL of ethanol-water (9:1) mixture for pouring directly onto the plate without a spreading apparatus. Korzun et al. (33) used a slurry of 120 g of alumina G in 110 mL of water to prepare 1 mm thick layers for preparative TLC. Cellulose powders in general contain impurities that are soluble in water or organic solvents, which should be removed by washing the cellulose powder several times with acetic acid (0.1 M), methanol, and acetone and drying before use. The layer is made by “turbo mixing” MN (Macherey-Nagel) cellulose-300 (15 g) for 10 min in distilled water (90 mL) and then spreading it to give a 0.25 mm thick layer. The layers are left overnight to dry.

The cellulose layers have several advantages; e.g., they are stable, they can be used with various specific reagents, and they give reproducible data. They are recommended particularly for quantitative evaluation by densitometry. The drawbacks of cellulose layers are that corrosive reagents cannot be used and the sensitivities of detection reactions of certain amino acids are lower than on silica gel layers.

The best known and most widely used adsorbents for TLC purposes are from Merck, but products of other firms can be used satisfactorily. Precoated plates are widely known, and an increasing number of workers use them for the investigation of amino acids and their derivatives. For example, ready-made cellulose layers from Macherey-Nagel (Germany) containing MN cellulose-300 in appropriately bound form are one of the best-known products. Chiralplate from the same firm, for the separation of enantiomers of amino acids and their various derivatives, contains a coating of reversed-phase silica gel impregnated with a chiral selector and copper ions. Use of homemade thin-layer plates has been found to be more convenient in our laboratory, and it is recommended that one not change the brand of adsorbent during a particular set of experiments.

2. Preparation of Thin Plates

A slurry of silica gel G (50 g) in distilled water (100 mL) is prepared and spread with the help of a Stahl-type applicator on five glass plates of 20×20 cm to obtain 0.5 mm thick layers. The plates are allowed to set properly at room temperature and are then dried (activated) in an oven at an appropriate temperature (60–90°C) for 6 h or overnight. The plates are cooled to room temperature before the samples are applied.

The same method has been used successfully to prepare plates with silica gel, silica gel-polyamide, and cellulose and with these adsorbents impregnated with a variety of reagents including HDEHP, TOPO, 8-hydroxyquinoline, dibenzoyl methane, and several metal salts (13–30). Brucine (20) and tartaric acid (22) were also mixed in slurries of silica gel as impregnating reagents to resolve enantiomers of amino acids and their PTH derivatives. Mixtures of H₂O– EtOH or other organic solvents can also be used depending on the nature of the impregnating reagents. Citrate (31) and phosphate (32) buffers have also been used for slurring silica gel in place of water. It is customary to use 0.25 or 0.50 mm thick layers in activated form, but for preparative purposes 1–2 mm thick layers are best (33).

3. Development of Chromatograms

Standard solutions of amino acids are prepared in a suitable solvent such as 70% EtOH or 0.1 N HCl in 95% ethanol. These solutions are applied generally as tight spots, 1–2 cm from the bottom of each layer, by using a glass capillary or Hamilton syringe. In the beginning, a higher concentration, e.g., 500 ng or more, is applied; however, the detection limits are determined for the system developed by repeating the experiment with lower concentrations.

The chromatograms are generally developed in rectangular glass chambers, which should be paper-lined for good chamber saturation and preequilibrated for 20–30 min with solvent prior to placing the plates inside. The time taken depends on several factors such as the nature of the adsorbent, the solvent system, and the temperature.

The developed chromatograms are dried in a chromatography oven between 60°C and 100°C, and the cooled plates are usually sprayed with ninhydrin reagent. Heating at 90–100°C for 5–10 min produces blue to purple zones of all amino acids except proline (yellow spot).

The same method is adopted for both one- and two-dimensional modes. The locating reagent is used after the second run, and a more polar solvent is generally used for developing the chromatogram in the second dimension.

4. Detection of Amino Acids on Thin-Layer Chromatograms

After drying the chromatogram it may be viewed under ultraviolet (UV) light if the absorbent had a fluorescent indicator or the compounds—such as dansyl amino acids—fluoresce. Solvent fronts may be seen, which indicate irregularity of solvent flow. Ninhydrin is the most commonly used reagent for the detection of amino acids, and a very large number of ninhydrin reagent compositions have been reported in the literature. The reagent may be made slightly acidic with a weak acid following the use of an alkaline solvent and vice versa. Constancy of color formed may be attained by the addition of complex-forming cations (Cu^{2+} , Cd^{2+} , or Ca^{2+}), and specific colors may be produced by the addition of bases such as collidine or benzylamine. Some of the ninhydrin compositions and their applications are described below.

1. A solution of ninhydrin (0.2% in acetone) is prepared with the addition of a few drops of collidine or glacial acetic acid. The chromatogram is dipped or sprayed with it and dried at 60°C for about 20 min or at 100°C for 5–10 min. Excessive heating causes a dark background. The sensitivity limit is 1 μg . Most amino acids give a violet color, whereas aspartic acid (Asp) gives bluish-red, and proline (Pro) and hydroxyproline (Hyp) give yellow. (See Fig. 1 for abbreviations for 20 common amino acids.)

2. Ninhydrin (0.3 g) in *n*-butanol (100 mL) containing acetic acid (3 mL) is sprayed on a dried, solvent-free layer, which is then heated for 30 min at 60°C or for 10 min at 110°C (34, 35). Detection limits range from 0.001 μg for alanine (Ala) to 0.1 μg for proline and aspartic acid (35).

3. Ninhydrin (0.3 g), glacial acetic acid (20 mL), and collidine (5 mL) are made up to 100 mL with ethanol (36) or ninhydrin (0.1% w/v) in acetone-glacial acetic acid-collidine (100:30:4) (37).

4. A solution of cadmium acetate (0.5 g) in water (50 mL) and glacial acetic acid (10 mL) is made up to 500 mL with acetone. Portions of this solution are taken, and solid ninhydrin is added to give a final concentration of 0.2% g. The chromatogram is sprayed

and heated at 60°C for 15 min. It is interesting to note the results immediately and again after 24 h, at room temperature (38). Alternatively, the layer is impregnated thoroughly with the reagent and the colors are allowed to develop in the dark at room temperature for 24 h (39). This reagent gives permanent colors, mainly red but yellow for proline. Sensitivity is 0.5 nmol.

5. Ninhydrin (1.0 g) in absolute ethanol (700 mL), 2,4,6-collidine (29 mL), and acetic acid (210 mL) has been used for spraying on solvent-free cellulose layers (40). The chromatogram is then dried for 20 min at 90°C.

6. Development of ion-exchange resin layers in ninhydrin (1%) in acetone containing collidine (10%) at room temperature for 24 h or at 70°C for 10 min has also been recommended (41).

7. A spray of ninhydrin (0.1% or 0.2%) in acetone on chromatograms followed by heating at 60°C or 90°C for 10–20 min has also been used (13, 20, 22–25).

8. Polychromatic reagents: Moffat and Lyttle (42) developed a polychromatic ninhydrin reagent. It consisted of (a) ninhydrin (0.2%) in ethanol (50 mL)+acetic acid (10 mL)+2,4,6-collidine (2 mL) and (b) a solution of copper nitrate (1.0%) in absolute ethanol. The two solutions are mixed in a ratio of 50:3 before use. Krauss and Reinbothe (43) replaced ethanol by methanol and also achieved polychromatic amino acid detection by joint application of ninhydrin and primary, secondary, or tertiary amines. The layers were first sprayed with diethylamine, dried for 3 min at 110°C, cooled, and then sprayed with 0.2% methanolic ninhydrin and heated for 10 min at 110°C, when the spots of amino acids appeared on a pale blue background. Use of ninhydrin (0.27 g), isatin (0.13 g), and triethylamine (2 mL) in methanol (100 mL) gave spots of amino acids on a yellow background.

Several other reactions have also been used for the detection of specific amino acids (Table 2). Oxalic acid (ethanolic 1.25%), dithiooxamide (ethanolic saturated), or dithizone followed by ninhydrin was used to aid identification and detect amino acids with various specific colors (54a). Acetyl acetone–formaldehyde detected amino acids as yellow spots under UV (54b). By using isatin–ninhydrin (5:2) in aqueous butanol (54c) or by modifying ninhydrin detection reagent by addition of D-camphor (54d) and various acids (54e), identification of amino acids was improved. Spraying of layers with 1,3-indanedione or *o*-mercaptobenzoic acid prior to ninhydrin improved sensitivity limits and color differentiation in amino acid detection (54f). 3,5-Dinitrobenzoyl chloride was used for detecting amino acids at a 3–4 μ g level (54g), and synchronization of timing was achieved by coupling pneumatic nebulization with optical fiber-based detection in a chemiluminescence TLC system to detect dansyl amino acids (54h). A new spray reagent, *p*-dichlorodicyanobenzoquinone, detected amino acids with 0.1–1 μ g detection limits and produced various distinguishable colors that facilitate identification (54i). Chromatograms sprayed with ninhydrin (0.3 g ninhydrin in 100 mL of *n*-butanol plus 3 mL of glacial acetic acid), air-dried for 5 s, resprayed, and heated in an oven at 110°C for 10 min gave the best sensitivity, stability, and color differentiation in comparison to different recipes of ninhydrin and fluorescamine sprays (72a).

D. TLC Systems for Amino Acids

An extensive bibliography of literature references from 1974 to 2000 on the TLC separation of amino acids has been provided by Sherma (55a–f), Sherma and Fried (56), and Zweig and Sherma (57). Silica gel and cellulose have been the major choice of adsorbents for one- or two-dimensional resolution of amino acids. These have been used as is (untreated) or impregnated with some other reagent employing a large number of solvents. Some of the successful systems for one- and two-dimensional resolution of amino acids are given in Tables 3 and 4, respectively. Sleckman and Sherma (69) compared the separation of amino acids on silica gel, cellulose, and ion-exchange thin layers using *n*-butanol–acetic acid–water (3:1:1) and discussed advantages and disadvantages

Table 2 Detection Reactions for Specific Amino Acids

Amino acid	Reagent	Reference
Arg	8 -Hydroxyquinoline	44
Arg	α -Naphthol, urea, Br ₂	45
Arg, His, Lys	BiI ₃	46
Asp	Ninhydrin, borate soln, HCl	47
Cys, Met	NaN ₃ , iodine	48
Gly	<i>o</i> -Phthalaldehyde, KOH	49
His	Sulfanilic acid	50, 51
Ser, Thr, Tyr	Sodium metaperiodate, Nessler reagent	53
Trp	<i>p</i> -Dimethylaminobenzaldehyde	54

Table 3 Some Solvent Systems for TLC of Amino Acids on Silica Gel

Solvent system	Ratio	Reference
Silica gel		
96% Ethanol–water	7:3	35
<i>n</i> -Propanol–water	7:3	
<i>n</i> -Butanol–acetic acid–water	4:1:1	
<i>n</i> -Propanol–34% NH ₄ OH	7:3	
<i>n</i> -Propanol–water	1:1	58
Phenol–water	3:1	

Isopropanol–water	7:3	59
Butyl acetate–methanol–acetic acid–pyridine	20:20:5:5	25
<i>n</i> -Butanol–formic acid–ethanol	3:1:1	24
<i>n</i> -Butanol–acetic acid–chloroform	3:1:1	22
<i>n</i> -BuOH–HOAc–EtOAc–H ₂ O	50:20:30:20	60
<i>n</i> -Propanol–H ₂ O	7:3	54a
<i>n</i> -BuOH–H ₂ O–HOAc	40:7:5	54b
Cellulose ³		
Propan-2-ol–butanone–1 M HCl	60:15:25	61
2-Methylpropan-2-ol–butanone–acetone– methanol–H ₂ O–conc. NH ₃	20:1:14:5	
Butanol–acetic acid–H ₂ O	4:1:5	63
Methanol–H ₂ O–pyridine	20:5:1	
Propan-1-ol–8.8% NH ₃	4:1	
Chloroform–MeOH–17% NH ₃	20:20:9	40
Butanol–acetone–Et ₂ NH–H ₂ O	10:10:2:5	
Phenol–water	3:1	
Ethyl acetate–pyridine–HOAc–H ₂ O	5:5:1:3	64
<i>n</i> -Butanol–acetic acid–H ₂ O–EtOH	10:1:3:0.3 or 4:1:10:1	65
Ethanol–conc. HCl	30:1	54c
<i>n</i> -BuOH–HOAc–H ₂ O	4:1:1	
Pyridine–acetone–NH ₄ OH–H ₂ O	26:17:5:12	65a
Propan-2-ol–formic acid–H ₂ O	25:3:2	

^aFor good separation, used in pairs for two-dimensional chromatography.

of each system. The hR_f values in these systems are given in Table 5. The data are of great value for separating and detecting amino acids by one-dimensional TLC.

Amino acids have also been grouped for the separation of 18-component mixtures (separation I) and essential amino acid mixtures (separation II) by calculating the resolution possibilities for each pair of acids (Table 6). Dale and Court (70), using Avicel F TLC plates (Analtech, Luton, UK), investigated six systems for one- or two-dimensional chromatography and reported hR_f values for 35 amino acids. Loads up to 0.05 M could be used for preparative work. Amino acids chromatographed in the presence of trichloroacetic acid (used in deproteinizing serum samples) show anomalous behavior, and this interference can be almost completely removed by predevelopment (two times) in ether saturated with formic acid (71). Separation of 18 amino acids on re

versed-phase (RP) thin layers including C₁₈ chemically bonded silica gel in *n*-propanol–H₂O (7:3) was reported by Sherma et al. (72), and it has been mentioned that the migration sequences on RP layers were generally the same as on cellulose and silica gel. Besides the above-mentioned ion-exchange systems (69, 72), sorbents with ion-exchange properties such as DEAE–cellulose have also been used as the stationary phase for TLC separation of amino acids. Verceanst et al. (73) used *n*-butanol–acetic acid–water (5:1:6, upper phase) and pyridine–water (4:1) in one- and

Table 4 Some Systems for Two-Dimensional TLC

Direction I	Direction II	Reference
Silica gel		
<i>n</i> -Butanol–HOAc–H ₂ O (4:1:5, upper phase)	Phenol–water (15:1, w/w)	66
Chloroform–MeOH–17% NH ₃ (2:2:1)	Phenol–H ₂ O (3:1)	67
<i>n</i> -Butanol–HOAc–H ₂ O (4:1:5, upper phase)	CHCl ₃ –MeOH–17% NH ₃ (2:2:1)	
Butanone–pyridine–H ₂ O–HOAc (70:15:15:2)	CHCl ₃ –MeOH–17% NH ₃ (2:2:1)	35
Cellulose		
Propanol–HCOOH–H ₂ O (40:2:10)	<i>t</i> -Butanol–methyl ethyl ketone–0.88 NH ₃ –H ₂ O (50:30:10:10)	68
Propan-2-ol–butan-2-one–1 M HCl (60:15:25)	2-Methyl propanol–butan-2-one–acetone–MeOH–H ₂ O (0.88) NH ₃ (10:4:2:1:3:1) or 2-Methyl propanol–butanone–propanone–methanol–H ₂ O (40:20:2:1:14:5)	61

Table 5 hR_f ($R_f \times 100$) Values for Amino Acids on Different Layers

	E						
	A	B	C	D	FX _A	FX _B	FX _C
Ala	41.9	29.0	32.4	28.8	50.9	51.2	53.6
Ser	26.9	16.1	26.4	24.1	67.1	64.1	67.1
Tyr	50.0	36.1	49.4	45.9	11.9	13.9	15.5
Glu	34.4	22.6	30.0	28.2	34.5	29.4	30.6
Asp	26.3	14.8	25.3	21.8	71.5	68.2	68.6
Arg	25.6	11.0	12.9	10.0	1.8	2.2	2.2
Gly	29.4	14.8	25.9	23.5	55.6	52.4	53.6

Leu	75.0	63.9	51.8	48.8	21.8	17.8	19.4
Ile	73.1	60.0	49.4	47.1	27.8	22.2	23.3
Trp	55.6	36.1	54.1	51.8	1.8	2.2	2.2
Met	41.0	22.5	47.3	43.5	28.0	27.2	25.0
Val	63.1	48.4	43.5	41.2	42.5	35.0	34.4
Lys	18.1	7.1	10.0	7.1	7.5	5.0	5.6
His	20.0	7.1	11.7	7.1	10.6	8.9	10.0
Phe	67.5	54.8	52.4	50.0	14.4	11.1	11.7
Thr	32.5	21.3	30.0	27.6	67.1	60.0	57.2
Cys	6.9	3.2	14.1	7.1	55.9	50.0	57.9
Pro	43.8	33.5	24.1	21.2	—	—	—
Time for 17 cm, h	7	11	4.5	7.5	6.5	6	2

A, Baker Flex cellulose sheets; B, Baker Flex microcrystalline cellulose sheets; C, Whatman K6 silica gel plates; D, Whatman high-performance silica gel plates; E, Fixion ion-exchange sheets (Na^+ form). FX_A , no prior treatment; FX_B , layer preequilibrated with equilibration buffer for 16 h; FX_C , layer preequilibrated as for FX_B but at 45°C. Solvent for A, B, C, D, 2-butanol–acetic acid–water (3:1:1); solvent for E and run buffer, 84 g citric acid +16 g NaOH +5.8 g NaCl +54 g ethylene glycol + 4 mL cone. HCl (pH 3.3); solvent equilibration buffer, run buffer diluted 30 times (pH 3.8).

Source: Ref. 69.

Table 6 Group Separation of Amino Acids

System as in Table 5	Separation ^a	Amino acids resolved
A	I	Leu, Phe, Trp, Ala, Glu, Ser, Lys, Cys, Tyr
	II	Leu, Phe, Trp, Thr, Lys
B	I	Leu, Phe, Tyr, Val, Glu, Asp, Lys
	II	Leu, Phe, Val, Trp, Thr, Lys
C	I	Trp, Ile, Val, Ala, Ser, Cys, Lys
	II	Trp, Ile, Val, Thr, Lys
D	I	Trp, Us, Val, Ser, Glu, Arg, Lys
	II	Trp, Ile, Val, Thr, Lys
FX_A	I	Thr, Gly, Val, Glu, Met, Leu, Phe, His, Lys, Arg
	II	Thr, Val, Met, Leu, Phe, His, Lys, Trp
FX_B	I	Asp, Thr, Gly, Val, Met, Leu, Tyr, His, Lys, Trp
	II	Thr, Val, Met, Leu, Phe, His, Lys, Trp

FX _C	I	Asp, Thr, Gly, Val, Met, Leu, Tyr, His, Lys, Trp
	II	Thr, Val, Met, Leu, Phe, His, Lys, Trp

^a Group I: 18-component mixture of amino acids. Group II: Mixture of essential amino acids.

Source: Adapted from Ref. 69.

two-dimensional chromatography of the main protein amino acids on Whatman DEAE-cellulose. Krafczyk and Helger (74) used a double layer consisting of a 2 cm band of cellulose+cation exchanger (45+5 g) in aqueous CM cellulose (0.05%), with the remaining portion of the layer prepared from cellulose SF suspension. A mixed layer of cellulose and the ion exchanger Amberlite CG-120 was effectively used in a similar way by Copley and Truter (75). A laboratory experiment was devised for students to illustrate qualitative determination of amino acids in egg lysozyme (75a). Amino acid separation on a newly synthesized support named aminoplast (75b) was compared with that of starch and cellulose using n-butanol–acetone–water (4:3:3) and propan-2-ol–formic acid–water (8:1:2). Nevertheless, silica gel continued to be the most widely used and most successful material.

In studies of collagen metabolism, proline and hydroxyproline were separated by TLC on silica-impregnated glass fiber sheets with 2-propanol–water (7:3), located by spraying with ethanolic ninhydrin reagent and autoradiography, and recovered by dialysis (75c). Amino acid mixtures were analyzed by separation on C₁₈ layers with MeOH–water (1:1, 1:3, 1:5) mobile phases, detection with ninhydrin, and derivative spectrometry of the colored reaction products (75d).

III. SEPARATION OF AMINO ACID DERIVATIVES

Separation and identification of derivatives of amino acids such as DNP, PTH, dansyl, and DABITC amino acids is very important, particularly in the primary structure determination of peptides and proteins. Adequate descriptions of the preparation of PTH (76–79), dansyl (80–82), and DNP amino acids (83–86) are available in the literature, and the methods of identification of N-terminal amino acids by TLC and other techniques have been reviewed by various workers (87–91). The present section describes briefly the preparation of such derivatives and TLC resolution data reported in recent years.

When an—NH₂ group of an amino acid at the N-terminal end of a polypeptide (or a free molecule) is coupled with phenyl isothiocyanate, the corresponding PTH derivative is obtained. The sequential degradation of amino acids as their PTH derivatives from a polypeptide followed by their identification is used to establish the primary structure of proteins (76). Both manual and automated and liquid-phase and solid-phase methods are currently used for small and large poly-peptides. During an automated degradation the sequencer can deliver several PTH amino acids in 24 h, which must be identified rapidly to match the output. In view of the limited space in this review, the method of formation of a PTH derivative from an amino acid and from the N-terminal end of a polypeptide is only briefly discussed in the following subsection. It follows the results of some successful TLC systems used for resolution and identification of PTH amino acids. The PTH amino acids are sensitive to light, and optically active derivatives racemize easily.

A. PTH Amino Acids

1. Preparation of PTH Amino Acids*

Amino acid (0.5–1.0 g) is added to aqueous pyridine (1:1) (25 mL) in a stoppered tube. The solution is adjusted to pH 9.0 with 1 N NaOH and placed in a water bath at 40°C. Phenyl isothiocyanate (1.2 mL) is added with shaking during a reaction time of about 30 min. Additional alkali is added to maintain the pH at 9. The mixture is extracted repeatedly with benzene to remove excess reagent and pyridine. When there is no further uptake of alkali, a slight excess of 1 N HCl is added to precipitate the PTC amino acid. The mixture is filtered and warmed with HCl (1 N, 30 mL) at 40°C for 2 h. The PTH derivative crystallizes upon cooling, and further yields are obtained by concentration of mother liquors. Most of the derivatives are recrystallized from aqueous acetic acid or ethanol.

The PTH derivatives of serine, threonine, and cystine are extremely labile. Ingram (92) applied milder conditions for serine and threonine. These were condensed with phenyl isothiocyanate at room temperature, and then the pH was brought to 1. Some pink oil was separated and discarded. The reaction was allowed to proceed for 2 days at room temperature, when PTH derivatives crystallized out. Sjoquist (78) described a method for microlevel preparation of PTH amino acids.

2. PTH Amino Acids from N-Terminal Polypeptides

Since the original report of Edman (76), many modifications to the experimental conditions have been reported (93–95). The technique developed by Fraenkel-Conrat and Harris (93) has been used successfully in this laboratory (96, 97) and is described below.

The peptide (0.2–0.3 mg) is dissolved in aqueous dioxane (50%, 4 mL), and the pH is adjusted to 8.7–9.0 with 0.01 N NaOH. The mixture is stirred for 1.5 h at 40°C with phenyl isothiocyanate (0.1 mL), keeping the pH constant. The reaction mixture is extracted seven times with benzene, and the aqueous solution is concentrated to dryness in vacuo.

The sodium salt of PTC peptide is redissolved in water (2–10 mL), and aliquots corresponding to 0.2–1.0 μ M are made 3 N with respect to hydrochloric acid and 0.2– 1.0×10^{-4} M with respect to peptide by addition of the correct amounts of water and 5.7 N HCl. The rate of release of phenylthiohydantoin can be determined by following the change of the absorption maximum of the solution (from 240 nm or lower to 265–270 nm) over a period of about 2 h. If the transformation takes place too slowly for a given peptide, the effect of increasing the temperature to 40–45°C should be investigated.

The PTH amino acids are extracted with ethyl acetate (with the exception of PTH arginine and PTH histidine), and residual peptide is recovered by concentration of the aqueous solution. The residue is redissolved in 50% aqueous dioxane and submitted to the same cycle of operations.

3. TLC Resolution, Detection, and Identification of PTH Amino Acids

Thin-layer chromatography has been used for the identification of PTH amino acids since Edman and Begg (98) used it in their classical work describing the automatic sequencer. TLC of PTH amino acids has been reviewed by Rosmus and Deyl (99), Niederwieser (100), Allen (101), and Bhushan and Reddy (102). Various TLC systems with different kinds of adsorbents, such as alumina, silica gel, and polyamide, have been reported. The methods of detection include (a) spraying a dilute solution of fluorescein on a plain layer of silica gel so the spots become visible

* After Ref. 76.

as dark areas against a yellow background in UV light; (b) exposing the dried chromatograms to iodine vapors to locate the spots as light brown compact zones (19, 21, 22, 26, 30); and (c) use of iodine azide solution, which causes bleached spots on a light brown background to be observed. The iodine azide method is considered less sensitive and causes difficulties in demarcating the exact spots and measuring the correct R_f .

Nakamura et al. (103) carried out two-dimensional TLC using plates coated with polyamide containing three fluorescent additives when all PTH amino acids showed colored spots under UV light. About 0.1 nmol of PTH amino acid could be detected, and characteristic changes in the colors of some derivatives were observed when the plate was heated after being sprayed with an alkaline solution. Typical results are given in Table 7. A rapid color-coded system was described by Walz and Reuterby (104) (Table 8). The colors produced allowed easy identification of those amino acids that had nearly identical R_f values, for example, Lys and Ser degradation products, Ala/Met/Phe, and Tyr/Thr. The method was considered significant because it gave positive identifications of PTH-Ser/Lys/Glu/Asp and their respective amides, which could not be identified by gas chromatography (GC). A compilation of solvent mixtures useful in TLC of PTH amino acids on various supports is given in Table 9.

Table 7 Characteristic Colors of PTH Amino Acids on Polyamide FM Plates Containing Mixed Fluorescent Additive

PTH amino acid	Color after	
	Second treatment	Alkaline treatment
Valine	Red	Red
Proline	Red	Red
Alanine	Red	Red
Glycine ^a	Red	Brownish red
Serine	Red	Brownish red (blue)
Asparagine ^a	Red	Greenish brown (bluish green) ^b
Aspartic acid	Red	Brownish red (dark brown)
Methionine ^a	Red	Brownish red

Leucine	Red	Brownish red
Isoleucine	Red	Red
Lysine	Red	Red
Tyrosine ^a	Red	Red (bluish green) ^b
Threonine ^a	Red	Bluish green (blue)
Glutamine ^a	Red	Greenish brown (white yellow)
Glutamic acid	Red	Red
Phenylalanine ^a	Red	Greenish red (white blue) ^b
Tryptophan ^a	Red	Greenish red (white blue) ^b
Histidine ³	Red	Blue (light blue) ^b
Arginine ^a	Red	Purple (blue) ^b
Cysteic acid	Red	Brownish red (dark brown)

^a Spots appear yellow, except glycine (pink).

^b Fluorescent.

Solvents: Toluene–*n*-pentane-acetic acid (6:3:2) and acetic acid–water (1:3) for first and second dimension, respectively. Alkaline treatment: Spraying of 0.05 M NaOH in methanol–water (1:1), heating at 150°C for 30 min, UV.

Source: Ref. 103.

Table 8 Characteristic Colors of PTH Amino Acids Following Ninhydrin Spray

PTH derivative	Color properties	NH ₄ OH color change
Proline	UV, colorless	Light blue after heating
Alanine	Purple	Deeper color
Glycine	Orange	
Serine	UV, purple	
Serine breakdown	Faint orange	Weak red
Asparagine	Yellow	More intense
Carboxymethylcysteine	UV, purple	
Methioninesulfone	Light tan	
Methionine	Faint tan	
Lysine	Very faint pink	Weak blue after heating
Tyrosine	UV, yellow before spray	Intense yellow
Threonine	Colorless	Light tan
Glutamine	Dark green	Dark blue

Phenylalanine	UV, colorless	Faint yellow
Tryptophan	UV, yellow before spray	Deep yellow
Aspartic acid	UV, pink	Darker
Glutamic acid	Gray	Dark blue

Silica gel plates, without fluorescent indicator, developed in heptane–CH₂Cl₂–propionic acid (45:25:30) and xylene–MeOH (80:10), sprayed with iodine azide and 1.7% ninhydrin in MeOH–collidine–HOAc (15:2:5), heated at 90°C for 20 min; color changes induced by blowing a saturated ammonia atmosphere over the ninhydrin plate.

Source: Ref. 104.

Resolution and identification of PTH amino acids on silica or polyamide layers by TLC as discussed above showed difficulties in achieving discrimination between derivatives of Leu/Ile (106) and resolution of complex mixtures without two-dimensional chromatography (113). Also, difficulties in resolving combinations of PTH-Phe/Val/Met/Thr (114, 115) and PTH-Asp and -Glu were observed. The use of chloroform–acetic acid (27:3) and chloroform–methanol (30:4) has been found extremely satisfactory for the discrimination between PTH-Asp and PTH-Glu, because the difference in their hR_f values was around 10 units (116). The difficulties, previously posed and as noted above, in resolving and identifying various combinations of PTH amino acids can be overcome by the use of certain solvent systems (30a, 30b) given in Table 9.

B. Dansyl Amino Acids

Derivatization of free amino group of amino acids with 5-methylamino naphthalene-1-sulfonyl (dansyl) chloride has become increasingly popular for N-terminal end determinations in proteins and for manual Edman degradation (91). The dansylation reaction has also been used as one of the most sensitive methods for quantitative amino acid analysis (117, 118).

1. Dansylation of Peptides*

The peptide is dissolved in a small volume of 1 % (v/v) aqueous triethylamine, and a small aliquot (1 μ L, 0.5 nmol) is transferred to a dansyl tube (4×50 mm) that has been preheated at 500–600°C overnight. The sample is dried, and sodium bicarbonate (0.2 M, 3 μ L) and dansyl chloride (3 μ L) solution (5 mg/mL in dry acetone) are added. The tube is sealed with parafilm and incu-

* Method of dansylation from Ref. 119.

Table 9 Various Solvent Systems for TLC of PTH Amino Acids

Solvent system	Ratio	Reference

A. Polyamide		
<i>n</i> -Heptane– <i>n</i> -BuOH–HOAc	40:30:9	105
Toluene– <i>n</i> -pentane–HOAc	60:30:35	106
Ethylene chloride–HOAc	90:16	107
Toluene– <i>n</i> -pentane–HOAc	60:30:35	93
EtOAc– <i>n</i> -BuOH–HOAc	35:10:1	108
<i>n</i> -BuOH–MeOH–HOAc (+30 mg butyl PBD-fluorescent reagent per liter)	19:20:1	109
B. Silica gel		
Heptane–CH ₂ Cl ₂ –propionic acid	45:25:30	104
Xylene–MeOH	80:10	
CHCl ₃ –EtOH and	98:2	110
CHCl ₃ –EtOH–MeOH (in the same direction)	89:25:0.75:10	
CHCl ₃ – <i>n</i> -butyl acetate	90:10	111, 112
Diisopropyl ether–EtOH	95:5	
CH ₂ Cl ₂ –EtOH–HOAc (or on cellulose)	90:8:2	
Petroleum ether (60–80°)–acetic acid	25:3	30a
<i>n</i> -Hexane– <i>n</i> -butanol	29:1	
<i>n</i> -Hexane– <i>n</i> -butyl acetate	4:1	
Pyridine–benzene	2.5:20	30b
MeOH–CCl ₄	1:20	
Acetone–dichloromethane	0.3:8	

bated for 30 min at 50°C, checking that the yellow color has disappeared. The contents are dried, and HCl (6 M, 5 μ L) is added. The tube is then sealed with a flame and incubated at 100°C for 6 h. The dansyl hydrolysate is ready for TLC after the addition of ethanol (95%, 3 μ L).

2. TLC of Dansyl Amino Acids

The two-dimensional TLC introduced by Woods and Wang (120), on polyamide sheets using water–formic acid (200:3) for the first-direction run and benzene–acetic acid (9:1) for rerun at right angles to the first run, has mostly been employed in conjunction with the Edman dansyl (121) technique for sequencing peptides. Hartley (122) reported the use of 1 N ammonia–ethanol (1:1) as a third solvent on two-dimensional chromatograms for the separation of especially basic dansyl amino acids. Gray (123) reported that the solvents of Wood and Wang could not resolve Dns-Glu/Asp, Dns-Thr/Ser, and α -Dns-

Lys/ ϵ -Dns-Lys/Arg/His. However, a third run in ethyl acetate–acetic acid–methanol (20:1:1) in the direction of solvent 2 resolved Dns-Glu/Asp, and Dns-Thr/Ser. A further run in the direction of solvents 2 and 3 using 0.05 M trisodium phosphate–ethanol (3:1) is supposed to resolve the monosubstituted basic dansyl amino acids. Most of the TLC systems reported up to 1978 required more than two runs for complete resolution of all dansyl amino acids. Bertrand et al. (124) reported two-dimensional TLC of 26 dansyl derivatives of amino acids on polyamide plates requiring caution with the spotting of the compounds. Mettrione (125) developed a few solvent systems to yield separations of basic, acidic, and hydroxyl derivatives in the presence of other amino acids without resorting to the “third solvent system”; the solvent systems and R_f values are given in Table 10. Additionally, a large number of solvent systems for one- or two-dimensional resolution of dansyl amino acids on silica gel or polyamide are summarized in Table 11. Bhushan and Reddy (126) worked out several successful and effective solvent systems for the resolution of almost all the dansyl amino acids on silica gel plates (Tables 12 and 13) and reviewed TLC of dansyl and DNP amino acids (126a).

Table 10 R_f Values for Dansyl Amino Acids in Various Solvent Systems on Polyamide Sheets

Dansyl amino acid	R_f in solvent system									
	A	B	C	D	E	F	G	H	I	J
1. Ala	0.53	0.48	0.49	0.69	0.69	0.57	0.81	0.68	0.43	0.79
2. Arg	0.05	0.03	0.03	0.91	0.39	0.09	0.76	0.22	0.01	0.06
3. Asp	0.08	0.07	0.10	0.69	0.88	0.10	0.88	0.37	0.12	0.19
4. Cys	0.03	0.03	0.04	0.19	0.43	0.22	0.78	0.09	0.03	0.06
5. Glu	0.15	0.10	0.15	0.66	0.88	0.02	0.88	0.34	0.05	0.30
6. Gly	0.32	0.21	0.32	0.69	0.63	0.48	0.80	0.48	0.28	0.69
7. His	0.07	0.05	0.13	0.96	0.76	0.32	0.84	0.36	0.06	0.18
8. Ile	0.77	0.54	0.65	0.40	0.57	0.71	0.78	0.76	0.60	0.84
9. Leu	0.70	0.49	0.59	0.34	0.57	0.71	0.78	0.75	0.54	0.80
10. Lys (mono)	0.35	0.21	0.38	0.22	0.09	0.63	0.72	0.58	0.09	0.79
11. Lys (di)	0.53	0.37	0.48	0.78	0.69	0.35	0.82	0.40	0.39	0.76
12. Met	0.52	0.36	0.51	0.43	0.59	0.68	0.80	0.62	0.55	0.81
13. Phe	0.57	0.38	0.53	0.31	0.43	0.68	0.77	0.62	0.51	0.81
14. Pro	0.85	0.66	0.71	0.55	0.74	0.46	0.84	0.75	0.69	0.90
15. Ser	0.12	0.07	0.16	0.81	0.71	0.49	0.82	0.42	0.10	0.44
16. Thr	0.15	0.10	0.26	0.81	0.74	0.57	0.82	0.56	0.16	0.56
17. Tyr	0.63	0.47	0.61	0.00	0.00	0.84	0.73	0.65	0.58	0.91

18. Val	0.72	0.56	0.61	0.47	0.67	0.71	0.81	0.80	0.61	0.88
19. Dns-OH	0.00	0.01	0.00	0.51	0.54	0.16	0.74	0.00	0.04	0.04
20. Dns-NH ₂	0.51	0.38	0.47	0.71	0.17	0.96	0.49	0.60	0.40	0.91

Solvent systems: A, benzene–acetic acid (9:1); B, toluene–acetic acid (9:1); C, toluene–ethanol–acetic acid (17:1:2); D, water–formic acid (200:3); E, water–ethanol–ammonium hydroxide (17:2:1); F, ethyl acetate–ethanol–ammonium hydroxide (20:5:1); G, water–ethanol–ammonium hydroxide (14:15:1); H, *n*-heptane–*n*-butanol–acetic acid (3:3:1); I, chlorobenzene–acetic acid (9:1); J, ethyl acetate–methanol–acetic acid (20:1:1). All of the proportions are based on volume.

In all cases, dansyl amino acids, because they are fluorescent, have been detected under a UV lamp (254 nm).

C. Dimethylamino Azobenzeneisothiocyanate Derivatives of Amino Acids

Dimethylamino azobenzene isothiocyanate (DABITC) reacts with the NH₂-terminal end of an amino acid in basic medium to give a DABTH amino acid via a DABTC derivative, in a manner similar to the Edman method, where PTH amino acid is obtained by the reaction of PITC. The use of DABITC reagent during amino acid sequencing of proteins (140) has distinct advantages over the use of dansyl chloride; for example, the color difference between DABITC, DABTC derivatives, and DABTH amino acid greatly facilitates direct visualization and identification with TLC. DABTH amino acids are colored compounds with absorption maxima at 520 nm in acid media ($\epsilon=47,000$). Thus, using the visible region, the quantification and identification of these derivatives become more convenient and sensitive (10 pmol by poly amide TLC).

1. Preparation of Standard α -Mono-DABTH Amino Acids*

Amino acids (0.5 mg) are dissolved in 100 μ L of triethylamine–acetic acid buffer (50 mL water +50 mL acetone+0.5 mL triethylamine +5 mL of 0.2 M acetic acid, pH 10.65) and treated with DABITC solution (50 μ L, 4 nmol/ μ L in acetone). The mixture is heated at 54°C for 1 h,

* Method of preparation from Refs. 141 and 142.

Table 11 Various Solvent Systems for TLC of Dansyl Amino Acids

Solvent systems ^a	Ratio	Reference
1. HCOOH	1.5%	120
Benzene–acetic acid	9:1	
2. Formic acid	1.5%	127
Benzene–acetic acid	4.5:1	

3. H ₂ O–pyridine–HCOOH	93:35:3.5	128
Benzene–acetic acid	4.5:1	
4. NH ₄ Cl+NH ₃ +ethanol	80 mg+22 mL+10 mL	124
Benzene–pyridine–HOAc	75:2:6	
5. H ₂ O–propanol–formic acid	100:5:2	129
Benzene–acetic acid	9:1	
6. Ethyl acetate–MeOH–HOAc	20:1:1	130
Benzene–HOAc–BuOH	90:10:5	
7. Formic acid	1.5%	131, 132
Benzene–acetic acid	9:2	
8. Benzene–anhydrous HOAc, followed by	9:1	133
EtOAc–MeOH–anhydrous HOAc in the same direction	20:1:1	
9. H ₂ O–pyridine–HCOOH	93:35:3.5	134
Benzene–acetic acid	4.5:1	
10. Formic acid	3%	135
Benzene–acetic acid	9:1	
11. Me acetate–iso–PrOH–NH ₃	9:7:4	136
CHCl ₃ –MeOH–HOAc	15:5:1	
CHCl ₃ –EtOAc–MeOH–HOAc	45:75:22.5:1	
Pet ether– <i>t</i> -BuOH–HOAc	5:2:2	
12. CHCl ₃ –MeOH	9:1	137
13. CCl ₄ –2-methoxyethanol	17:3	138
14. Benzene–pyridine–acetic acid	80:20:2	139

^a1–8: two-dimensional TLC on polyamide layers; 9–14: one-dimensional TLC on silica gel layers.

dried under vacuum, and then redissolved in water-acetic acid (40 μ L+80 μ L) saturated with HCl (alternatively, 100 μ L of 50% TFA can be used instead of this aqueous acid mixture). The acid solution is heated at 54°C for 45 min and then dried again under vacuum. The dried DABTH amino acid (about 200 nmol) is dissolved in a suitable volume of 90% ethanol and stored at –20°C for TLC analysis. The presence of excess of free amino acid does not, in any case, interfere with the analysis. The pH of solutions of histidine, aspartic acid, and glutamic acid usually falls below 8 and should be adjusted to 10 (by addition of 1 M NaOH) before adding DABITC.

2. DABTH Amino Acids from NH₂-Terminal Proteins or Peptides

Peptide or protein (0.1–1 nmol) is dissolved in water or a suitable solvent (20 μ L) and treated with freshly prepared DABITC solution (40 μ L, 10 nmol/ μ L in pyridine). The coupling reaction is done at 70°C for 60–70 min, and then the reaction mixture is extracted with four portions of 250 μ L of heptane–ethyl acetate (2:1) by centrifuging. The aqueous phase is dried under vacuum and then redissolved in water–acetic acid (20 μ L, +40 μ L) saturated with HCl for the cleavage reaction, this being performed at 54°C for 50 min. The sample is dried under vacuum and redissolved in water (50 μ L). The released DABTH is extracted twice with butyl acetate (200 μ L and

Table 12 R_f Values of 10 Dansyl Amino Acids on Silica Gel Thin Layers

Sample no.	Dansyl amino acid	Solvent system ^a				
		S ₁	S ₂	S ₃	S ₄	S ₅
1	Dansyl L-alanine	62	61	60	50	27
2	Dansyl L-isoleucine	80	92	85	85	49
3	Dansyl L-leucine	83	85	80	89	65
4	Dansyl L-methionine	86	64	62	55	31
5	Dansyl L-proline	60	84	72	30	39
6	<i>N</i> - <i>O</i> -Didansyl L-tyrosine	55	73	40	60	18
7	<i>N</i> - α -Dansyl L-tryptophan	51	53	46	40	21
8	Dansyl L-phenylalanine	77	76	74	52	40
9	Dansyl L-valine	72	88	65	48	35
10	Dansyl L-norvaline	75	81	68	45	37

^aS₁, *n*-Heptane–BuOH–HOAc (20:8:3). S₂, dichloromethane–MeOH–propionic acid (30:1:0.5). S₃, chloroform–HOAc–ethyl acetate (24:5:4). S₄, chloroform–MeOH–ethyl acetate (23:8:2). S₅, chloroform–propionic acid–ethyl acetate (23:6:4).

R_f values are average of five determinations.

Source: Ref. 126.

100 μ L), and the extract is evaporated and redissolved in ethanol (10–20 μ L) for TLC. In some cases the dried acid sample can be dissolved directly in ethanol for analysis.

3. TLC of DABTH Amino Acids

Two-dimensional TLC on polyamide sheets by ascending solvent flow is used to identify all DABTH amino acids except DABTH-Ile/Leu. No phase equilibrium is necessary, and

H₂O–acetic acid (2:1) is used for the first dimension and toluene–n-hexane–acetic acid (2:1:1) for the second

Table 13 R_f Values of 10 Dansyl Amino Acids on Silica Gel Thin Layers

Sample no.	Dansyl amino acid	Solvent system ^a				
		A ₁	A ₂	A ₃	A ₄	A ₅
1	<i>N</i> - α -Dansyl L-asparagine	56	75	53	30	35
2	Dansyl L-aspartic acid	66	72	60	64	30
3	α -Dansyl L-arginine	7	12	3	2	3
4	<i>N,N</i> -Didansyl L-cystine	84	83	68	85	18
5	Dansyl L-cysteic acid	82	80	25	15	11
6	Dansyl L-glutamic acid	80	90	84	74	55
7	Dansyl L-glutamine	62	77	63	41	40
8	<i>N</i> -Dansyl L-lysine	16	20	10	6	8
9	<i>N</i> -Dansyl L-serine	72	85	72	58	32
10	Dansyl L-threonine	76	88	76	68	45

^aA₁, Dichloromethane–MeOH–propionic acid (21:3:2). A₂, ethyl acetate MeOH–propionic acid (22:10:3). A₃, chloroform–MeOH–HOAc (28:4:2). A₄, chloroform–acetone–HOAc (20:8:4). A₅, chloroform–acetone–propionic acid (24:10:5).

R_f values are average of five determinations.

Source: Ref. 126.

dimension. The sheet is dried after the second run and exposed to HCl vapors until all yellow spots turn red or blue. For discrimination between DABTH-Ile and DABTH-Leu, one-dimensional separation on poly amide (143) using formic acid–ethanol (10:9) or one-dimensional separation on silica gel (Merck) using (144) chloroform–ethanol (100:3) is carried out. The successful identification of DABTH amino acids relies on the skillful running of the small polyamide sheet and interpretation of the pattern of spots (141, 145).

D. Dinitrophenyl Amino Acids

Use of dinitrophenyl (DNP) amino acids, formed by condensation of 1-fluoro-2,4-dinitrobenzene (FDNB) with the free amino group of an amino acid, was first described by Sanger in 1945 (83). Sanger identified DNP amino acids by paper chromatography. Since then many modifications to the methods of obtaining derivatives of amino acids for sequence analysis and to the identification of such derivatives have been reported, and the use of DNP amino acids for sequencing purposes is rapidly going out of date. Nevertheless, the importance of DNP amino acids is not yet lost. In view of the limited applications of DNP amino acids at present, the methods of preparing these derivatives

from standard amino acids or peptides are not described here. However, the details of those procedures can be obtained from Rosmus and Deyl (88) and Bailey (146).

In addition to the references cited previously (83–91), Kirchner (147) presented considerable information on TLC analysis of DNP amino acids based on the literature available up to 1970. Grant and Wicken (148) prepared thin layers (5 plates of 20×20 cm×0.25 mm) from a mixture of 10 g of cellulose MN-300 and 4 g of silica gel H (Merck), homogenized in 80 mL of water. The plates were dried overnight at 37°C and developed in the first dimension in two solvents successively: isopropanol–acetic acid–H₂O (75:10:15) for 15 min, then *n*-butanol–0.15 N ammonium hydroxide (1:1, upper phase). The dried chromatograms were developed in 1.5 M sodium phosphate buffer (pH 6.0) in the second dimension.

In almost all the methods reported, the separation was carried out in groups of water-soluble and ether-soluble DNP amino acids, and for each group mostly two-dimensional TLC was performed. In 1988, Bhushan and Reddy (29) reported a few solvent systems for one-dimensional resolution of DNP amino acids on silica gel plates (Table 14).

The DNP amino acids have been visualized by UV light (360 nm with dried plates; 254 nm with wet ones) or by their yellow color, which deepens upon exposure to ammonia vapors. Thin layers of silica gel usually give an intense purple fluorescence for DNP amino acids under UV light, which masks the presence of very faint spots and decreases the color contrasts. The cellulose–silica mixed layers (148) gave much lower fluorescence and preserved the color contrasts among the various derivatives.

Because of the photosensitivity of these derivatives, it is advisable to carry out their preparation and chromatography in the absence of direct illumination.

IV. RESOLUTION OF AMINO ACIDS AND DERIVATIVES ON IMPREGNATED LAYERS

Thin-layer chromatography of amino acids and derivatives on impregnated plates was reviewed by Bhushan and Parshad (30c) and Bhushan and Martens (30e), and chromatography on thin layers impregnated with organic stationary phases was reviewed by Gasparic (150a); see also Chapter 17 on enantiomer separations in this handbook. The reagents and methods used for impregnation are not to be confused with locating or spray reagents, because the latter are required for identification even on impregnated plates. The various methods used for impregnation include mixing the impregnation reagent with the inert support, spraying it onto the plate, exposing the layer to the vapors of the impregnating reagent, immersing or dipping the plate in the solution of reagent, or allowing the solution to ascend or descend in a normal manner of development. Chemical reaction between the inert support and a suitable reagent can also be considered impregnation. The various explanations given to the role of the impregnating agent in the resolution process include ion pairing, complex formation, ligand exchange, coordination bonds, charge transfer, ion exchange, and hydrogen bonding.

Table 14 hR_f Values of DNP Amino Acids on Silica Gel Thin Layers

Sample no.	N-DNP-L-amino acid	Solvent system ^a				
		S ₁	S ₂	S ₃	S ₄	S ₅
1	Phenylalanine	53	48	85	70	55
2	Isoleucine	68	82	96	97	60
3	Tyrosine	25	30	60	52	36
4	Alanine	40	36	68	50	42
5	Glycine	28	17	35	25	27
6	Leucine	65	73	93	90	52
7	Tryptophan	48	33	53	47	34
8	Methionine	45	40	75	57	42
9	Valine	62	65	90	85	47
10	Proline	41	45	74	60	38
11	Norvaline	61	62	88	83	45

^aS₁, *n*-Heptane-*n*-butanol-acetic acid (20:4:1); S₂, chloroform-propionic acid (26:2); S₃, chloroform-acetic acid (21:1); S₄, chloroform-ethanol-propionic acid (30:2:1); S₅, benzene-*n*-butanol-acetic acid (34:1:1). R_f values are average of five determinations.

Sample no.	N-DNP-L-amino acid	Solvent system ^a				
		A ₁	A ₂	A ₃	A ₄	A ₅
12	N-DNP-L-serine	51	68	70	70	70
13	N-DNP-lysine	21	26	11	7	27
14	N,S-di-DNP-L-cysteine	82	87	77	85	85
15	N-DNP-L-glutamic acid cyclohexylamine salt	67	80	83	92	82
16	N-DNP-L-aspartic acid	38	70	75	60	60
17	N-DNP-L-asparagine	30	64	45	38	55
18	N-DNP-L-arginine	10	6	5	3	18
19	N,N-di-DNP-L-cystine	48	70	55	65	82

^aA₁, Chloroform-methanol-acetic acid (25:5:1); A₂, chloroform-propionic acid-methanol (15:10:1); A₃, *n*-heptane-butanol-acetic acid (16:8:4); A₄, *n*-butanol-ethyl acetate-acetic acid (20:8:2); A₅, *n*-butanol-methanol-propionic acid (18:8:2).

Source: Ref. 29.

Resolution of amino acids has been reported to be very rapid and to be improved by using copper sulfate and polyamide (13); halide ions (22); zinc, cadmium, and mercury salts

(18); and alkaline earth metal hydroxides (24) as impregnating materials. Some of the results are described in Tables 15–17. The chromatograms developed in these systems provide compact spots, without lateral drifting of the solvent front. The C₁₈ layers impregnated with dodecylbenzenesulfonic acid were helpful in confirming the presence of an unknown amino acid in a sample, and the migration sequence on these impregnated plates was reversed, probably due to an ion-exchange mechanism (72). Separation of α -amino acids with *n*-butanol–acetic acid–water (3:1:1), *n*-butanol–acetic acid–chloroform (3:1:1), and *n*-butanol–acetic acid–ethyl acetate (3:1:1) on plain and nickel chloride—impregnated plates (30d) was reported. The partition and adsorption coefficients for the amino acids under study were determined on both untreated and impregnated (with Ni²⁺) silica

Table 15 hR_f of Amino Acids in Presence of Halides

Sample no.	Amino acid	Control plate	Amino acids pretreated with			Plates impregnated with		
			Cl [−]	Br [−]	I [−]	Cl [−]	Br [−]	I [−]
1	Gly	07	08	09	12	07	08	09
2	Tyr	30	35	40	47	29	30	31
3	Pro	12	15	19	22	08	09	10
4	Thr	15	14	15	19	13	14	16
5	Cys	22	22	25	27	19	20	22
6	Leu	32	40	47	50T	50T	55T	60T
7	Met	23	35	36	37	22	23	24
8	Ile	30	38	44	44	30	30	31
9	Ala	15	19	13	16T	16T	16	16
10	Trp	35T	40	50T	53	30	31	34
11	Phe	36T	41	48	48	36T	37	38
12	Val	19	32	25	29	25	26	26
13	Asp	08	13	14	15	08	09	10
14	Ser	09	13T	13	14T	08	08	09
15	His	01	03	04	05	02	02	02
Time (min)		50	64	67	67	50	50	50

Solvent system: *n*-Butanol–acetic acid–chloroform (3:1:1); temp. 25±2°C.

Source: Ref. 23.

gel in a batch process, and correlations were drawn between TLC separation of amino acids on impregnated silica gel with adsorption and/or partition characteristics. The

results indicated a predominant role of the partitioning phenomenon in the TLC of amino acids on plates impregnated with metal ions. Application of antimony (V) phosphate-silica gel plates in various aqueous and nonaqueous and mixed solvent systems has also been reported (150b). Some impregnated TLC systems for resolution of amino acids are summarized in Table 18.

Thin-layer chromatographic separation of several amino acids was achieved (30f) below their pI on silica gel plates by using various ammonium salts as the impregnating reagents so that there was little scope of any complex formation with the cationic component of the impregnating reagents and the amino acids, and only the ion-pair or electrostatic behavior prevailed. Amino acids examined were kept in cationic form by keeping the pH of the sample solutions below their respective isoelectric points. The pH of the solvent systems, (I) *n*-butanol-methanol-acetic acid (8:1:3) and (II) *n*-butanol-carbon tetrachloride-acetic acid (8:3:1), was nearly 2 and 3, respectively. Sulfate, oxalate, nitrate, acetate, and chloride of ammonium were selected for impregnation, and the effect of varying concentrations (0.1, 0.2, 0.3, 0.4, and 0.5%) was studied. Aliphatic amino acids (Ala, Val, Leu, Ile, and Pro) that did not resolve on untreated plates separated on chloride-impregnated plates in solvent system I and on chloride-, nitrate-, and sulfate-impregnated plates in solvent system II. Thus certain other combinations of acidic, aliphatic, and sulfur-containing amino acids that did not separate on untreated plates were separated on such impregnated plates. The treatment also resolved combinations such as Ser/Asp/Pro and Tyr/Trp that were unresolved in the earlier report (23). Typical results on ammonium sulfate-impregnated plates are shown in Table 19.

Advantage is taken of the zwitterionic characteristic of amino acids in causing the formation of ion pairs. Experiments showed that impregnation with different anions influenced the chromatographic behavior of the amino acids due to formation of an ion pair between the impregnated anion and the amino acid in cationic form below its pI . The solubility of the ion pairs so formed

Table 16 hR_f Values for Amino Acids on Copper Sulfate and Polyamide Mixed Silica Gel Plates

Amino acid	A	B	C
L-Leucine (Leu)	65	63	71
D,L-Isoleucine (Ile)	66	72	81
D,L-Tryptophan (Trp)	63	68	75
D,L-Methionine (Met)	64	64	72
D,L-Valine (Val)	64	60	77
L-Lysine · HCl (Lys)	16T	12	33
L-Histidine · HCl(His)	22T	20	39
D,L- β -Phenylalanine (Phe)	64	65	82
D,L-Threonine (Thr)	50	51	67
D,L-Alanine (Ala)	46	45	64

D,L-Serine (Ser)	40	43	56
L-Tyrosine (Tyr)	58	61	71
L-Glutamic acid (Glu)	41	48	58
D,L-Aspartic acid (Asp)	28	25	44
L-Arginine HCl (Arg)	24T	19	39
Glycine (Gly)	36	46	49
L-Proline (Pro)	37	36	58
L-Cysteine HCl (Cys)	20T	17	29
D,L-2-Aminobutyric acid (Aba)	51	54	61
L-Ornithine	27T	23	35

The values are the average of two or more identical runs, 10 cm in 35 min. T, tailing; A, untreated silica gel plate; B, copper sulfate—impregnated silica gel; C, polyamide mixed silica gel layers. Solvent, methanol–butyl acetate–acetic acid–pyridine (20:20:10:5).

Source: Ref. 13.

in the mobile phase (i.e., the new solvent systems worked out), the hydrophobic interactions between the silica gel and the amino acid molecule, and the polarity and flow of the mobile phase were responsible for improved separations.

Except for Glu, amino acids with an acidic or amide side chain (Glu/Asp/Gln/Asn) moved very little from the baseline when solvent system II (*n*-BuOH–MeOH–HOAc, 8:1:3) was used on plain and impregnated plates. Solvent system I (*n*-BuOH–CCl₄–HOAc, 8:3:1), which was relatively more polar than II, was successful in resolving this group of four amino acids on sulfate- and oxalate-impregnated plates, which was not resolved on plain plates.

Separation of amino acids on silica gel layers impregnated with Cu(II) with acetate buffer (0.3 M, pH 6)–acetonitrile–1-butanol (12:5:10) mobile phase were compared with those obtained by RP-HPLC (30 g).

Certain difficulties, as mentioned in Section III.3, in resolving or identifying various PTH amino acid combinations have successfully been removed with the application of silica gel layers impregnated with various metal salts including transition metals and other reagents such as (+)-tartaric acid and (–)-ascorbic acid for the identification and resolution of PTH amino acids in multicomponent mixtures and enantiomeric mixtures (18, 19, 21, 22, 26, 30). The methods reported provide very effective resolution and compact spots (by exposure to iodine vapors) and can be applied to the identification of an unknown PTH amino acid; some of these are given in Tables 20–22. Some of the successful solvent systems for TLC of PTH amino acids on impregnated plates are summarized in Table 23.

Table 17 hR_f Values of 15 Amino Acids on Silica Gel Impregnated with Zn, Cd, and Hg Salts

	A	B	C	D	E	F	G	H	I	J
Thr	25	55	42	41T	35	36	42	33	50	40
Ser	12	38	39	28T	32	29	31T	15	40	31T
Gly	10	35	29	23T	28	25	28	16	35	27T
Lys	03	13	07	05	51	08	05	04	10	05
Ala	30	48	40	31	38	36	38	20	45	35
Tyr	60	60	52	50	48	45	51	62	55	56
Ile	55	67	56	52	50	48	54	50	60	53
Leu	50	65	55	55	52	50	56	47	65	55
Cys	00	00	00	00	00	00	00	00	00	00
Met	45	62	48	48	48	42	48	39	54	45
Glu	1ST	43	38	36T	34	27	38T	18	36	34T
Trp	57	60	53	51	51	44	54	45	60	47
Phe	54	67	57	55	55	46	57	58	68	52
Val	50	63	45	50	52	42	56	47	57	45
Arg	07	19	13	13	09	11	11	10	15	08

Solvent, butyl acetate–methanol–acetic acid–pyridine (20:20:5:5). Developing time, 30 min. Detection limit, 10^{-4} M. Solvent front, 10 cm. A, plain silica gel; B, C, D, 0.5%, 0.2%, 0.1% Zn^{2+} -impregnated, respectively; E, F, G, 0.5%, 0.2%, 0.1% Cd^{2+} -impregnated, respectively; H, I, J, 0.5%, 0.2%, 0.1% Hg^{2+} , respectively.
Source: Ref. 25.

V. HPTLC/OPTLC

Improvements in all practical aspects of the TLC process culminated in a performance break-through, resulting in an increase in separation efficiency, sample detectability limits, and reduced analysis time; the specific advance in instrumentation was termed HPTLC. Chapters 5 and 7 in this volume describe basic and theoretical aspects of the application of instrumentation in TLC and OPLC. That HPTLC could be used with advantage for the separation of PTH amino acids was recognized by Bucher (150m) and Yang (150n). But they could not achieve separation of all 20 common PTH amino acids. Schuette and Poole (150o) used continuous multiple development on silica gel and were able to separate 18 samples and standards simultaneously using five development steps with four changes in mobile phase and scanning densitometry; typical results are given in Table 24. Butler et al. (150p) separated PTH-Leu/Ile/Pro by HPTLC using multiwave-

length detection. Fater (150q) carried out separation and quantification of PTH amino acids by OPLC using chloroform–ethanol–acetic acid (90:10:2) for the resolution of polar PTH derivatives and CH_2Cl_2 –ethyl acetate (90:10) for the resolution of nonpolar PTH amino acids. The method was claimed to be superior to HPTLC methods (150m, 150n, 150p) in having relatively increased migration distance resulting in the resolution of complex mixtures containing a large number of derivatives. OPTLC (149) and HPTLC on RP-8, RP-18, and homemade ammonium tungstophosphate layers (150) have also been used for the analysis of DNP amino acids.

Norfolk et al. separated 18 amino acids on cellulose, silica gel, and chemically bonded C_{18} modern HPTLC plates, all of which except the cellulose contained a preadsorbent zone (72a); quantification was carried out by scanning standard and sample zones at 610 nm. R_f values of amino acid standards on reversed-phase and normal-phase layers in various solvents are given in Tables 25 and 26, respectively. Amino acids were identified in water conditioned by four strains of snails using cellulose HPTLC plates developed with 1-propanol–water (2:3), and detection with ninhydrin spray reagent and valine content was quantified by densitometric scanning at 610 nm (72b).

Table 18 TLC of Amino Acids on Impregnated Silica Gel Layers

Solvent system	Ratio	Impregnation	Ref.
Isoamyl alcohol– H_2O –HOAc	6:5:3	Pyridinium tungstoarsenate	150c
H_2O –EtOAc–MeOH	64.3:5.7:30	Silanized silica and triethanolamine, SDS, sodium dioctyl sulfonate, dodecylbenzenesulfonic acid	150d
0.1 M HOAc in aq. 50% MeOH		Dodecylbenzenesulfonic acid	150e
aq. MeOH+ I_2 (KCl or HO Ac added)		Ammonium tungstophosphate and dodecylbenzene sulfonic acid	150f
aq. NH_4NO_3 or HNO_3 or H_2O –HOAc–MeOH (79:1:20)		Ammonium tungstophosphate	150g
H_2O		Polyamide	150h
H_2O –butanol–anhyd. HOAc	4:4:2	Kieselguhr or cellulose	150i
<i>n</i> -Butanol–acetic acid–water	4:1:5	Starch-agar (1:1)	150j
Propan-2-ol–EtOAc–acetone–methanol– <i>n</i> -pentyl alcohol–aq, 26%	9:3:3:1:1:3:3	Cellulose	150k
NH_3 –water, in first direction; and Butanol–acetone–propan-2-ol–formic acid–water, in second direction	18:8:8:3:6		
1 M NH_4NO_3 –0.1 M HNO_3		Ammonium tungstophosphate	150l
MeOH–butyl acetate–HOAc–	4:4:2:1	Copper sulfate and polyamide	13

pyridine			
<i>n</i> -Butanol–acetic acid–CHCl ₃	3:1:1	Cl [−] , Br [−] , I [−]	23
<i>n</i> -Butanol–acetic acid–ethanol	3:1:1	Hydroxides of Mg, Ca, Ba, Sr	24
Butyl acetate–MeOH–HOAc–pyridine	4:4:1:1	Zn ²⁺ , Cd ²⁺ , Hg ²⁺	25

VI. RESOLUTION OF ENANTIOMERIC MIXTURES OF AMINO ACIDS AND THEIR DERIVATIVES

The measurement of specific rotation is a common and accepted method for evaluating the enantiomeric purity of chiral compounds. The determination of the enantiomeric excess (ee) value is influenced by the presence of impurities and changes in concentration, solvent, and temperature (151) and requires the $[\alpha]_D$ value for the pure enantiomer. The availability of a reliable optically pure standard would depend on the analytical method by which it had been resolved from the enantiomeric or racemic mixture of the compound in question. Though TLC provides a direct method for resolution and analytical control of enantiomeric purity, there are few reports on thin-layer chromatographic separation of enantiomers.

In general, three approaches have been applied to the TLC resolution of enantiomers: use of a chiral selector as impregnating reagent mixed with the adsorbent, e.g., silica gel; immersing or developing the plate in a solution of chiral selector prior to sample application; and use of a chiral mobile phase. Yuasa et al. (152) reported the TLC separation of D,L-tryptophan on a crystalline cellulose-coated plate. Weinstein (153) resolved nine dansyl amino acids as follows. Reversed-phase TLC plates from Whatman were developed prior to application of dansyl amino acids in buffer A (0.3 M sodium acetate in 40% acetonitrile, and 60% water adjusted to pH 7 by acetic acid). After “fan-drying,” the plates were immersed in a solution of 8 mM *N,N*-di-*n*-propyl-L-alanine and 4 mM cupric acetate in 97.5% acetonitrile and 2.5% water for 1 h or overnight and left to dry in the air. After the samples were applied, the plates were developed in buffer A with or without *N,N*-di-*n*-propyl-L-alanine (4 mM) and cupric acetate (1 mM) dissolved in it. The enantiomers were detected by irradiating with UV light (360 nm) to yield fluorescent yellow-green spots. Use of 25% acetonitrile was preferred for glutamic and aspartic acids and serine and threonine derivatives. *N,N*-Di-*n*-propylalanine can be prepared by following Bowman and Stroud's

Table 19 Typical Results Showing hR_f Values of Amino Acids on Plain and Impregnated Plates

Amino acid	Solvent I		Solvent II	
	Plain	SO_4^{2-}	Plain	SO_4^{2-}
Ala	39	47	15	15
Val	65	68	35	67
Leu	63	73	34	73
Ile	63	67	35	64
Pro	30	35	11	10
Ser	25T	32	11T	02
Thr	33	42	15T	07
Cys	16	50	08T	17ST
Met	78	62	25	58
Glu	47	69	39	54
Asp	16	38	02	06
Gln	22	31	04	04
Asn	15	28	07	03
Trp	62	69	40	70
Phe	64	67	40	66
Tyr	45	71	37	64

Solvent I: *n*-Butanol–methanol–acetic acid (8:1:3), solvent front 10 cm in 80 min.

Solvent II: *n*-Butanol–carbon tetrachloride–acetic acid (8:3:1), solvent front 10 cm in 80 min.

T=Tailing; ST=slight tailing.

Detection: Ninhydrin 0.2% in acetone.

Source: Ref. 30f.

procedure (154): L-Alanine (17.8 g) is dissolved in ethanol (200 mL) and 10% palladium on activated charcoal catalyst (3 g), and propionaldehyde (43 mL) is added. The mixture is hydro-genated for 48 h at 40–50°C at an initial hydrogen pressure of 50 psi. The catalyst is removed using a sintered glass filter, and the filtrate is evaporated to dryness. The reaction product (*N,N*-di-*n*-propyl-L-alanine) is crystallized from chloroform, and the purity may be confirmed by TLC, NMR, and C, H, N analysis.

A. Resolution of Enantiomers of Dansyl DL-Amino Acids

Grinberg and Weinstein (155) reported a two-dimensional RP-TLC technique for the resolution of complex mixtures of dansyl amino acids. The Dns derivatives were first

separated in a nonchiral mode using 0.3 M sodium acetate in H₂O-acetonitrile (80:20, pH 6.3) to which 0.3 M sodium acetate in H₂O-acetonitrile (70:30) was added to give a final acetonitrile concentration of 38% or 47%. For the second dimension the mobile phase was 8 mM *N,N*-di-*n*-propyl-L-alanine and 4 mM copper(II) acetate dissolved in 0.3 M sodium acetate in H₂O-acetonitrile (70:30, pH 7). The plates were developed in the second dimension using a temperature gradient. The method is reported to be applicable for the resolution of amino acids in a protein hydrolysate and quantification by densitometry.

Alak and Armstrong (156) resolved enantiomers of dansyl amino acids and β -naphthylarnide amino acids using β -cyclodextrin (β -CD) plates. The plates were prepared by mixing 1.5 g of β -CD bonded silica gel in 15 mL of 50% methanol (aq.) with 0.002 g of binder (ASTE—all-solvent binder) and acetate in 50/50 MeOH–1% aqueous triethylammonium acetate (pH 4.1). Some of these results are shown in Table 27.

Table 20 hR_f Values of PTH Amino Acids on Fe²⁺-, Co²⁺-, Ni²⁺-, and Zn²⁺-Impregnated Silica Plates

Sample no.	PTH amino acid	Alone	Fe ²⁺		Co ²⁺		Ni ²⁺		Zn ²⁺	
			0.2%	0.3%	0.05%	0.1%	0.1%	0.2%	0.2%	0.3%
1	Alanine	60	42	41	57	51	38	40	50	43
2	Aspartic acid	0	0	0	0	0	0	0	0	0
3	Glycine	39	26	21	44	38	29	30	32	27
4	Glutamic acid	0	0	0	0	0	0	0	0	0
5	Isoleucine	90	84	75	72	90	65	71	81	72
6	Leucine	95	87	71	82	81	70	76	85	76
7	Lysine	23	8	6	15	17	7	4	10	8
8	Methionine	70	54	47	81	62	58	51	57	58
9	Phenylalanine	75	61	49	77	68	52	55	66	58
10	Proline	97	89	89	84	76	83	90	96	89
11	Serine	13	5	5	11	9	11	12	8	5
12	Tyrosine	96	86	69	68	95	85	78	83	78
13	Tryptophan	95	91	70	91	97	77	82	88	81
14	Threonine	86	78	57	94	83	60	63	78	70
15	Valine	85	75	73	96	79	57	58	76	67

Solvent, chloroform–ethyl acetate (29:3); developing time, 35 min; solvent front, 10 cm.

Source: Ref. 21.

A macrocyclic antibiotic, vancomycin, was used as a chiral mobile-phase additive (179) for TLC resolution of 6-aminoquinolyl-*N*-hydroxy succinimidyl carbamate (AQC)

derivatized amino acids, and dansyl amino acids on chemically bonded diphenyl-F reversed-phase plates. Both the nature of the stationary phase and the composition of the mobile phase have a strong influence on enantiomeric resolution; typical results are given in Table 28.

The enantiomeric resolution of some of the dansyl DL-amino acids was achieved (156a) on thin silica gel plates impregnated with (1*R*,3*R*,5*R*)-2-azabicyclo[3, 3, 0]octan-3-carboxylic acid, which is an industrial waste material, and a proline analog nonproteinogenic α -amino acid. Different combinations of 0.5 M NaCl–MeCN were found to be successful in resolving dansyl DL-amino acids; addition of methanol was required in some cases. Spots were visualized using a fixed wavelength (254 nm) ultraviolet chamber (some results are shown in Table 29).

Direct racemic resolution of dansyl DL-amino acids was accomplished (156c) by normalphase TLC on silica gel plates impregnated with vancomycin (0.34 mM) as chiral selector. The mobile phases enabling successful resolution of most of the racemic dansyl amino acids were acetonitrile–0.5 M NaCl (aq.), 10+4, and 14+3 (v/v). Spots were detected by use of a fixed dual-wavelength ($\lambda=254$ nm) ultraviolet light. The minimum amount detected was 2.1 μ g. Typical results with vancomycin and erythromycin are shown in Table 30.

The macrocyclic antibiotics erythromycin (156b) and vancomycin (156c) were successfully used for the resolution of enantiomers of dansyl DL-amino acids by mixing them during plate making. Effect of change in temperature, chiral selector, concentration, pH, and amount of organic modifier added to the mobile phase were studied. Experiments showed that the surface of the stationary phase of a vancomycin-impregnated plate appeared light blue under UV irradiation after it was developed without spotting any of the DL samples; thus vancomycin used to impregnate the plates was immobilized on the stationary phase. Resolutions were significantly affected by variations in the solution environment, which in turn affects the chiral antibiotic, which is ionizable, contains hydrophilic and hydrophobic moieties, and is somewhat flexible. The resolution

Table 21 hR_f Values of PTH Amino Acids on Untreated Plates and Plates Impregnated with Sulfates of Mg, Mn, Fe, and Co

PTH amino acid	S ₁ (heptane–butyl acetate, 15+5)					S ₂ (heptane–propionic acid, 20+4)					S ₃ (benzene–ethyl acetate, 15+3) ^a	
	PS ₁	M ₁	M ₂	M ₃	M ₄	PS ₂	M ₁	M ₂	M ₃	M ₄	PS ₃	M ₄
Methionine	28	30	26	32	31	43	45	30	32	35	62	78
Phenylalanine	30	35	29	37	34	50	52	36	38	40	67	80
Tryptophan	63	61	51	60	57	71	67	55	55	57	82	94
Valine	49	46	40	51	47	66	62	52	50	55	73	85
Isoleucine	62	62	50	62	59	77	72	61	60	62	78	65

Tyrosine	66	64	53	64	61	80	74	64	64	65	84	89
Threonine	57	52	45	56	53	63	64	53	53	53	72	83
Alanine	23	25	23	26	25	32	34	27	25	29	50	67
Serine	55	55	42	55	51	48	46	38	49	40	70	44
Leucine	69	65	54	63	56	76	71	62	60	67	80	96
Lysine	06	04	02	03	05	06	10	04	06	07	18	35
Glycine	17	15	13	15	15	17	20	15	15	18	37	55
Glutamic acid	04	06	05	06	06	04	04	06	06	05	0	14
Aspartic acid	05	07	06	07	07	05	08	07	07	06	0	22
Proline	44	33	31	34	32	44	42	35	35	45	79	96

^aCompounds moved to solvent front on plates impregnated with surfaces of Mg, Mn, and Fe. PS₁, PS₂, PS₃, untreated plates; M₁, M₂, M₃, M₄, treated with sulfates of Mg, Mn, Fe, and Co, respectively. *R_f* values are the average of at least five determinations. Developed in 30–40 min at 25±2°C and exposed to iodine vapors to locate the spots.

Source: Ref. 26.

with macrocyclic antibiotics is a complex phenomenon owing to possible changes in conformational structure of the molecule with change in temperature, pH, etc.; both antibiotics and dansyl DL-amino acids contain ionizable groups, and their charges and spatial interactions may vary with mobile-phase pH. Therefore, any change in pH from the optimum might result in ionization of either or both the antibiotic (e.g., vancomycin) and the dansyl DL-amino acid, and as a result no enantiomeric resolution might be observed under the changed conditions. Thus, the enantiomer separation could be a result of the formation of a hydrophobic pocket (using the C-shaped aglycone basket), Coulombic interactions between the charged species of the chiral selector and the analyte, and any one of the other factors discussed above.

Though cyclodextrins (CDs) have been used extensively as mobile-phase additives for chiral resolution of a number of compounds, including amino acids and derivatives, they have been used sparingly as impregnating agents. Only β -CD has been used for resolution of enantiomers by TLC. γ -CD has not been used or recommended for TLC because of its high cost. Impregnation of silica gel with β -CD (using β -CD bonded silica gel mixed with a suitable binder) provided resolution of enantiomers of dansyl DL-amino acids (156) by forming a reversible inclusion complex of different stability. Dansyl DL-amino acids are successful because compounds containing two or more rings are most effectively separated with β -CD. Cyclodextrins, being cyclic oligo-glucose molecules, have hydrogen atoms and glycosidic oxygen groups located inside the molecule, which is like a truncated cone with both ends open, forming the relatively hydrophobic cavity, which interacts with organic molecules to form inclusion complexes.

Table 22 hR_f Values of PTH Amino Acids on Silica Plates Impregnated with Zinc Salts

PTH amino acid	S ₁ (heptane–butyl acetate, 15+5)				S ₂ (heptane–propionic acid, 20+4)				S ₃ (benzene–ethyl acetate, 15+3)			
	L ₁	L ₂	L ₃	L ₄	L ₁	L ₂	L ₃	L ₄	L ₁	L ₂	L ₃	L ₄
Methionine	17	22	18	25	33	33	29	33	42	57	48	58
Phenylalanine	22	25	25	28	37	38	35	36	47	60	52	60
Tryptophan	36	41	41	51	40	50	40	55	67	74	61	82
Valine	40	35	44	42	52	53	54	52	54	68	64	69
Isoleucine	50	46	55	54	60	62	63	63	62	79	74	74
Tyrosine	55	48	59	56	62	64	75	65	64	84	79	78
Threonine	47	40	52	48	53	51	52	56	57	72	72	67
Alanine	23	17	21	22	29	27	23	27	35	44	38	44
Serine	49	45	42	50	38	36	37	39	52	66	60	64
Leucine	55	51	55	59	61	60	67	60	65	81	77	76
Lysine	03	02	03	03	04	05	04	07	6	7	6	8
Glycine	15	10	12	13	16	15	15	15	24	29	25	31
Glutamic acid	0	04	0	04	03	02	02	04	0	0	0	0
Aspartic acid	0	05	0	05	04	03	03	05	0	0	0	0
Proline	38	25	32	30	40	40	40	42	70	77	83	72

L₁, L₂, L₃, L₄, plates impregnated with Cl[−], , SO₄^{2−}, CH₃COO[−], and PO₄^{3−} of zinc, respectively. Other conditions as in Table 14.

Source: Ref. 26.

B. Resolution of Enantiomers of PTH Amino Acids

Enantiomeric PTH amino acids were resolved (22) by mixing (+)-tartaric acid or (+)-ascorbic acid with silica gel during plate making, which resulted in the formation of diastereomers during development of the chromatogram, i.e., in situ rather than by prior treatment of racemic mixture with a chiral selector. Thus, a difference in the solubility of diastereomers so formed was the basis of achieving resolution (Table 31).

Table 23 TLC of PTH Amino Acids on Impregnated Silica Gel Layers

Solvent system	Ratio	Impregnation ^a	Reference
CHCl ₃ –H ₂ O–EtOAc	28:1:1	Zn ²⁺ , Cd ²⁺ , Hg ²⁺	18
CHCl ₃ –MeOH–benzene	19:1:9		
CCl ₄ –HOAc	19:1		
CHCl ₃ –benzene–EtOAc	25:5:3	Fe ²⁺ , Co ²⁺ , Zn ²⁺	19
CHCl ₃ –EtOAc	29:3	Fe ²⁺ , Co ²⁺ , Ni ²⁺	21
<i>n</i> -Heptane– <i>n</i> -butyl acetate	15:5		26
<i>n</i> -Heptane–propionic acid	20:4	Cl [–] , CH ₃ COO [–] , of zinc, or SO ₄ ^{2–} of	
Benzene–EtOAc		Mg ²⁺ , Mn ²⁺ , Fe ²⁺ , Co ²⁺	
CHCl ₃ – <i>n</i> -butyl acetate	10:5		30
CHCl ₃ –EtOAc	25:2		

^aSeparation data provided for various concentrations of each of the impregnating reagent.

Table 24 Optimum Experimental Conditions for the Separation of PTH Amino Acids by Continuous Multiple Development HPTLC

Step	Mobile phase	Plate length (cm)	Time (min)	PTH amino acid identified
1	CH ₂ Cl ₂	3.5	5	Pro
2	CH ₂ Cl ₂ –iso-PrOH (99:1)	7.5	10	Pro, Leu, Ile, Val, Phe
3	CH ₂ Cl ₂ –iso-PrOH (99:1)	7.5	10	Pro, Leu, Ile, Val, Phe, Met, Ala/Tip, Gly, Lys, Tyr, Thr
4	CH ₂ Cl ₂ –iso-PrOH (97:3)	7.5	10	Pro, Met, Lys, Tyr, Thr, Ser, Glu
5	C ₂ H ₅ COOCH ₃ –CH ₃ CN–CH ₃ COOH (74.3:20:0.7)	7.5	10	Asn, Glu/Gln, Asp, Cm-Cys, His, Arg

Source: Ref. 150o.

Table 25 hR_f Values of Amino Acid Standards on Reversed-Phase Layers

Amino acid	TLC system					
	1	2	3	4	5	6
Aspartic acid	30	59	72	60	83	73
Arginine	28	4	35	28	86	82
Serine	55	36	69	50	82	73
Glycine	50	38	62	45	69	54
Tyrosine	91	77	88	68	77	67
Alanine	78	59	71	63	71	54
Glutamic acid	82	70	86	67	83	69
Proline	56	69	64	40	65	46
Cystine	11	12	39	33	85	84
Methionine	90	74	75	59	75	61
Lysine	31	84	27	24	74	79
Tryptophan	90	77	85	63	72	63
Valine	90	74	75	59	75	61
Threonine	78	52	68	50	72	57
Histidine	21	3	29	23	77	68
Phenylalanine	90	76	83	65	72	61
Leucine	90	77	81	62	75	63
Isoleucine	91	77	81	62	74	61

Layers: 1, 2, Whatman C-18; 3, 5, Merck RP-18; 4, 6, Merck RP-18W. Mobile phases: 1, 3, 4: *rc*-Butanol–glacial acetic acid–water (3:1:1); 2, 5, 6: *n*-Propanol–water (7:3).

Source: Ref. 72a.

Table 26 hR_f Values of Amino Acid Standards on Normal-Phase Layers

Amino acid	TLC system			
	1	2	3	4
Aspartic acid	28	27	26	58
Arginine	18	18	17	2
Serine	26	30	27	40

Glycine	26	32	28	43
Tyrosine	46	58	53	78
Alanine	38	32	32	55
Glutamic acid	69	56	50	64
Proline	45	32	28	50
Cystine	10	11	9	30
Methionine	60	59	51	72
Lysine	15	13	10	4
Tryptophan	55	63	57	82
Valine	60	56	49	68
Threonine	34	32	32	53
Histidine	14	14	11	17
Phenylalanine	68	61	55	80
Leucine	79	61	55	78
Isoleucine	78	59	54	77

Layers: 1, Cellulose; 2, 4, Whatman silica gel; 3, Merck silica gel. Mobile phases: 1, Butan-2-ol-glacial acetic acid-water (3:1:1); 2, 3, *n*-butanol-glacial acetic acid-water (3:1:1); 4: *n*-propanol-water (7:3).

Source: Ref. 72a.

Table 27 Separation Data for Enantiomeric Compounds on β -CD-Bonded Phase Plates

Sample no.	Compound, D,L mixture	R_f		Mobile phase ^a	Detection method
		D	L		
1	Dns-leucine	0.49	0.66	40/66	Fluorescence
2	Dns-methionine	0.28	0.43	25/75	Fluorescence
3	Dns-alanine	0.25	0.33	25/75	Fluorescence
4	Dns-valine	0.31	0.42	25/75	Fluorescence
5	Alanine- β -naphthyl amide	0.16	0.25	30/70	Ninhydrin
6	Memionine- β -naphthylamide	0.16	0.24	30/70	Ninhydrin

^aVolume ratio of methanol to 1% triethylammonium acetate (pH 4.1).

Source: Ref. 156.

Table 28 RP-TLC Enantiomeric Separation Using Vancomycin as Chiral Mobile-Phase Additive^a

Compound ^b	hR_f		Vancomycin conc. (M)
	L	D	
AQC- <i>allo</i> -isoleucine	14	21	0.025
AQC-methionine	19	23	0.025
AQC-norleucine	13	16	0.025
AQC-norvaline	21	25	0.025
AQC-valine	23	27	0.025
Dansyl glutamic acid	21	23	0.04
Dansyl leucine	03	09	0.04
Dansyl methionine	05	12	0.04
Dansyl norleucine	03	07	0.04
Dansyl norvaline	05	12	0.04
Dansyl phenylalanine	03	05	0.04
Dansyl serine	15	20	0.04
Dansyl threonine	13	17	0.05
Dansyl tryptophan	01	03	0.04
Dansyl valine	06	10	0.04

^aMobile phase: Acetonitrile–0.6 M NaCl (2:10). Plates developed at room temperature (22°C) in cylindrical glass chambers. Time: 1–3 h for 5×20 cm plates.

Visualization: UV.

^bAQC: 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, a fluorescent tagging agent. Reaction mixture of AQC and amino acid was used without purifying the derivatives.

Source: Ref. 179.

Table 29 Results from Resolution of Dansyl DL-Amino Acids^a

Dansyl amino acid	hR_1			Solvent system (v/v) 0.5 M NaCl-MeCN
	From DL mix		Pure L	
	D	L		
Dns-Phe	65	50	50	15+2 15+1.5
Dns-Val	49	38	38	15+1

Dns-Trp	34	23	23	20+0.5
Dns-aspartic acid	67	55	55	15+2
	70	61	61	18+2
	60	52	52	15+1
	52	30	30	20+0.5
Dns-Leu	68	64	64	10+4+1 MeOH
				9+3+0.5 MeOH
Dns-Norvaline	61	56	56	17+2+0.4 MeOH
				16+2+0.25 MeOH

^aDetection at 254 nm.

Table 30 Typical Results for hR_f Values of Dansyl DL-Amino Acids on Plates Impregnated with Macrocyclic Antibiotics

Dansyl DL-amino acid	Erythromycin			Vancomycin			
	hR_f in solvent I			hR_f in solvent II		hR_f in solvent III	
	D	L		D	L	D	L
Ser	64	68	10:4:1	94 ^a	82 ^a	—	—
	36	30	15:1:1				
Phe	65	50	15:2:0	—	—	79	68
	27	20	15:1:0				
Val	30	22	15:1:0	87	73	63	59
Leu	32	24	15:1:0	87	73	88	75
Trp	47	38	18:1:0.25	87	73	80	69
α -Amino-n-butyric acid	51	42	12:1:0	87	73	88	75
Norleucine	71	63	16:1:0.4 HOAc	87	73	88	75

Solvent I: 0.5 M aq. NaCl–MeCN–MeOH; temp. 25±2°C; solvent front 10 cm in 20–25 min; detection at 254 nm.

Solvent II: MeCN–0.5 M aq. NaCl (10:4).

Solvent III: MeCN–0.5 M aq. NaCl (14:3).

Solvents II, III, and IIa: Temp. 18±2°C; solvent front 8.5 cm in 10–15 min; detection 254 nm.

^aSolvent IIa: MeCN–0.5 M aq. NaCl–2-propanol (10:4:1).

Source: For erythromycin, Ref. 156b. For vancomycin, Ref. 156c.

Table 31 hR_f of Pure and Resolved Enantiomers of PTH Amino Acids, for Tartaric Acid–Impregnated Plate^a

PTH amino acid in D,L mixture	hR_f of pure L	hR_f	
		D	L
Met	83	18	83
Phe	85	15	85
Trp	95	—	95
Val	80	21	80
Ile	92	15	92
Tyr	95	16	95
Thr	85	30	85
Ala	55	12	55
Ser	84	10	84

^aSolvent: Chloroform–ethyl acetate–water (28:1:1). Development time, 35 min. Solvent front, 10 cm. Room temperature, 25±1°C.

Impregnation with (+)-ascorbic acid resolved D,L mixtures of PTH-Met, -Phe, -Val, -Thr, -Ala, -Ser.

Source: Ref. 22.

Application of microcrystalline cellulose triacetate mixed with silica gel for TLC resolution of enantiomers of PTH amino acids and N- and C-substituted amino acids has been reported (179a, 179b) using 2-propanol or ethanol–water mixtures as mobile phase. The experiments showed that retention of solutes increased as the percentage of alcohol was reduced, in accordance with the general behavior of reversed-phase chromatography, and the analytes with an aromatic group usually resulted in better resolution than those with a nonaromatic group. Further, the compounds with a stereogenic center on a conformationally more rigid substrate (PTH-Phe and PTH-Tyr) were found to resolve efficiently into their enantiomers. The resolution data for these compounds are shown in Table 32.

C. Resolution of DL-Amino Acids

Günther et al. (157) covered the glass plates with silicic acid that had been made hydrophobic (RP 18-TLC), immersed (1 min) in a 0.25% copper(II) acetate solution (MeOH–H₂O, 1:9), dried, and then immersed in a 0.8% methanolic solution of the chiral selector (1 min); the chiral selector was (2*S*,4*R*,2'*RS*)-4-hydroxyl-1-(2'-hydroxydodecyl)proline. Using these plates they reported the resolution of racemic α -amino acids, as shown in Table 33. Using the same approach, described as ligand-exchange TLC, Günther, Martens, and coworkers were able to develop ready-to-use

Chiralplate, marketed by Macherey-Nagel (158). Martens et al. (159) and Günther et al. (160) reported the resolution of DL-methyl DOPA, and DL-DOPA on Chiralplate using methanol–H₂O–acetonitrile (50:50:30) as the mobile phase and ninhydrin as the detecting reagent. The R_f values for L-DOPA and D-DOPA were reported to be 0.47 and 0.61, respectively, and the system was capable of resolving enantiomers in trace amounts, with the lowest level of detection for the D enantiomers in L-DOPA samples being 0.25% (160). The resolution of enantiomers of α -substituted α -amino acids (161) and racemic mixtures of natural and nonnatural amino acids, TV-methylated and *N*-formylated amino acids, and various other derivatives of amino acids (162) has also been reported by Günther et al. (161) on a ready-to-use Chiralplate; typical results are presented in Tables 34 and 35. Enantiomers of unusual aromatic amino acids were analyzed on Macherey-Nagel Chiralplates with MeCN–MeOH–water (4:1:1 or 4:1:2) or MeCN–MeOH–water–diisopropyl ethylamine (4:1:2:0.1) as mobile phase and ninhydrin as detection reagent (162a). TLC on Chiralplates with acetonitrile–methanol–water (4:1:1) mobile phase and ninhydrin detection reagent was used in the quality control of L-tryptophan (162b).

As noted above, the resolution of enantiomers of amino acids and certain of their derivatives was achieved by Martens et al. (28, 159) and Günther and coworkers (157, 158, 160–162), by the immersion technique and ligand exchange. Bhushan (20, 22, 163, 163a) reported resolution of enantiomers of amino acids by mixing the chiral selector with silica gel slurry, e.g., (–)-brucine for the resolution of enantiomers of amino acids (Table 36), and impregnating the silica gel plates with Cu–L-proline complex for the resolution of DL-Phe, -Tyr, -Ile, and -Trp using different solvents (163a). TLC resolution of enantiomers of amino acids and their various derivatives has been

Table 32 Resolution Data for Separation of Enantiomers of PTH Amino Acids and N- and C-Substituted Amino Acids

Compound	Mobile phase	hR_f	
		L	D
PTH-Phe	Ethanol-water (80:20)	31	36
PTH-Tyr	2-Propanol-water (80:20)	53	64
<i>N</i> -CBZ-Phe-ONp	2-Propanol-water (80:20)	27	24
<i>N</i> -t-Boc-Phe-ONp	Methanol-water (80:20)	32	34

Migration distance: 15 or 13 cm; detection at 254 nm.

Source: Ref. 179d.

Table 33 Enantiomeric Resolution of Amino Acids by Thin-Layer Chromatography^a

Amino acid	R_f value (configuration)	Mobile phase ^b
Isoleucine	0.37 (2 <i>R</i> ,3 <i>R</i>)	A
	0.44 (2 <i>S</i> ,3 <i>S</i>)	
Phenylalanine	0.38 (<i>R</i>)	A
	0.45 (<i>S</i>)	
Tyrosine	0.26 (<i>S</i>)	B
	0.34 (<i>R</i>)	
Tryptophan	0.39 (<i>R</i>)	A
	0.45 (<i>S</i>)	
Proline	0.40 (<i>R</i>)	B
	0.59 (<i>S</i>)	
Glutamine	0.37 (<i>S</i>)	A
	0.53 (<i>R</i>)	

^aDevelopment distance, 14 cm; saturated chamber.^bA, methanol–water–acetonitrile (50:50:200); B, methanol–water–acetonitrile (50:50:30).

Source: Ref. 157.

reviewed by Bhushan (163) and Martens and Bhushan (164, 165, 165a). A novel chiral selector from (1*R*,3*R*,5*R*)-azabicyclo-[3,3,0]-octan carboxylic acid was synthesized and used as a copper(II) complex for the impregnation of RP-18 plates for ligand exchange TLC separation of amino acids, and the results were comparable to those on Chiralplate (28). A review on the resolution of enantiomers along with their possible explanations using an achiral stationary phase in conjunction with a mobile phase having no external chiral compound was also published (165b). In spite of

Table 34 Enantiomeric Resolution of α -Dialkylamino Acids by TLC^a

Parent amino acid	R ₁	R ₂	R_f value (configuration)	Mobile phase ^b
Asp	CH ₂ CO ₂ H	CH ₃	0.52 (D), 0.56 (L)	A
Glu	(CH ₂) ₂ CO ₂ H	CH ₃	0.58 (L), 0.62 (D)	A
Leu	CH ₂ CH(CH ₃) ₂	CH ₃	0.48, 0.59	A
Phe	CH ₂ C ₆ H ₅	CH ₃	0.53 (L), 0.66 (D)	A
Ser	CH ₂ OH	CH ₃	0.56 (L), 0.67 (D)	B

Try	CH ₂ -3-indolyl	CH ₃	0.54, 0.65	A
Tyr	CH ₂ -(p-OH-C ₆ H ₄)	CH ₃	0.63 (D), 0.70 (L)	A
Val	CH(CH ₃) ₂	CH ₃	0.51, 0.56	A
α -Aminobutyric acid	CH ₂ CH ₃	CH ₃	0.50, 0.60	A
Phe	CH ₂ C ₆ H ₅	CHF ₂	0.63, 0.70	A
Phe	CH ₂ C ₆ H ₅	CH ₂ CHCH ₂	0.57, 0.63	A
Phe	CH ₂ C ₆ H ₅	CH ₂ CH ₂ SCH ₃	0.57, 0.62	A

^aDevelopment distance 13 cm; saturated chamber.

^bMobile phase A, methanol–water–acetonitrile (1:1:4); B, methanol–water–acetonitrile (5:5:3), ratios by volume.

Source: Ref. 161.

Table 35 Enantiomeric Resolution of Racemates by TLC^a

Racemate	R_f value (configuration)	Mobile phase ^b
Valine	0.54 (D), 0.62 (L)	A
Methionine	0.54 (D), 0.59 (L)	A
<i>allo</i> -Isoleucine	0.51 (D), 0.61 (L)	A
Norleucine	0.53 (D), 0.62 (L)	A
2-Aminobutyric acid	0.48, 0.52	A
<i>O</i> -Benzylserine	0.54 (D), 0.65 (L)	A
3-Chloroalanine	0.57, 0.64	A
<i>S</i> -(2-Chlorobenzyl)-cysteine	0.45, 0.58	A
<i>S</i> -(3-Thiabutyl)-cysteine	0.53, 0.64	A
<i>S</i> -(2-Thiopropyl)-cysteine	0.53, 0.64	A
<i>cis</i> -4-Hydroxyproline	0.41 (L), 0.59 (D)	A
Phenylglycine	0.57, 0.67	A
3-Cyclopentylalanine	0.46, 0.56	A
Homophenylalanine	0.49 (D), 0.58 (L)	A
4-Methoxyphenylalanine	0.52, 0.64	A
4-Aminophenylalanine	0.33, 0.47	A
4-Bromophenylalanine	0.44, 0.58	A
4-Chlorophenylalanine	0.46, 0.59	A

2-Fluorophenylalanine	0.55, 0.61	A
4-Iodophenylalanine	0.45 (D), 0.61 (L)	A
4-Nitrophenylalanine	0.52, 0.61	A
<i>O</i> -Benzyltyrosine	0.48 (D), 0.64 (L)	A
3-Fluorotyrosine	0.64, 0.71	A
4-Methyltryptophan	0.50, 0.58	A
5-Methyltryptophan	0.52, 0.63	A
6-Methyltryptophan	0.52, 0.64	A
7-Methyltryptophan	0.51, 0.64	A
5-Bromotryptophan	0.46, 0.58	A
5-Methoxy tryptophan	0.55, 0.66	A
2-(1-Methylcyclopropyl)-glycine	0.49, 0.57	A
<i>N</i> -Methylphenylalanine	0.59 (D), 0.61 (L)	A
<i>N</i> -Formyl-tert-leucine	0.48 (+), 0.61 (–)	A
<i>N</i> -Glycylphenylalanine	0.51 (L), 0.57 (D)	B

^aDevelopment distance, 13 cm; saturated chamber.

^bA, methanol–water–acetonitrile (50:50:200); B, methanol–water–acetonitrile (50:50:30).

Source: Ref. 162.

being contradictory to the generally accepted rules of stereochemistry that no discrimination of optical isomers is possible in an achiral environment, chromatography has been reported to be successful in providing resolution of enantiomeric mixtures (nonracemic) of certain compounds. Nevertheless, extensive studies to obtain stronger explanations for such chiral resolutions are required.

Thin-layer chromatographic resolution of enantiomers from racemic amino acids was achieved (165c) on silica gel plates impregnated with optically pure (–)-quinine. The successful solvent systems were butanol–chloroform–acetic acid (3:7:5) for DL-methionine, 6:8:4 for alanine, and 10:1:4 for threonine and ethyl acetate–carbon tetrachloride–propionic acid (10.5:6.5:3.5) for valine (Table 37). Minimum detection limits were found to be different for each of the amino acids,

Table 36 Resolution Data for Enantiomers of Amino Acids from Brucine-Impregnated Plates^a

Sample no.	DL-Amino acid	hR_f , pure L	Resolved hR_f	
			D	L
1	Alanine	53	18	53
2	2-Aminobutyric acid			

3	Isoleucine	35	16	35
4	DOPA			
5	Leucine			
6	Methionine	29	18	29
7	Norleucine			
8	Phenylalanine	40	27	40
9	Serine	50	12	50
10	Threonine	29	16	29
11	Tryptophan	31	17	31
12	Tyrosine	29	22	29

^aSilica plates impregnated with (–)-brucine, developed in *n*-butanol–acetic acid–chloroform (3:1:4), up to 10 cm.

Source: Ref. 20.

ranging between 0.9 and 3.7 μg . The effects of concentration of impregnating reagent, temperature, and pH on resolution of enantiomers have been studied in detail.

Direct enantiomeric resolution of DL-arginine, DL-histidine, DL-lysine, DL-valine, and DL-leucine into their enantiomers was achieved (165d) by TLC on silica gel plates impregnated with optically pure (1*R*,3*R*,5*R*)-2-azabicyclo[3,3,0]octan-3-carboxylic acid (0.011 M) as a chiral selector, which is a waste of the pharmaceutical industry. Various combinations of acetonitrile–methanol–water were found to be successful in resolving the DL-amino acids (Table 38). The spots were detected by ninhydrin (0.2% in acetone), and the detection limit was 0.66 μg .

Table 37 hR_f ($R_f \times 100$) Values of Resolved DL-Amino Acids on Silica Gel Plates Impregnated with (–)-Quinine (0.1%)

Sample no.	DL-Amino acid	Solvent system	hR_f Value from racemic mix			
			Ratio (v/v)	Pure L	D	L
1	Methionine	S ₁	3:7:5	50	25	50
2	Alanine	S ₁	6:8:4	16	7	16
3	Threonine	S ₁	10:1:4	11	5.5	11
4	Valine ^a	S ₂	10.5:6.5:3.5	19.3	11.8	19.3
5	Leucine	S ₂	10.5:4:7	13.8	6.4	13.8
6	Isoleucine	S ₂	10.5:4:7	12.9	6.4	12.9

Time, 40–45 min; solvent front, 10 cm; detection, ninhydrin solution; temp., $25 \pm 2^\circ\text{C}$.

^aTemperature for resolution of valine is $18 \pm 2^\circ\text{C}$.

S₁, *n*-Butanol–chloroform–acetic acid; S₂, ethyl acetate–carbon tetrachloride–propionic acid.

Source: Ref. 165c.

Table 38 hR_f ($R_f \times 100$) Values of Enantiomers of DL-Amino Acids Resolved on Plates Impregnated with (1*R*,3*R*,5*R*)-2-Azabicyclo[3,3,0]octan-3-Carboxylic Acid

DL-Amino acid	Solvent system, acetonitrile-methanol-water	Pure L	hR_f Value from DL mixture	
			L	D
Arginine	7:6:3	13	13	6
Histidine	7:6:2	25	25	18
	3:1:1 ^a	36	36	11
Lysine	10:5:2	31	31	15
Leucine	10:4:3	21	21	11
Valine ^b	10:5:2	50	50	38
Serine	7:1:1 ^a	18	18	06
Tryptophan	8:1:1 ^a	62	63	38

Time: 30–35 min; solvent front, 8.5 cm; detection, ninhydrin (0.2% in acetone); temp., $26 \pm 2^\circ\text{C}$.

^aTime: 25–30 minutes for 10 cm run (from Ref. 156a).

^bAt $20 \pm 2^\circ\text{C}$ (from Ref. 165d).

A new chiral reagent, NSP-C1, was synthesized and used to derivatize amino acids, and the resulting diastereomers were resolved by TLC (166). Chiralplate with MeCN–MeOH–H₂O (4:1:1) as mobile phase was used to evaluate reaction products in the synthesis of modified Phe and Tyr derivatives (167) and also to separate aspartame and its precursor stereoisomers (168). Tryptophans and substituted tryptophans were separated on cellulose layers developed with copper sulfate solutions (169) when excess Cu²⁺ ions decreased the chiral discrimination of the system, and with aqueous α -cyclodextrin (1–10%) plus NaCl solutions (0.1, 0.5, 1.0 M) when the best results were obtained with aqueous 4% α -cyclodextrin–1 M NaCl solution (170), comparable to Chiralplate. It was observed that chiral effects are essentially additive (for cellulose and α -cyclodextrin) and there is a strong temperature dependence for the chiral separations.

Enantiomeric RP-TLC separations of amino acids and derivatives were obtained with α - and β -cyclodextrins (171), hydroxypropyl- β -cyclodextrin (172), and bovine serum albumin (173–176) in the mobile phase. Chiral monohalo-*S*-triazines were used for the TLC resolution of DL-amino acids (177). Racemic dinitrophenyl, dinitrophenyl, and

dinitrobenzoyl amino acids were separated on RP-TLC plates developed with aqueous organic mobile phases containing bovine serum albumin as a chiral agent (178).

VII. QUANTIFICATION

Thin-layer chromatography is supplemented with spectrophotometric methods for the quantification of amino acids and their PTH and DNP derivatives.

A. Amino Acids^{*}

1. Materials

Materials consist of standard solutions of amino acids, acetate buffer (4 M, pH 5.5), ethanol (50%), methyl Cellosolve (ethylene glycol monomethyl ether), and ninhydrin reagent (0.9 g of ninhydrin and 0.12 g of hydrantin dissolved in 30 mL of methyl Cellosolve and 10 mL of acetate buffer, prepared fresh).

^{*} Based on Ref. 180.

2. Method

Six to eight standard dilutions in an appropriate concentration range for each amino acid are prepared; 2 mL of amino acid solution and 2 mL of buffered ninhydrin are mixed in a test tube, heated in a boiling water bath for 15 min, and cooled to room temperature, and 3 mL of 50% ethanol is added. The extinction is read at 570 nm (or 440 nm for proline) after 10 min. Standard plots of concentration versus absorbance are drawn for each amino acid. The scraped layer corresponding to each spot is extracted with 70% ethanol in a known minimum volume, and ninhydrin reaction is performed followed by spectrophotometry. The concentration of unknown samples is read from the standard plots. TLC with densitometry was used to determine 0.5 mg/L of phenylalanine in blood serum as an indicator of phenylketonuria (181).

B. PTH Amino Acids

The quantification of PTH amino acids is carried out in situ or after elution. For in situ determination, the fluorescence-quenching areas of PTH derivatives are usually measured, against the fluorescent background, at 254 nm. Pataki and coworkers (182, 183) used a Turner fluorimeter fitted with a door for scanning chromatoplates and found that the position of the scanner, the standardization of time between scanning and the end of chromatography, the loading volume, the developing distance, and the layer thickness were the important influencing factors for reproducibility. The quantification of PTH amino acids is also carried out by measuring their UV absorbance after they have been eluted from the layer (184). The scraped layer is extracted with methanol overnight and centrifuged for 30 min at 300 rpm, and the spectra of the extracts are recorded in the

range from 320 nm to about 230 nm. To obtain reproducible UV absorbances the layers must be washed with methanol prior to development and with chloroform after the separation has been carried out. The quantification of PTH amino acids in our lab during sequence determination of certain plant proteins (96, 97) has been practiced as follows. The developed chromatograms are exposed to iodine vapors, and the brownish spots of PTH amino acids are scraped off and thoroughly eluted with 95% ethanol or ethyl acetate, and the iodine is removed by warming the sample tubes in a warm water bath. The optical densities are read at 269 and 245 nm, appropriate blank determinations are carried out, standard plots are drawn, and concentrations of unknown samples are calculated.

C. DNP Amino Acids*

The layer is scraped off the plate and extracted for 5 min with 1 mL of 0.05 M Tris buffer of pH 8.6 at room temperature. Then the slurry is removed by centrifugation, and the clear liquid is evaluated by measuring the optical density at 360 or 385 nm for DNP-proline. For a blank, a similar extract is obtained from a clear spot on the same layer. Pataki and Wang (183) recommended the use of direct fluorimetric quantification (fluorescence quenching) in situ. Silica gel G plates were developed in chloroform–benzyl alcohol–acetic acid (70:30:3) and *n*-propanol–ammonia (7:3), and polyamide plates were developed in benzene–acetic acid (4:1). The spots were scanned by using a Camag/Turner scanner, after being dried in a stream of air, at the scanning speed of 20 mm/min and an excitation wavelength of 254 nm.

VIII. AMINO ACIDS AS CHIRAL SELECTORS

As already noted above, impregnation of adsorbent of thin layers with various types of compounds has resulted in improved separation of various classes of compounds. One of the most interesting features of impregnation is the enantiomeric resolution of a variety of compounds using optically pure selectors as the impregnating reagents. Because the focus of this chapter is on TLC of amino acids, it was considered worthwhile to draw the reader's attention to the application of optically pure isomers of amino acids as suitable chiral selectors for direct enantiomeric separation and

* Based on Ref. 185.

Table 39 Amino Acids as Chiral Selectors

Sample no.	Racemate resolved	Chiral selector	hR_f		Ratio of solvents	Detection limit	Ref.
			(+)	(-)			
1	(±)-Ibuprofen	L-Arginine	80	77	5:1:1	0.1 µg of racemic mixture	186
2	Atropine	L-Histidine	4.6	9.2	11.3:5.4:0.6	6 µg	187
3	(±)-Atenolol	L-Lysine	10	03	16:4, 16:2, 15:2	2.6 µg	188

		L-Arginine	12	18	15:5, 14:6		
4	(±)-Propranolol	L-Lysine	13	04	15:2, 16:2	2.6 μ g	188
		L-Arginine	26	07	15:3, 16:4		
5	(±)-Metoprolol	L-Lysine	15	11	15:4, 15:5	0.26 μ g	188
		L-Arginine	23	12	15:3, 16:4		

Detection: Iodine vapor. Solvent system: Samples 1 and 2, MeCN-MeOH-H₂O; samples 3-5, MeCN-MeOH.

determination of enantiomeric purity of certain pharmaceutically important compounds marketed as racemic mixtures. During the last few years, there have been reports on the resolution of (±)ibuprofen, (±)- β -blockers, atropine, etc., into their enantiomers using thin silica gel plates impregnated with one or the other pure isomer of an amino acid. Some of these results are summarized in Table 39.

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15

Antibiotics

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I. INTRODUCTION

Antibiotics are chemical substances produced by living organisms (generally microorganisms) or derivatives of these substances that suppress the growth of or kill other microorganisms in low concentrations. Nowadays the term antibiotics is extended to synthetic antibacterial agents such as the sulfonamides or quinolones.

Antibiotics are very common substances in the microbial world, but it was not until the 1940s that their true potential was acknowledged and large-scale production was developed. Penicillin was the first antibiotic, discovered by Alexander Fleming in 1928. The first sulfonamide drug, tested in 1932 by Gerhard Domagk and patented in 1935 by Bayer (Germany), was a red azo dye called prontosil. In 1939 René Dubos obtained tyrothricin, a mixture of two peptide antibiotics, gramicidin and tyrocidine. That same year penicillin was isolated by Ernst Chain, Howard Florey, and others. In 1944 Selman Waksman and Albert Schatz isolated streptomycin from *Streptomyces griseus*. The Nobel prizes for medicine were awarded to Domagk (1939); Fleming, Chain, and Florey (1945); and Waksman (1952). Most classes of antibiotics were discovered in the 1940s, 1950s, and 1960s.

The large number of antibiotics currently available can be classified in a variety of ways, e.g., by their chemical structure, their microbial origin, or their mode of action. They are also designated by their effective range: broad-spectrum antibiotics such as tetracyclines, medium-spectrum such as penicillins, and narrow-spectrum such as polymyxins. The most common classification is based on chemical structure and mechanism of action, as follows (1):

1. *Bactericidal cell wall agents* inhibit synthesis of bacterial cell walls (penicillin, cephalosporins, cycloserine, vancomycin, bacitracin, and the imidazole antifungal agents miconazole, ketoconazole, and clotrimazole).
2. *Bactericidal cell membrane agents* act directly on the cell membrane of microorganisms, affecting membrane permeability and leading to leakage of intracellular compounds (polymyxin, colistimethate, and the polyene antifungal agents nystatin and amphotericin B).

3. *Bacterial protein synthesis inhibitors* include

- a. Bactericidal agents that alter protein synthesis, leading to cell death (the aminoglycosides streptomycin, neomycin, kanamycin, gentamicin, tobramycin, amikacin, and kasugamycin).
 - b. Bacteriostatic agents that inhibit protein synthesis (chloamphenicol, the tetracyclines, and the macrolides).
4. Bactericidal agents that affect nucleic acid metabolism (rifamycins and quinolones).
 5. Bacteriostatic antimetabolites, which block metabolic steps essential to microorganisms (trimetoprim and the sulfonamides).
 6. Nucleic acid analogs that halt viral replication (zidovudine, gancyclovir, vidorabine, and acyclovir).

The arrival of antibiotics was recognized as a beneficial turning point in medicine, which seemed finally to have prevailed over bacterial diseases. However, two serious disadvantages have occurred. One is connected with toxic side effects of many antibiotics, e.g., allergy, blood and nervous system diseases, hepatic injury, nephropathy, cardiotoxicity, retinotoxicity, and ototoxicity. Most antibiotics cause changes in the intestinal bacterial population and can result in infections from other microorganisms such as fungi as well as in colitis and diarrhea. The second disadvantage is connected with the emergence of antibiotic-resistant bacteria. Bacterial resistance is due to random genetic mutations that alter bacterial sensitivity to the drug and to chemically similar drugs. Many bacteria transfer their resistance to other bacteria of the same or different species. Indiscriminate use of antibiotics, overprescription, and failure to finish courses of drugs are considered among the reasons for the rise of drug-resistant bacteria. Another source of resistance seems to be overuse of antibiotics in veterinary medicine and animal husbandry. Antibiotics are used in prevention and treatment of animal diseases and, which is more dangerous, as growth promoters. All that results in the appearance of unsafe antibiotic residues or their metabolites in food. The monitoring of antibiotic residues should be an important task for government authorities. Antibiotic resistance is connected with increased mortality and health care costs. The postantibiotic era has approached in which infectious diseases we thought were under control are coming back. Prevention and control of these infections will demand the development of new antibiotics and/ or vaccines and reasonable use of existing antibiotics.

The analytical methods for determining antibiotics can be based on microbiological, immunochemical, and physicochemical principles. The most popular methods of the latter group are chromatographic ones, mainly liquid chromatography, including high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). HPLC offers high sensitivity and separation efficiencies. Usually, before HPLC analysis tedious sample pretreatment is necessary; this may consist of protein precipitation, ultrafiltration, partitioning, metal chelate affinity chromatography (MCAC), dialysis, matrix solid-phase dispersion (MSPD), or solid-phase extraction (SPE). TLC is cheaper and less complicated than HPLC, provides high sample throughput, and usually requires limited sample pretreatment. However, the method is generally less sensitive and less selective and gives poor resolution. Some of these problems can be solved by high-performance thin-layer chromatography (HPTLC) or forced-flow planar chromatography (FFPC). Reliability of TLC can also be achieved through automation, applying special

techniques of development and scanning densitometry as a detection method, spraying the plate after development with appropriate reagents, or bioautography. There is also the possibility of coupling TLC with mass spectrometry (MS).

There are many reviews on chromatography of antibiotics (2–8) and on thin-layer chromatography (9–11) where TLC of antibiotics is included. The broadest review on TLC of antibiotics was written by Kreuzig (12).

This chapter deals with antibiotics used in both human and veterinary medicine. The chapter includes quinolones, nitrofurans, and sulfonamides, which are synthetic chemotherapeutic agents. Many antibiotics are obtained semisynthetically. Some of those of natural origin are currently synthesized for economical reasons. According to Lambert and O'Grady (13), the name “chemotherapeutics” can be used in parallel with “antibiotics,” because old definitions cannot withstand the development in this field of pharmacotherapy.

My initial intention was to describe only recent papers. However, to give a full survey, some older but historically valid papers are also presented.

II. TLC OF ANTIBIOTICS

A. Penicillins

In 1928, Sir Alexander Fleming, a Scottish biologist, observed that *Penicillium notatum*, a common green mold, had destroyed *Staphylococcus* bacteria growing on a germ culture medium. A decade later a scientific team lead by Howard Florey, Ernst Chain, and Norman Heatley isolated and purified the ingredient responsible, penicillin, and established its effectiveness against many serious bacterial infections. In 1941 the first doses of an injectable form of the drug were available for therapeutic use, and by the end of World War II industrial production had begun. The basic structure of penicillins is a thiazolidine ring linked to a β -lactam ring to form 6-aminopenicillanic acid, the so-called penicillin nucleus. This acid, obtained from *Penicillium chrysogenum* cultures, is a precursor of semisynthetic penicillins (ampicillin, amoxicillin, oxacillin, cloxacillin, dicloxacillin, and methicillin) produced by attaching different side chains to the “nucleus.” Benzylpenicillin (penicillin G) and phenoxymethylpenicillin (penicillin V) are naturally occurring penicillins.

The most widely used stationary phase for analysis of penicillins is silica gel, but reversed-phase (RP) or cellulose plates are also used. It is advantageous to add acetic acid to the mobile phase and/or to spot acetic acid before sample injection in order to avoid the decomposition of β -lactams on silica gel. RP phases usually contain pH 5–6 buffer and organic solvents.

Penicillin V in fermentations (14) was controlled by chromatography on silica gel HPTLC plates with toluene–ethyl acetate–acetic acid (40:40:20) as the mobile phase. After scanning at 268 nm, R_f values for 4-hydroxyphenicillin V, penicillin V, and phenoxyacetic acid were calculated as 0.34, 0.50, and 0.60, respectively.

Hendrickx et al. (15) described identification of 18 penicillins on silica gel and on silanized silica gel, using 35 mobile phases. They concluded that TLC on silica gel was not a valuable general method, because RP systems gave better results. No system could

separate all 18 penicillins, but it was easy to find systems appropriate for groups of related products.

Two-dimensional TLC on cyano phases with dichloromethane-hexane-acetic acid (9:10:1) in the first dimension and acetonitrile-methanol-water (40:37:32) in the second was used for the separation of a *Penicillium* fungal extract (16). Detection was carried out under UV light at 254 and 366 nm.

Dhanesar (17) described the use of scanning densitometry for direct quantification of six penicillins on a hydrocarbon-impregnated silica gel HPTLC plate without solvent elution. Penicillin standards and samples were dissolved in water and spotted onto the plate. The sample remained as a single spot centered at the point of application, thereby facilitating direct quantification by densitometry at different wavelengths. The detection level was 0.1 ng.

For the quantitative analysis of ampicillin in urine (18), the following method was used. Ampicillin was isolated from urine by extraction with chloroform containing benzalkonium chloride. Extracts were evaporated and resolved in chloroform. The obtained samples and standards were then developed with dioxane-water-1-butanol-formic acid (75:15:15:1.25) on HPTLC SiF₂₅₄ plates. Scanning was performed at 480 nm after spraying the plate with ninhydrin and heating it at 110°C for 5 min. The limit of detection was 0.05 mg/mL.

Saesmaa (19) described a number of TLC systems and spray reagents that are capable of separating the cation and anion parts of benzathine and embonic acid salts of ampicillin, amoxicillin, cefalexin, and 3 embonate. These TLC methods were used for assessment of the purity of the synthesized embonate and benzathine salts of β -lactam antibiotics.

The residues of penicillins in food can be dangerous for a consumer because of possible allergic reactions. Sensitive persons may suffer an anaphylactic reaction after consumption of a single bite of meat contaminated with penicillin, which usually occurs when the producer does not follow prescribed withdrawal periods for the animal (20). The traces of penicillins in meat were extracted with methanol and analyzed by TLC bioautography (TLC-B) on silica gel or cellulose using polar mobile phases and detection with *Bacillus subtilis* (21–23). Different solvents and sorbents were tested for their suitability for TLC-B of several antibiotics—among others, penicillin, ampicillin, and amoxicillin (24). The best results for these antibiotics were obtained on silica gel and alumina tested with *Escherichia coli*. The solvents usually used for TLC were tested, and only three of them gave inhibition zones. They were tetrahydrofuran, 5% sulfuric acid, and 5% hydrogen chloride. Recently, a new bioautographic test kit was produced by Merck and tested by Eymann and Hauck (25) for procaine penicillin G, monensin, and chlorotetracycline, with good results.

The retention behavior of some penicillin and cephalosporin derivatives was studied by Kovács-Hadady and Szilágyi (26) by means of overpressured layer chromatography (OPLC) on silica gel impregnated with tricaprylmethylammonium chloride (TCMA) using methanol-water mobile phases. The retention increased with increasing TCMA concentration on the plate. The basis of the retention was not ion pairing but hydrophobic interactions due to the caprylic group of TCMA. The pH and ionic strength of the eluent had no effect on retention.

A review paper of chromatographic methods for the analysis of penicillins in food animal tissues was written by Boison (8). The review includes microbiological and immunochemical tests, gas chromatography, HPLC, and TLC.

B. Cephalosporins

Other β -lactam antibiotics are cephalosporins derived from natural cephalosporin C produced by the *Cephalosporium acremonium* fungus. They possess a cephem nucleus (7-aminocephalosporanic acid) substituted with two side chains. Chemically they are closely related to penicillins and exhibit the same mechanisms of action, i.e., they inhibit bacterial cell wall synthesis. They kill both gram-positive and gram-negative bacteria and are used mainly for treating staphylococcal and streptococcal infections in patients who cannot use penicillins. Cephalosporins are commonly divided into three generations that differ slightly in their spectrum and toxicity.

Cephalosporins can be analyzed by both normal- and reversed-phase TLC. More efficient separation is obtained on silanized gel than on bare silica gel. Mobile phases are polar and similar to those used for penicillins. Cephalosporins can be detected by bioautography and by UV detection at 254 nm. The detection limit can be diminished by applying a reagent such as ninhydrin, iodoplatinate, chloroplatinic acid, or iodine vapor.

Cephalosporin C was separated from its by-products formed during fermentation (desacetyl-cephalosporin C, desacetoxyccephalosporin C, and penicillin N) by RP-TLC with water or aqueous phases (27).

Quintens et al. (28) described a procedure that enables identification of 30 cephalosporins on TLC silanized silica gel plates containing a fluorescence indicator. The mobile phases were composed of a buffer (15% w/v of ammonium acetate adjusted to pH 6.2 with glacial acetic acid) mixed with various organic modifiers:

- A. Buffer-methanol (85:15)
- B. Buffer-acetonitrile (85:15)
- C. Buffer-methanol-acetonitrile (85:10:5)
- D. Buffer-acetone (85:15)
- E. Buffer-tetrahydrofuran (90:10)
- F. Buffer-ethanol (85:15)
- G. Buffer-methyl acetate (85:15)

Table 1 contains hR_f values of 30 cephalosporins developed with phases from A–G. No system separated all the cephalosporins, but 12 could be separated from all others with at least one mobile phase. The others could be identified when supplementary information was obtained from color reactions and/or an additional TLC system.

With the OPLC system described above (26), cephalosporin C, 7-aminocephalosporanic acid, 7-aminodesacetoxyccephalosporanic acid, a new cephalosporin BK-218, and cefalotin can be separated. Cephalosporins were also separated using ion-pair TLC on silanized plates. Detection was done under a UV lamp and by iodine vapor (29).

Misztal et al. (30) developed new solvent systems, both normal- and reversed-phase, for the separation of seven cephalosporins: cefoxitin sodium (A), cefsulodin sodium (B), cefalotin sodium (C), cefatoxime sodium (D), ceftriaxone sodium (E), cefalexin

monohydrate (F), and cefamandole naphthate (G). The R_f values on silica gel plates for the best NP solvents are presented in Table 2. The first phase separated six analyzed drugs; the second, five. The best RP solvents, offering good separation of all analyzed substances on RP C-18, were phosphate buffer pH 2.65–tetrahydrofuran (9:1, 8:2, or 7:3); and phosphate buffer pH 2.65–methanol (8:2). All cephalosporins could be detected at 254 nm. Eight other modes of detection are presented in Table 3.

Bushan and Parshad (31) used Na_2EDTA as an impregnating agent to resolve some cephalosporins. Changes in the concentration of the impregnating reagent and in the composition of the mobile phase influenced the resolution. Three new solvent systems were successful:

Table 1 Cephalosporin hR_f Values on Laboratory Made Silanized Silica Gel Plates

No.	Generic name	Mobile phase ^a						
		A	B	C	D	E	F	G
1	Cephalosporin C	80	85	80	91	89	86	91
2	Cefadroxil	72	77	72	79	67	76	78
3	Cefatrizine	52	61	51	58	36	57	58
4	Cefaloglycin	26	39	28	47	37	34	46
5	Cefaclor	43	51	44	57	46	49	56
6	Cefalexin	39	53	42	59	50	47	60
7	Cefradine	33	50	36	55	45	40	57
8	Cefonicid	61	52	53	56	37	63	49
9	Cefamandole	21	24	18	27	15	27	23
10	Cefamandole naphthate	07	09	06	09	06	10	07
11	Cefsulodin	74	69	69	80	73	77	79
12	Ceforanide	60	64	56	71	61	65	72
13	Cefalotin	14	20	12	21	12	19	17
14	Cefoxitin	33	35	30	40	26	39	32
15	Cefaloridine	22	29	22	37	28	29	32
16	Cefalonium	41	36	36	41	32	47	38
17	Cefapirin	14	22	14	23	20	18	26
18	Cefazolin	35	40	34	42	33	43	44
19	Cefuroxime	32	34	31	35	22	39	28
20	Cefotiam	40	44	39	54	48	49	53
21	Cefotaxime	35	40	34	44	31	43	41

22	Cefmenoxime	32	33	29	37	22	40	32
23	Ceftazidime	50	64	54	73	63	58	71
24	Ceftizoxime	57	59	56	63	47	62	58
25	Ceftriaxone	52	60	51	67	50	59	62
26	Cefixime	59	66	58	67	51	62	66
27	Cefotetan	70	68	67	75	65	74	74
28	Cefoperazone	15	18	13	20	12	26	18
29	Moxalactam ^b							
30	Flomoxef	53	40	45	45	34	58	37

^aThe results given are the mean values of at least two experiments. A–G: See text listing.

^bThis substance gave a spot that tailed from the origin.

Source: Ref. 28.

Table 2 Chromatographic Systems and R_f Values on Silica Gel Plates

No.	Mobile phase (by volume)	hR_f^a						
		A	B	C	D	E	F	G
1	Ethyl acetate–acetic acid–water (3:1:1)	51	0.0	62	40	26	49	23
2	<i>n</i> -Butanol–acetic acid–water (20:1:2)	31	0.0	41	19	0.0	26	14

^aA–G: See text listing.

Source: Ref. 30.

Table 3 Detection of Cephalosporins on Silica Gel Plates by NP-TLC and RP-TLC

No.	Reagent	Limit of detection ^a (μg)						
		A	B	C	D	E	F	G
1	Dragendorff reagent (after Amelink)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
2	Iodine vapor	0.6	0.6	0.8	0.5	0.6	0.8	0.8
3	0.2% Ninhydrin solution in ethanol	0.8	0.8	0.8	0.8	0.8	0.8	0.6
4	0.25% Ninhydrin solution in 1% acetic acid	0.5	0.8	0.8	0.8	0.8	0.8	0.8
5	Hexacyanoferrate(II) and ferric chloride(III)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
6	2% <i>p</i> -Dimethylamine–benzaldehyde in ethanol	2.0	2.0	2.0	2.0	2.0	2.0	2.0
7	Potassium iodoplatinate (acidified with HCl)	1.5	1.5	1.5	1.5	1.5	1.5	1.5
8	Sulfuric acid–formaldehyde Marquisa reagent	1.0	1.0	0.8	0.8	0.8	0.8	0.8

^aA–G: See text listing.

Source: Ref. 30.

1. Propionic acid–2-propanol–water (6:3:3)
2. Ethyl acetate–2-propanol–water (3:5:3)
3. *n*-Butanol–water–acetic acid (5:4:2)

Qureshi et al. (32) used layered double hydroxide (LDH) anion exchanger mixed with silica gel as the stationary phase for separation of ceftriaxone, cefuroxime, cefotaxime, ceftazidime, cefadroxil, and cefalexin. LDHs consist of positively charged brucite-like layers with partial substitution of M(II) cations by M(III); their formula is $\text{Mg}_{2.44}\text{Al}(\text{OH})_{0.88}(\text{CO}_3)_{0.5} \cdot n\text{H}_2\text{O}$. Eighteen mobile phases were tested, and the best were buffer–methanol (2:8), buffer–methanol–acetonitrile (85:10:5), buffer–methyl acetate (85:15), and buffer–diethyl ether (85:15). The buffer solution consisted of a 15% w/v solution of ammonium acetate adjusted to pH 6.2 with glacial acetic acid. The spots were detected with iodine vapor, and R_f and R_M were calculated. For the phase buffer–methanol, R_f and R_M values vs. methanol concentration were established. For quantitative work the regions containing the cephalosporins were scraped from the plate and eluted with distilled water, and the content was spectrophotometrically determined. Recovery was close to 100%.

Eric-Jovanovic et al. (33) presented the method of quantitative determination of ceftriaxone, cefixime, and cefotaxime, third-generation cephalosporins, in dosage forms. The standards and sample solutions were injected into the concentrating zones of silica gel HPTLC plates and developed with the mobile phase ethyl acetate–acetone–methanol–water (5:2.5:2.5:1.5). The densitograms were produced at 270 nm. The calibration curves were established in the concentration range 125–500 ng per spot, for all cephalosporins analyzed. The limits of detection for ceftriaxone, cefixime, and cefotaxime were found to be 19.1, 18.4, and 16.7 ng, respectively.

Dhanesar described the use of scanning densitometry for direct quantification of ceftriaxone (34, 35) and 12 other cephalosporins (36) on hydrocarbon-impregnated silica gel plates without prior solvent elution. The method was similar to the one described above for quantification of penicillins (17).

C. Aminoglycosides

Aminoglycosides consist of two or more amino sugars joined via glycosidic bonds to an amino-cyclitol nucleus. Streptomycin, the first known aminoglycoside, was isolated in 1943 by Selman Waksman and Albert Schatz from *Streptomyces griseus*; then others were discovered in different *Streptomyces* strains. Some aminoglycosides are semisynthetic derivatives of natural fermentation products; e.g., amikacin is derived from kanamycin A, arbekacin and dibekacin from kanamycin B, isepamicin from gentamicin B, netilmicin from sisomicin, and dihydrostreptomycin from streptomycin, although the last is also produced naturally (5). Aminoglycosides are particularly active against aerobic microorganisms and against *Tubercle bacillus*, but because of their potential ototoxicity and nephrotoxicity and because of the emergence of resistant bacterial strains that produce aminoglycoside-modifying enzymes, they should be carefully administered. Aminoglycosides, due to their extremely polar, hydrophilic character, are analyzed

mainly on silica gel. Polar organic solvents (methanol, acetone, chloroform) mixed with 25% aqueous ammonia are the most popular mobile phases. Because the majority of aminoglycosides lack UV absorption, they must be derivatized by spraying or dipping after development with, for instance, fluorescamine, vanillin, or ninhydrin solutions. Bioautography with *Bacillus subtilis*, *Sarcina lutea*, and *Mycobacterium phlei* is also possible. Shaikh and Allen (37) presented an overview of physicochemical methods, including 13 concerning TLC, for determining aminoglycosides in tissues and fluids of food-producing animals.

A mixture of nine aminoglycosides were almost separated on HPTLC silica gel plates using the eluent 25% ammonia solution–methanol (2:3) and detection with ninhydrin (38) (see Fig. 1). Impurities of netilmicin were separated by chloroform–methanol–25% ammonia solution (10:8:5). The gentamicin components were additionally separated with methanol–chloroform–25% ammonia solution (1:1:1) (lower layer) (see Fig. 2).

A similar mobile phase, i.e., ethanol–chloroform–25% ammonia solution (9:10:19) (lower layer), for separation of gentamicin components was used earlier by Funk et al. (39). Several derivatization methods were applied, for example, dipping into vanillin solution in 2-propanol or an ethanolic KOH solution and heating for 10 min at 110°C or dipping in a fluorescamine solution.

Funk et al. (40) quantitatively determined neomycin A, B, and C by TLC on silica gel 60 F₂₅₄ HPTLC or TLC plates at 50°C with methanol–25% ammonia solution–acetone–chloroform (35:20:20:25). Derivatization was done with fluorescamine. Fluorescence intensity increased by a factor of 5 when the developed chromatogram was dipped in a solution of dichloromethane–triethanolamine–light liquid paraffin (8:1:1).

Relative amounts of the B and C components of neomycin were determined by Roets et al. (41). The stationary phase was silica gel, and the mobile phase was methanol–20% sodium chloride solution (15:85). Fluorescence detection was performed after derivatization with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). A number of commercial samples were analyzed.

Argekar et al. (42) established the HPTLC method for quantitative determination of amikacin in parenteral dosage forms. HPTLC was done on aluminum-backed silica gel 60 F₂₅₄ plates;

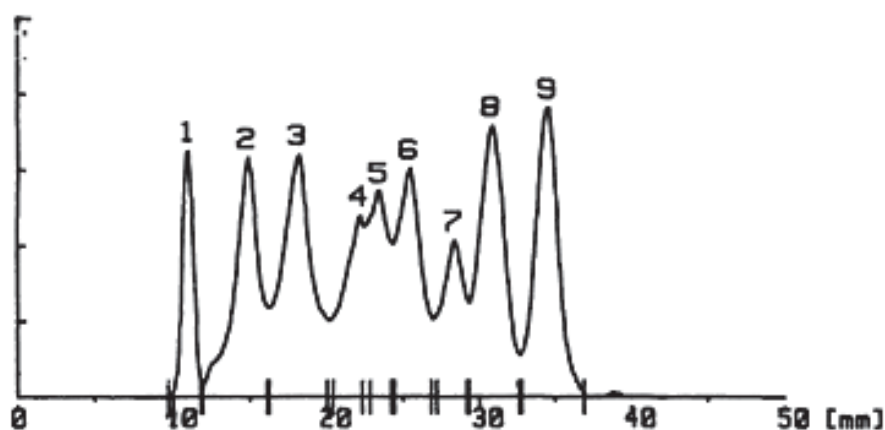


Figure 1 Chromatogram of an aminoglycoside antibiotic mixture: 1,

streptomycin; 2, amikacin; 3, neomycin; 4, paromomycin; 5, kanamycin; 6, tobramycin; 7, gentamicin; 8, sisomicin; 9, netilmicin. (From Ref. 38.)

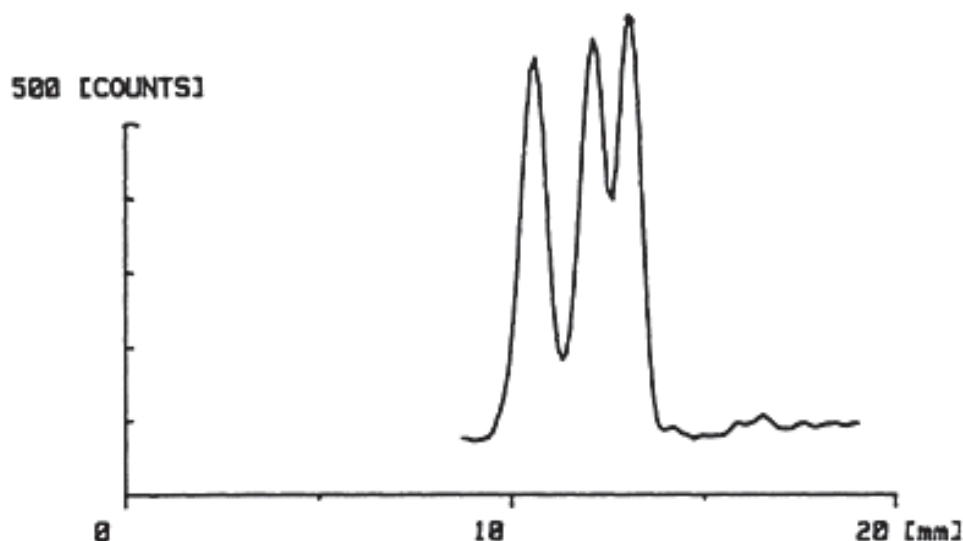


Figure 2 Separation of gentamicin components C_{1a} , C_2/C_{2a} , and C_1 . (From Ref. 38.)

methanol–25% ammonia–chloroform (10:10:2) was used as the mobile phase. The spots were derivatized with ninhydrin. Quantification was linear over the range 500–3000 ng/ μ L.

The separation of nebramycin components (apramycin, carbamoyl kanamycin B, and carbamoyl tobramycin) by silica gel TLC using the mobile phase acetone–ethanol–25% ammonia (1:1:1) was studied (43). Spots were detected by charring followed by scanning at 450 nm. The effect of ammonia content on the R_f values of nebramycin components was investigated. The procedure can be used to control fermentation processes.

A simple and reliable method was developed for the estimation of gentamicin as the bulk drug and gentamicin from plasma and urine (44). The aminoglycoside components gentamicin C_1 , C_2 , and C_{1a} separated on silica gel plates using chloroform–methanol–20% ammonium hydroxide (2.4:2.2:1.5) as the mobile phase were reacted with NBD- C_1 to yield highly fluorescent derivatives. They were quantified by in situ fluorodensitometry. The same components of gentamicin were determined in chicken meat by Vega et al. (45). The homogenized sample was extracted with acetonitrile–4% aqueous KCl (9:1), defatted with hexane, and purified on an RP-18 column. The solvent was 20% aqueous solution of K_2HPO_4 . Derivatization was done with fluorescamine or ninhydrin. The plates were scanned at 366 nm after excitation in the fluorescence mode for fluorescamine derivatives and at 510 nm in the absorbance mode for ninhydrin derivatives.

Netlimicin in serum was isolated by solid-phase extraction (SPE) on C-18 cartridges and then analyzed by HPTLC followed by derivatization with a mixture of diphenylboronic anhydride and salicylaldehyde (46). SPE on copolymeric bonded silica (with C₈ and sulfonic groups) was used for the isolation of hygromycin B from serum and plasma (47). Sample eluates were spotted on silica gel TLC plates with concentrating zones and developed with acetone–ethanol–99.9% ammonium hydroxide (1:1:1). Hygromycin B was visualized by derivatization with fluorecamine. Hygromycin added to bovine plasma was detectable at 25 ppb and added to swine serum at 50 ppb. Neomycin and gentamycin from aqueous solutions could be estimated in a similar way at a level of 50 ppb. Gentamicin, neomycin, spectinomycin, hygromycin B, and streptomycin were separated with this TLC system.

Similar chromatographic conditions were used for determination of hygramycin B isolated from biological fluids (48). Derivatization with fluorecamine was replaced by spraying with 0.2 M citrate buffer and UV detection at 365 nm. Hygramycin was isolated, as previously, on copolymeric resin. Then further purification was done using affinity chromatography. Recently, a thorough review on aminoglycosides was published that included many analytical methods among them thin-layer chromatography (5).

D. Macrolides

Macrolides are bacteriostatic antibiotics composed of a macrocyclic lactone ring containing 14, 15, or 16 atoms with one or more deoxy sugars attached to it via glycosidic bonds. The main representative of the class, erythromycin, was discovered in 1952 as a metabolic product of *Streptomyces erythreus*. Except for rosaramicin and mirosamycin, isolated from *Micromonospora*, macrolides are obtained from *Streptomyces*. The others are semisynthetic derivatives of erythromycin A. Macrolide antibiotics are active against gram-positive and some gram-negative bacteria and some fungi. They are used in the treatment of pneumonia caused by fungi, streptococcus, and syphilis infections, especially when the patient is allergic to penicillin. Erythromycin is highly active against many new, dangerous bacteria such as *Campylobacter* or *Legionella*. Spiramycin and tylosin are used almost exclusively in veterinary medicine. Separation of macrolides is performed on silica gel, kieselguhr, cellulose, and reversed-phase layers. Silica gel and polar mobile phases are frequently used, usually with the addition of methanol, ethanol, ammonia, or sodium or ammonium acetate. Because macrolides lack chromophore groups, bioautography or post-chromatographic derivatization is often used.

The early papers on macrolide antibiotics, mainly erythromycin, were published in the 1960s and considered paper chromatography or TLC on silica gel and kieselguhr with methanolic mobile phases and detection with charring and bioautography. These papers are summarized, together with several newer ones, in a review by Kanfer et al. (7).

Various commercially available macrolides were separated on silica gel using ethyl acetate–ethanol (or 2-propanol)–15% ammonium acetate (9:4:8), pH 9.6. The components of erythromycin as well as various macrolides were separated on silanized silica gel with methanol–water–ammonium acetate (5:2:1), pH 7.0. Eight spraying reagents were described, e.g., sulfuric acid, anisaldehyde, or Dragendorff reagent (49).

Kibwage et al. (50) developed a TLC method for the separation of erythromycins using silica gel and diisopropyl ether–methanol–25% ammonia (75:35:2). The results obtained with several other phases were also discussed. Detection was achieved by spraying with anisaldehyde–sulfuric acid–ethanol (1:1:9) and heating. A similar TLC method was described for semiquantitative analysis of erythromycin A and its metabolites in rat urine and feces (51). Similar phases were also used for the separation of erythromycins, pseudoerythromycins, and erythromycin A enol ether and *N*-oxide, degradation products possibly present in preparations of erythromycin (52). Spots were sprayed with 4-methoxybenzaldehyde–sulfuric acid–ethanol (1:1:9) and heated.

Dichloromethane–methanol–25% ammonia (90:9:1.5) was used for quantitative analysis of bulk erythromycin (53). Detection by postchromatographic color reaction was achieved by spraying with 0.15% xanthidrol in hydrochloric acid–acetic acid (90:7.5) and heating. Spots were quantified by scanning at 525 nm, using troleandomycin as an internal standard.

Separation of erythromycin, tylosin, oleandomycin, and spiramycin in livestock products has been reported (54). The TLC system was silica gel and *n*-butanol–water–acetic acid (6:2:2). The plates were sprayed with xanthidrol or anisaldehyde–sulfuric acid–ethanol (1:1:9) and heated at 110°C. Semiquantitative analysis was carried out by densitometry at 525 nm and bioautography using *Bacillus subtilis*.

Flurithromycin, a novel macrolide antibiotic, was analyzed by TLC and HPLC by Colombo et al. (55). Silica gel plates were developed with methylene chloride–methanol–ammonium hydroxide or methylene chloride–methanol. The spots were visualized by exposure to iodine vapor or by spraying with an anisaldehyde–sulfuric acid–glacial acetic acid–methanol mixture and heating.

The TLC method of monitoring mycinamycins in raw materials was coupled with preparative and semipreparative HPTLC methods (56). TLC was done on silica gel using chloroform–methanol–ammonium hydroxide as a mobile phase and with bioautography as a detection method.

Vega et al. (57) established a method for determining erythromycin and tylosin in chicken meat. The antibiotics were extracted with a mixture of acetonitrile and aqueous solution of potassium chloride (9:1), defatted with hexane, and reextracted with chloroform–ethyl acetate (2:1). The extract was spotted on silica gel HPTLC plates and developed with ethyl acetate–

Table 4 Quantification of Erythromycin^a

Sample no.	Expected (%)	Found (%)	Recovery (%)
1	5.0	4.14	82.20
2	5.0	4.49	89.80
3	5.0	4.24	84.80
4	5.0	4.17	83.40
5	5.0	4.45	89.00
Mean	5.0	4.30	85.80

^aFive samples of chicken meat spiked with 5 ppm of erythromycin and tylosin.

Source: Ref. 57.

acetic acid (7:3) for cleanup. After drying, the plate was developed with ethyl acetate–acetic acid–water (6:2:2). Tylosin spots could be observed under UV light at 254 nm. For observation of erythromycin the plate was dipped in a solution of anisaldehyde and heated. Quantification was done with a scanner, for tylosin at 302 nm and erythromycin at 517 nm. The results are presented in Tables 4 and 5.

E. Tetracyclines

Tetracyclines, consisting of octahydronaphthacene skeletons, are broad-spectrum antibiotics, active against gram-positive and gram-negative bacteria. The first member of this group, chlortetracycline (CTC), was discovered in 1948. Tetracyclines are produced by *Streptomyces* or are obtained semisynthetically. Seven tetracyclines, besides chlortetracycline, are commercially available: tetracycline (TC), oxytetracycline (OTC), doxycycline (DC), minocycline (MINO), methacycline (MTC), demeclocycline (DMCTC), and rolitetracycline, methylpyrrolidine substituted tetracycline (PRMTC) (2). Tetracyclines are amphoteric compounds and form hydrates and salts with both acids and bases that are stable as powders. In solution and by exposure to light, tetracyclines are subject to rapid degradation. Tetracyclines have a propensity to form chelation complexes with metal ions, sample matrix proteins, and silanol groups. These characteristics make separation of tetracyclines and their isolation from matrixes complicated (4). Tetracyclines can be separated in both RP and NP systems. In both cases mobile phases should contain chelating agents such as Na₂EDTA, citric acid, or oxalic acid. Silica gels impregnated with EDTA or Na₂EDTA are usually used as stationary phases in normal-phase TLC. Impregnation is necessary due to strong interactions of tetracyclines with the silica gel surface. Tetracyclines give fluorescent spots that can

Table 5 Quantification of Tylosin^a

Sample no.	Expected (%)	Found (%)	Recovery (%)
1	5.0	3.70	74.00
2	5.0	4.60	90.20
3	5.0	4.30	86.10
4	5.0	4.17	83.40
5	5.0	4.23	84.60
Mean	5.0	4.38	83.70

^aFive samples of chicken meat spiked with 5 ppm of erythromycin and tylosin.

Source: Ref. 57.

be detected by UV lamp fixed at 366 nm or by densitometry. Spraying with reagents such as diazonium salts or magnesium chloride provides lower detection levels. Tetracyclines can also be detected by mass spectrometry and bioautography.

The earliest works, not reviewed here, were carried out mainly on kieselguhr containing EDTA. Later, from the mid-1970s, cellulose was used and then replaced almost exclusively by silica gel. A review on the analysis of tetracyclines in foods written by Oka et al. (2) contains many TLC examples.

A TLC method was described in which tetracyclines were separated on a cellulose layer by development with aqueous solutions of divalent metal salts. The spots exhibited good fluorescence in UV light. This fluorescence could be used for direct photometric determination of the tetracyclines (58). Four tetracyclines, i.e., TC, CTC, ETC, and ATC were separated on cellulose containing EDTA and developed with chloroform saturated with EDTA (59).

Oka and coworkers wrote a series of publications on TLC analysis of tetracyclines titled "Improvement of the Chemical Analysis of Antibiotics." The papers concern both normal-phase and reversed-phase systems as well as the reference compounds and the tetracyclines isolated from different matrices. Some papers give comparisons to RP-HPLC systems.

The first paper (60) reported the separation of doxycycline, oxytetracycline, chlortetracycline, and their degradation products on silica gel HPTLC plates that were predeveloped with a saturated aqueous Na₂EDTA solution, dried, and activated at 130°C for 2 h (60). The mobile phases used were (a) chloroform-methanol-5% aq. Na₂EDTA (65:20:5) (lower layer), (b) 2-propanol-diethyl ether-5% aq. Na₂EDTA (3:4:7) (upper layer), and (c) acetone-5% aq. Na₂EDTA (10:1). Scanning was done at 360 nm for TC, OTC, DC, and 4-epitetracycline (ETC) and at 450 nm for 4-epianhydrotetracycline (EATC) and anhydrotetracycline (ATC). Those seven substances can be determined quantitatively using two or three solvents.

In the next experiment the seven tetracyclines were divided into two groups (61). The first group (OTC, TC, CTC, DC) was separated on a C-8 TLC plate with methanol-acetonitrile-0.5 M aq. oxalic acid, pH 2.0 (1:1:4), and the second (ETC, TC, CTC, EATC, ATC) on a C-18 TLC plate with methanol-acetonitrile-0.5 M aq. oxalic acid, pH 2.0 (1:1:2).

Based on the analytical methods described above, semiquantitative screening methods for the tetracyclines were established (62). The detection reagent Fast Violet B salt was used, after which the silica gel HPTLC plates were heated, whereas the RP-TLC plates were sprayed with pyridine. The colored spots were evaluated visually, giving semiquantitative results comparable with UV densitometric values. Recovery from spiked food samples (fish, egg, milk, meat) was established using NP-TLC and both detection methods. For the measurement of impurities in tetracycline drugs using RP-TLC, the detection methods were also compared, and similar results were obtained.

In the tenth paper of the cycle, an analytical method for eight commercially available tetracyclines was established that used a combination of silica gel HPTLC and C-8 RP-TLC (63). Detection was done using UV densitometry at 360 nm or spraying with Fast Violet B (followed by pyridine in the case of RP-TLC) and heating at 120°C. The results were compared with HPLC on a C-8 column. For the determination of rolitetracycline, it was effective to measure it after its conversion to tetracycline by incubating for 5 min in methanol at 50°C.

Tetracyclines were also isolated from fortified honey (64) and animal tissues and liver (65), using SPE with a C-18 cartridge. OTC, TC, DC, CTC, MTC, MINO, and DMCTC

were analyzed in honey, whereas OTC, TC, DC, and CTC were analyzed in tissues and liver. In both papers, HPTLC plates predeveloped with a saturated aqueous Na_2EDTA solution and developed with chloroform–methanol–5% aq. Na_2EDTA (65:20:5) (lower layer) were used as well as RP-8 plates developed with methanol–acetonitrile–0.5 M aq. oxalic acid, pH 2.0 (1:1:4). Recovery from beef and pork tissues was established for the TLC methods and compared with HPLC results [RP-8 column, methanol–acetonitrile–0.01 M aq. oxalic acid (1:1.5:3)].

To confirm tetracyclines on TLC plates, thin-layer chromatography coupled with fast atom bombardment mass spectrometry (TLC-FAB-MS) was introduced by Oka and coworkers for the residual analysis of TCs in food (66–68). In this method, the developed and dried TLC plate is inserted into the TLC-FAB-MS ion source, and the FAB mass spectrum of the desired compound is measured directly on the plate. In contrast to LC-MS, nonvolatile components of the TLC system, such as oxalic acid and Na_2EDTA , do not cause any problems, because they remain on the TLC plate. To prevent diffusion of the analyte and to obtain high sensitivity from the TLC-FAB-MS, a sample condensation technique was developed (3).

Naidong et al. (69) described a procedure for identification of tetracyclines—CTC, TC, OTC, DC, DMCTC, MTC, and MINO—by TLC on silica gel previously sprayed with a 10% solution of Na_2EDTA with dichloromethane–methanol–water (59:35:6) as the mobile phase (69). The same chromatographic system was then used for assay and purity control of oxytetracycline and doxycycline (70) and tetracycline (71, 72). Results were compared with those obtained previously by HPLC on a poly(styrene-divinylbenzene) phase. Chlortetracycline was assayed using the same system, whereas for demeclocycline the proportions of the mobile phase were changed to 60:35:5 (73). All the major impurities were well separated from the main components and from each other. The results were compared with those obtained on silanized silica gel with a methanol–acetonitrile–0.5 M oxalic acid (pH 2) (1:1:6) mobile phase and with HPLC on a poly(styrene-divinylbenzene) phase; good correlation was obtained. The mobile phase dichloromethane–methanol–water (59:35:6 or 60:35:5) was used for purity control by semiquantitative TLC of six tetracyclines: DC, CTC, OTC, TC, MTC, and DMCTC (74). After development, the plates were dipped in a 30% solution of liquid paraffin in hexane and inspected under UV light at 365 nm. The fluorescence was stable for long periods of time.

Minocycline was separated from impurities using the above-described TLC method. The stationary phase was silica gel impregnated with Na_2EDTA , and the mobile phase was dichloromethane–methanol–water (57:35:8) (75). 6-Deoxy-6-demethyltetracycline was selectively determined by fluorescence densitometry, and other impurities and MINO were quantified by UV densitometry. Results were compared with those obtained by HPLC on the poly(styrene-divinylbenzene) phase. A similar method was described for the assay and purity control of metacycline (76).

Using NP-TLC, TC, DC, OTC, and CTC were separated on silica gel with the aid of various mobile phases. The influence of impregnation with Na_2EDTA and activation of chromatographic plates was discussed (77). The same tetracyclines were isolated from milk and separated on silica gel plates impregnated with 5% aq. Na_2EDTA . Samples of milk spiked with tetracyclines were spotted into the middle of specially prepared trapezoidal regions created by making incisions in the concentrating zones of the plate.

Development with the mobile phase chloroform–methanol– 5% aq. Na₂EDTA (65:20:5) (lower phase) was preceded by predevelopment in the same direction with hexane and acetone (78).

Direct analysis of tetracycline and its impurities on the TLC plate was done by TLC/MALDI-MS (79). Using the electrospray method, the limit of detection for tetracycline from a TLC plate was found to be 1 ng.

Xie et al. (80) presented a TLC-fluorescence densitometry method for the determination of OTC, TC, and DC in honey, serum, and urine. Silica gel TLC plates impregnated with Na₂EDTA were used, and the solvent system was chloroform–methanol–acetone–1% aq. ammonia (10:22:50:18). Kang and Ebel (81) described the separation of eight tetracyclines on cyanophase HPTLC plates developed with methanol–acetonitrile–0.5 M oxalic acid (3:1:6).

F. Quinolones

Quinolone and fluoroquinolone antibiotics are a group of relatively new broad-spectrum synthetic antibiotics. Nalidixic acid, discovered in 1962 by Lescher and coworkers, was the first member of this class, though of rather minor importance. In the 1980s, synthetic fluoroquinolones were developed and became valid antibiotics with high potency and of good tolerance. Quinolone antibiotics consist of a 1-substituted 1,4-dihydro-4-oxopyridine-3-carboxylic moiety combined with an aromatic or heteroaromatic ring. Quinolones are sometimes divided into three generations. First-generation quinolones, e.g., nalidixic and oxolinic acids, have poor anti-gram-positive activity and are predominantly used to treat urinary tract infections. The second-generation quinolones, e.g., norfloxacin and ciprofloxacin, are active against both gram-positive and gram-negative bacteria, whereas those of the third generation, e.g., temafloxacin, have potency against *Staphylococcus aureus* and also against the anaerobic bacteria *Chlamydia* and *Mycoplasma*. Quinolones are polar, amphoteric compounds that are strongly adsorbed on silica gel. Therefore, silica gel plates for quinolone analysis are often impregnated with Na₂EDTA or K₂HPO₄. Multicomponent organic mobile phases are used, usually with the addition of aqueous solutions of ammonia or an acid. Densitometry or fluorescence densitometry is possible, sometimes preceded by postchromatographic derivatization.

Oxolinic acid was quantified in fish feed in the presence of erythromycin and oxytetracycline (82). TLC of oxolinic acid was done on HPTLC silica gel plates impregnated with 0.1 M K₂HPO₄ ethanolic solution. The mobile phase was chloroform–acetone (9:10). Spots were detected by scanning at 265 nm.

Oxolinic acid and flumequine were separated on HPTLC plates prepared as described above using toluene–ethyl acetate–90% formic acid (60:30:10) as solvent (83). The procedure was used for analysis of fish feed and for residual analysis of fish meat. Nalidixic acid was used as an internal standard. The sensitivity of detection of oxolinic acid could be increased ten-fold by derivatization with sulfuric acid–hydrochloric acid reagent before scanning.

High-performance TLC analysis was used for qualitative determination of seven quinolones—enrofloxacin, ciprofloxacin, danofloxacin, norfloxacin, flumequine, oxolinic acid, and nalidixic acid—in pork muscle (84). Extraction from the spiked tissues was followed by SPE on a C-8 cartridge. Then samples were spotted on HPTLC silica gel

plates with a concentrating zone and developed with methanol–ammonia (85:15). The plate was dried and monitored under 312 nm light, sprayed with terbium chloride solution, heated for 5 min at 100°C, and monitored again under 312 nm light. The method was validated to a level of 15 ppb for enrofloxacin, ciprofloxacin, danofloxacin, and norfloxacin and 5 ppb for flumequine, oxolinic acid, and nalidixic acid.

A TLC procedure was established for qualitative and quantitative monitoring of the degradation of ciprofloxacin in aqueous solutions irradiated by a high-pressure mercury lamp (85). Qualitative TLC analysis was done using aluminum-backed silica gel 60 sheets. Mixtures of acetonitrile and various aqueous phases containing ammonia served as eluents. Quantitative experiments were done on silica gel HPTLC plates prewashed with methanol and developed with eluent comprising mixtures of acetonitrile and ammonium chloride buffer. The degradation products were easily separated from each other and from the parent compound.

An HPTLC method with direct fluorescence measurement for determination of norfloxacin on stainless steel pharmaceutical equipment surfaces was described (86). The marked surface was wiped with bands of cotton wet with 0.005 M NaOH in water–methanol (1:1). Norfloxacin was then extracted from the cotton with the same solution and spotted on an HPTLC silica gel plate prewashed with methanol. The plate was developed with methanol–chloroform–cone, ammonia (51:34:15), dried, then immersed for 4 h in liquid paraffin-*n*-hexane (1:2). The plate was scanned in the fluorescence/reflectance mode at 313 nm. The method was validated for the monitoring of norfloxacin at the allowed limit of 10 mg/m².

Fleroxacin, sparfloxacin, and cinoxacin in tablets were separated and determined by HPTLC on silica gel plates developed with dichloromethane–2-propanol–25% ammonia (4:5:2) (87). The compounds were completely separated with *R_f* values of 0.55, 0.46, and 0.40 for fleroxacin, sparfloxacin, and cinoxacin, respectively (Fig. 3). Tablets were extracted with chloroform, methanol, and methanol–acetone (1:1) for fleroxacin, sparfloxacin, and cinoxacin, respectively. Quantification was done by videodensitometry at 254 nm (Fig. 4). The calibration curves obtained by plotting peak area against drug concentration were linear in the range 0.08–0.48 µg/µL (corresponding to 0.4–2.4 µg per band). Detection by UV irradiation was compared with other methods (see Table 6).

Six chinolones used in veterinary therapy, i.e., ciprofloxacin, enrofloxacin, difloxacin, sarafloxacin, norfloxacin, and flumequine, were separated on diol-HPTLC plates in an ion-association system with di(2-ethylhexyl)orthophosphoric acid (HDEHP, an ion-pairing agent) (88). Chromatograms were developed with solutions of HDEHP (1%, 2.5%, 5%) in acetone, with solutions of HDEHP (5%, 10%, 15%) in ethyl acetate, and with ethyl acetate–HDEHP–methanol (9:1: 1.25). The spots were detected under UV light at 254 nm.

Wang et al. (89) described a TLC-fluorescence densitometry method for the determination of norfloxacin, pefloxacin, and ciprofloxacin on silica gel plates impregnated with Na₂EDTA. The

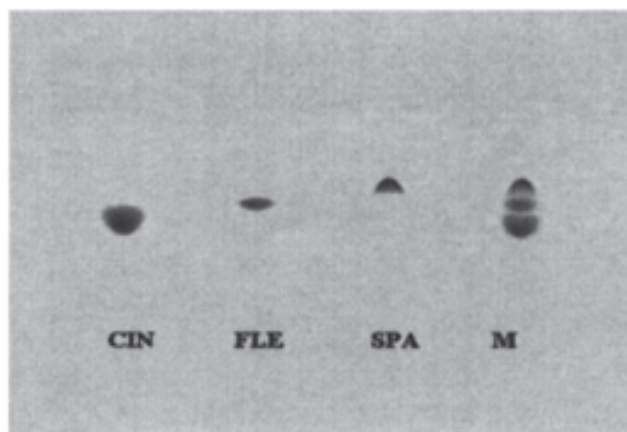


Figure 3 Separation of sparfloxacin (SPA), fleroxacin (FLE), cinnoxacin (CIN), and their mixture on silica gel plate with dichloromethane–2-propanol–25% ammonia (4:5:2). (From Ref. 87.)

mobile phase was chloroform–methanol–toluene–dichloromethane–aq. ammonia (2.7:4.6:1.7:0.5:0.5). The recovery of the three antibiotics in body fluids ranged from 96% to 108%.

Ofloxacin and enoxacin were determined on silica gel plates impregnated with various concentrations of Na₂EDTA solution at various pH values (90). The mobile phase was chloroform–methanol–ethyl acetate–tetrahydrofuran–aq. ammonia (6:4.6:1.5:0.8:1.5). The mean recovery of the two antibiotics in body fluid ranged from 95.4% to 105.2%.

G. Peptides

Peptide antibiotics consist of peptide chains covalently linked to other chemical entities. Most peptides are toxic and are poorly absorbed from the alimentary tract. Peptides are difficult to analyze in biological matrices because they are very similar to the matrix components. They can be separated on silica gel, amino silica gel, and silanized silica gel plates. A variety of mobile phases are used, from simple ones such as chloroform–methanol up to multicomponent ones such as *n*-butanol–butyl acetate–methanol–acetic acid–water. The detection methods used are densitometry and fluorescence densitometry and/or spraying of the plate with reagents such as ninhydrin or fluorescamine. Bioautographic detection can also be used with *Bacillus subtilis* and *Mycobacterium smegmatis*.

The antitumor antibiotic bleomycin is a mixture of closely related glycopeptides that differ only in their terminal amines. The main components of bleomycin are bleomycin A₁, A₂, A₅, and B₂; demethylbleomycin A₂; and bleomycin acid. For separation of bleomycins, three TLC systems

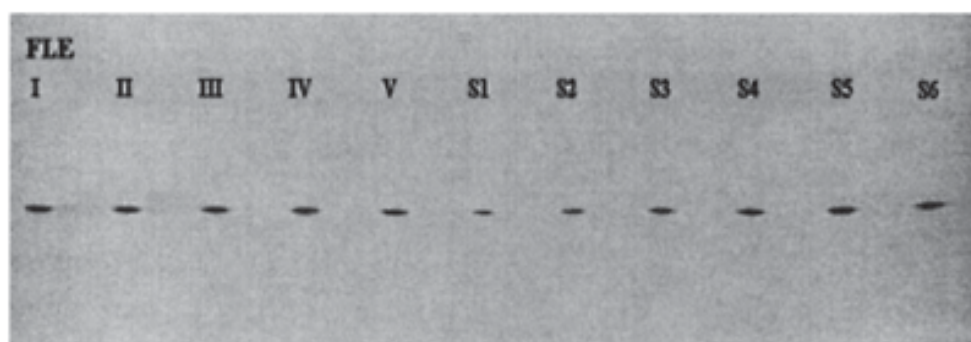


Figure 4 Results of HPTLC of fleroxacin on silica gel plate with dichloromethane–2-propanol–25% ammonia (4:5:2). S1–S6, standard solutions (0.4–2.4 μg); I–IV, sample solutions (1.4 μg). (From Ref. 87.)

Table 6 Detection Modes of the Quinolone Drugs on HPTLC Plates

No.	Detection reagent	Limit of detection ^a (μg)		
		SPA	FLE	CIN
1	UV irradiation ($\lambda=254\text{ nm}$) on Si 60 F ₂₅₄	0.10	0.10	0.01
2	Acidified phosphomolybdenate reagent	0.10	0.10	—
3	Folin–Ciocalteu reagent	0.50	0.50	—
4	Alkaline solution of KMnO ₄	0.50	0.50	—
5	Iodine reagent	0.50	0.20	0.10
6	Dragendorff reagent (after Amelink)	0.05	—	—
7	Forrest reagent	0.05	0.05	0.05
8	15% FeCl ₃ in 5% HCl solution	0.10	0.10	0.05

^aSPA, sparfloxacin; FLE, fleroxacin; CIN, cinoxacin.

Source: Ref. 87.

were used (91): (a) silica gel developed with 0.19 M (NH₄)₂HPO₄–methanol (1:1); (b) silanized silica gel developed with 0.357 M NH₄NO₃–methanol (7:3); and (c) silanized silica gel developed with 0.1 M hexane sulfonic acid–methanol (1:1). After development in a saturated tank, plates were dried at 100°C for 30 min, sprayed with ninhydrin, and heated at 100°C for 2 min. The bleomycin components appeared as purple spots on a white background. The first TLC system was combined with bioautographic detection using *Bacillus subtilis* or *Mycobacterium smegmatis*.

Vancomycin, discovered in 1956, is a major antibiotic for the treatment of life-threatening gram-positive bacterial infections. Four TLC systems were developed for the separation of vancomycin, related antibiotics, and degradation products (92):

1. Reversed-phase plates developed with 5% NH_4HCO_3 –dioxane (3:7)
2. Silica gel developed with 1% NH_4HCO_3 –methanol (9:1)
3. Silica gel developed with 1% $(\text{NH}_4)_2\text{CO}_3$ –2-propanol (9:11)
4. Carboxymethylcellulose developed with 0.9% $(\text{NH}_4)_2\text{HPO}_4$, pH 8.3

The plates were dried and sprayed with a freshly prepared aqueous solution of *p*-nitrobenzene-diazonium tetrafluoroborate. Alternatively, bioautography was performed with *Bacillus subtilis*. To enhance the contrast between the zones of inhibition and the background, the bioautogram was sprayed with *p*-iodonitrotetrazolium.

Gramicidin in fermentation samples and bulk products was quantified on HPTLC silica gel plates developed with the solvent acetic acid–butyl acetate–1-butanol–methanol–water (20:40:7.5:2.5:12) (93). The plates were dried for 15 min at 115°C, sprayed with 4-dimethyl-aminobenzaldehyde, heated for 3 min at 90°C, and scanned at 570 nm.

A variety of RP-TLC systems were investigated to find optimal conditions for analysis of 19 peptide-type antibiotics: cycloserine, hadacidin, azaserine, viomycin, echinomycin, polymyxin B₁, colistin S, actinomycin C₂, bacitracin A, phleomycin, bleomycin S, thiostrepton, saramycetin, gramicidin A, cinnamycin, duramycin, neocarzinostatin, restrictocin, and largomycin F-II (94). For that purpose, 27 mobile phases were used, representing three organic modifiers, three buffers, and three pH values. With only slight modifications of mobile phases, those systems were used for RP-HPLC.

Virginiamycin fermentation broth contains seven antibiotics and a number of pigments as contaminants. The HPTLC method using silica gel plates prewashed with methanol–chloroform (1:1) and developed with chloroform–methanol (92:8) was established for their separation (95). The developed plates were dried and quantified at 235 nm. Because of the lack of commercially available standards, they were isolated from fermentation broth and purified. Their purity (98%) was confirmed by UV spectrophotometry and HPLC. The chromatograms obtained for standards and components of fermentation broth are presented in Fig. 5. Separation and quantification of

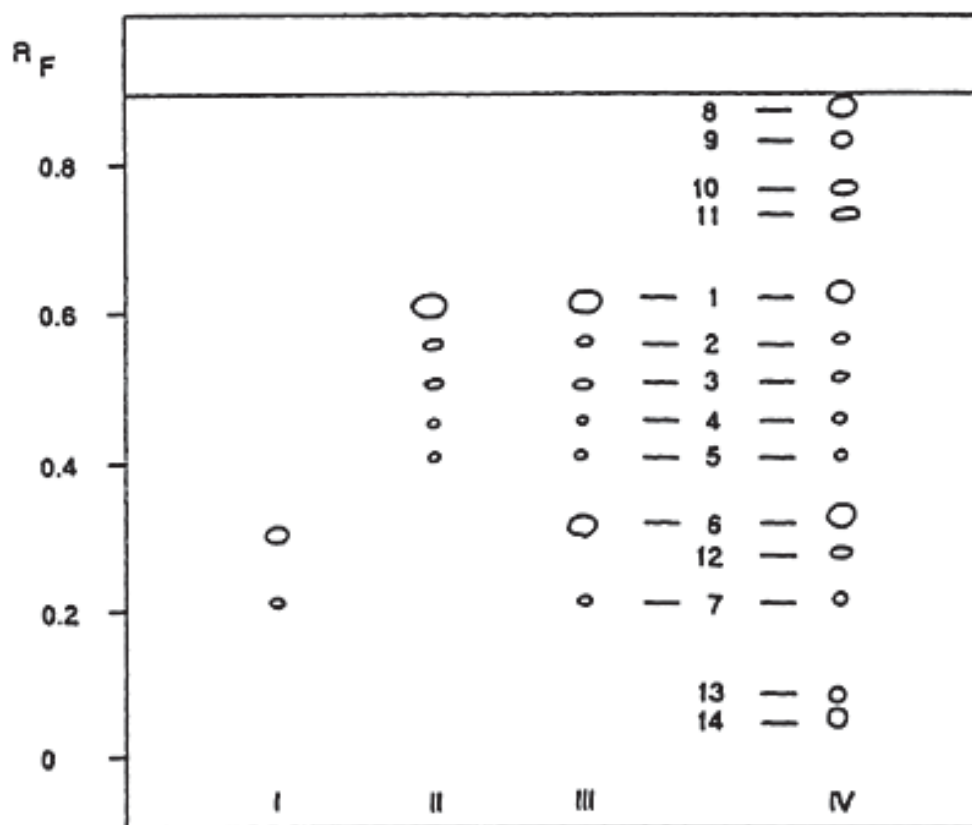


Figure 5 Chromatographic separation of virginiamycins. I, V[M]; II, V[S]; III, mixture of V[M]+ V[S]; IV, sample of fermentation broth. 1, V[S1]; 2, V[S2]; 3, V[S3]; 4, V[S4]; 5, V[S5]; 6, V[M1]; 7, V[M2], and 8–14, unknown components of fermentation broth. (From Ref. 95.)

the virginiamycins were also done by HPLC. Table 7 lists the TLC and HPLC results obtained for major components of the fermentation broth, virginiamycins VM1 and VS1, in several samples. Comparison of HPTLC and HPLC data showed good correlation ($r=0.993$).

H. Sulfonamides

Sulfonamide drugs, often called sulfa drugs, are synthetic compounds and were the first chemotherapeutic agents discovered. The first sulfonamide drug, discovered in 1932 by the German doctor Domagk, was a red azo dye, 2,4-diaminobenzene-4'-sulfonamide, called prontosil rubrum. The sulfonamides include sulfanilamide (4-aminobenzenesulfonamide) and numerous compounds closely related to it (derived from substitution in the sulfamide group). Other sulfonamide drugs are probenecid, used in

treating gout; acetazolamide and furosemide, which are diuretics; the hypoglycemic tolbutamide; and chlorothiazide and hydrochlorothiazide, which are both diuretic and antihypertension agents. Bacteriostatic sulfonamide drugs are used mainly in the treatment of infections in livestock and, at subtherapeutic doses, to promote the growth of food animals. To a lesser extent they are also used in the treatment of human infections such as bronchitis and urinary tract infections, diabetes, edema, hypertension, and gout. The sulfonamides are toxic, and some patients are hypersensitive to them. The common side effects are nausea, vomiting, mental disturbance, anemia, leukopenia, kidney dysfunction, fever, and skin allergy. Some sulfonamides could be carcinogenic.

Table 7 Results Obtained by Use of Densitometry and HPLC for Quantitative Analysis of Virginiamycins M1 and S1 in Fermentation Broths

No.	Antibiotic	Densitometry			HPLC		
		X ^a	SD ^b	CV ^c	X ^a	SD ^b	CV ^c
1	V(M1)	3.40	0.085	2.50	3.50	0.126	3.60
	V(S1)	1.30	0.050	3.90	1.30	0.040	3.20
2	V(M1)	2.80	0.050	1.90	2.90	0.087	3.00
	V(S1)	1.00	0.036	3.60	0.95	0.038	4.00
3	V(M1)	4.40	0.097	2.20	4.20	0.151	3.60
	V(S1)	1.30	0.034	2.60	1.30	0.036	2.80
4	V(M1)	4.00	0.144	3.60	3.90	0.121	3.10
	V(S1)	1.40	0.039	2.80	1.50	0.058	3.90
5	V(M1)	3.70	0.074	2.00	3.50	0.115	3.30
	V(S1)	1.20	0.040	3.30	1.20	0.036	3.00
6	V(M1)	3.90	0.156	4.00	4.00	0.116	2.90
	V(S1)	1.10	0.044	4.00	1.00	0.038	3.80
7	V(M1)	2.60	0.091	3.50	2.40	0.096	4.00
	V(S1)	1.00	0.031	3.10	1.05	0.038	3.70
8	V(M1)	2.50	0.052	2.10	2.60	0.100	3.90
	V(S1)	0.80	0.024	3.00	0.75	0.030	3.60
9	V(M1)	4.30	0.120	2.80	4.20	0.071	1.70
	V(S1)	1.40	0.036	2.60	1.45	0.043	3.00
10	V(M1)	3.80	0.144	3.80	3.90	0.156	4.00
	V(S1)	1.20	0.025	2.10	1.20	0.046	3.80

^aMean concentration (mg/mL) ($n=3$; $P=0.95$).

^bStandard deviation.

^cCoefficient of variation.

Source: Ref. 95.

Sulfonamides can be analyzed both by normal-phase TLC (on silica gel, alumina, polyamide, and Florisil layers) and by reversed-phase TLC (on silanized silica, RP-2, RP-8, and RP-18 layers). Both aqueous and nonaqueous eluents are used. Sulfonamides can be detected on fluorescent layers at 254 nm and after derivatization with fluorescamine solution at 366 nm.

Lepri et al. (96) reported R_f values for 20 Sulfonamides and sulfanilic acid obtained with both aqueous and nonaqueous eluents on RP-12 and RP-18 layers. Additionally, two-dimensional chromatography was used for the separation of 19 Sulfonamides on RP-18 layers eluted in the first direction with benzene–ethyl acetate–acetic acid (80:18:2) and in the second direction with 1 M acetic acid in 20% methanol. The separation was satisfactory, because 16 compounds were distinguished.

Relationships between R_M values of 15 Sulfonamides and solvent composition were determined on TLC plates coated with either normal-phase layers (silica gel, alumina, polyamide, and Florisil) or reversed-phase layers (silanized silica, RP-2, RP-8, and RP-18) (97). Spots were detected under UV light at 254 nm. The behavior of the Sulfonamides was investigated using aqueous and nonaqueous binary solvent systems as mobile phases. Modifiers and diluents were chosen so that TLC experiments could be extended to HPLC. The influence of the different types of sorbents and organic modifiers on retention and selectivity was compared by graphical correlation (see Figs. 6 and 7). The results show the usefulness of all the tested sorbents for the chromatographic analysis of the Sulfonamides.

Thin-layer chromatography and HPLC based on aminopropyl-modified silica gel using aqueous and nonaqueous mobile phases containing organic modifiers and cationic or anionic ion-pairing reagents were used for the determination of R_f and k' values of some sulfonamides (98). The influence of the different organic modifiers and ion-pairing reagents on retention was compared. The TLC data using sandwich quasi-column development can be extended to HPLC.

The simultaneous HPTLC quantification of two sulfonamides in pharmaceutical preparations consisting of sulfameter (I) and sulfamethoxazole (II) or sulfameter (I) and sulfoxazole (III) was described (99). Standards and acetone–chloroform extracts from the tablets were spotted on silica gel HPTLC plates developed with chloroform–ethyl acetate (4:6). Densitograms obtained from the sulfonamide mixtures are presented in Fig. 8. The measurements were done at 270 nm in absorbance/reflectance mode. The method was tested on mixtures prepared from known amounts

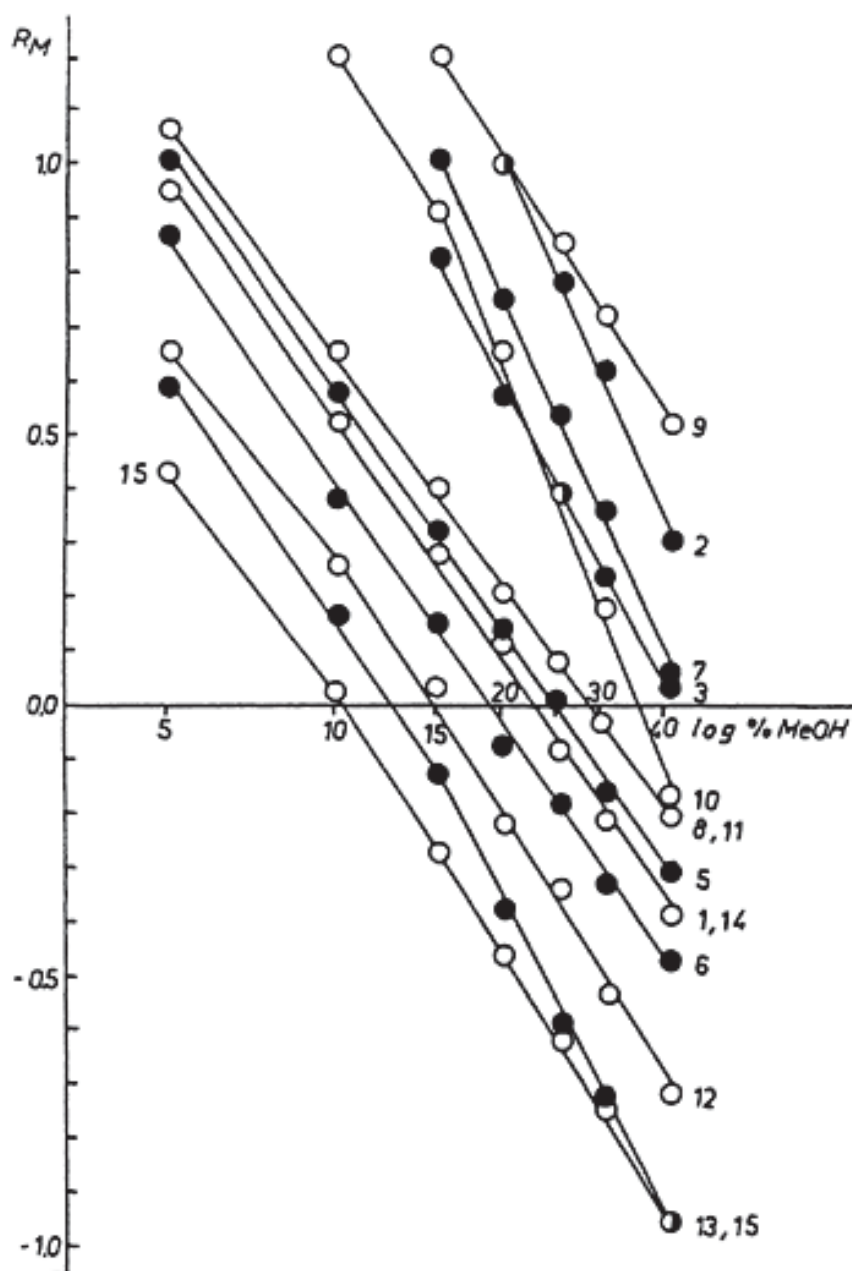


Figure 6 Dependence of sulfonamide drug R_M values on $\log C$ (% v/v) for TLC on Florisil eluted with chloroform–methanol. The sulfonamides examined were (1) sulfanilamide, (2) sulfacarbamide, (3) sulfaguanidine, (4) sulfacetamide, (5) sulfamethoxazole, (6) sulfadiazine, (7) sulfafurazole, (8) sulfathiazole, (9) sulfamethizole, (10) sulfaproxyline,

(11) sulfadiazine, (12) sulfamerazine, (13) sulfadimidine, (14) sulfisomidine, and (15) sulfadimethoxine. (From Ref. 97.)

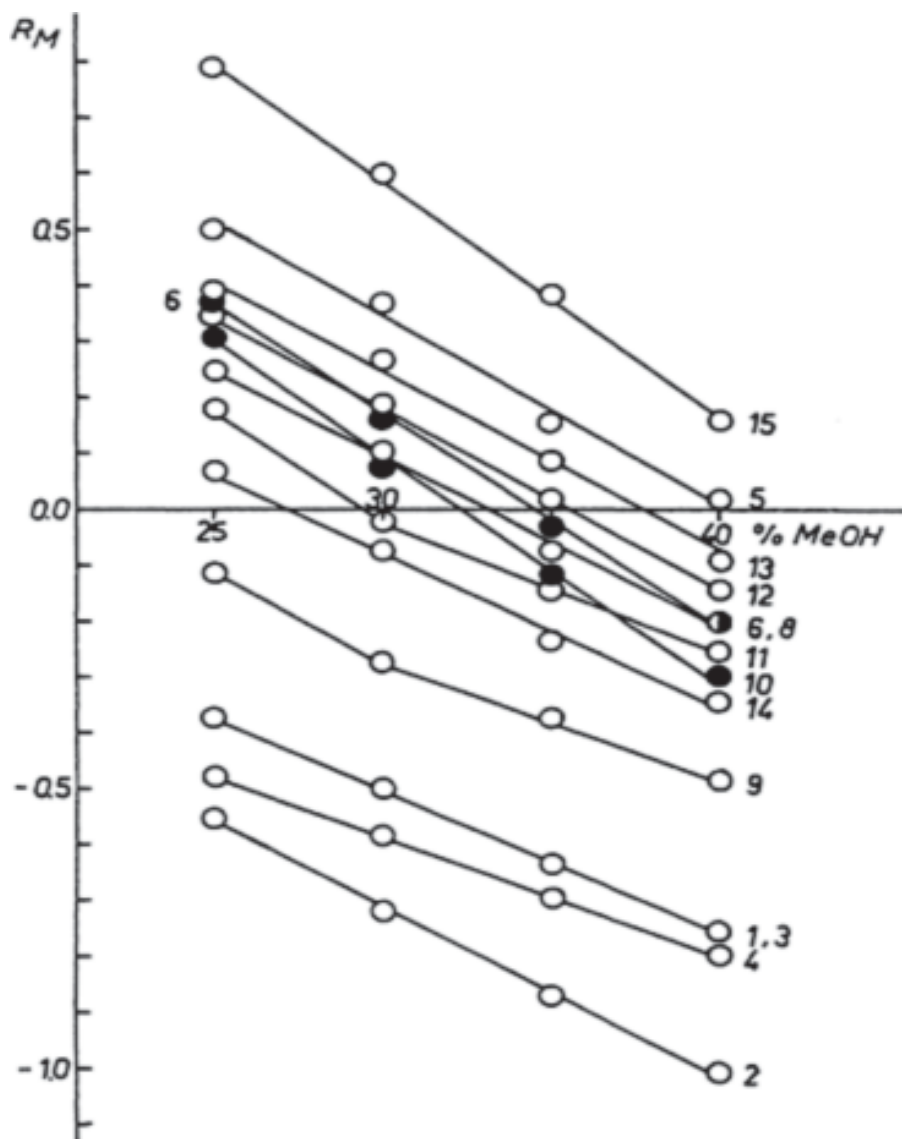


Figure 7 Dependence of sulfonamide drug R_M values on $\log C$ (% v/v) for TLC on silanized silica gel eluted with chloroform-methanol. Compounds as listed in Fig. 6. (From Ref. 97.)

of the sulfonamides. The amounts found were close to 100%. Table 8 contains data from HPTLC and spectrophotometry.

Abjean (100) described the method of determining sulfanilamide, sulfadoxine, sulfadiazine, sulfadimidine, sulfamethoxypyridazine, sulfadimethoxine, and sulfaquinoxaline in animal tissue. The extracts from tissues and standards were spotted on the concentrating zone of a silica gel plate and developed with chloroform-*n*-butanol (4:1). The plates were then treated with fluorescamine solution, and the sulfonamide spots were revealed by UV irradiation at 365 nm.

Evaluation tests were carried out on eight sulfonamides, using bacteriological, TLC, and HPLC methods (101). TLC silica gel 60 F₂₅₄ plates were used, with a solution of ethyl acetate—

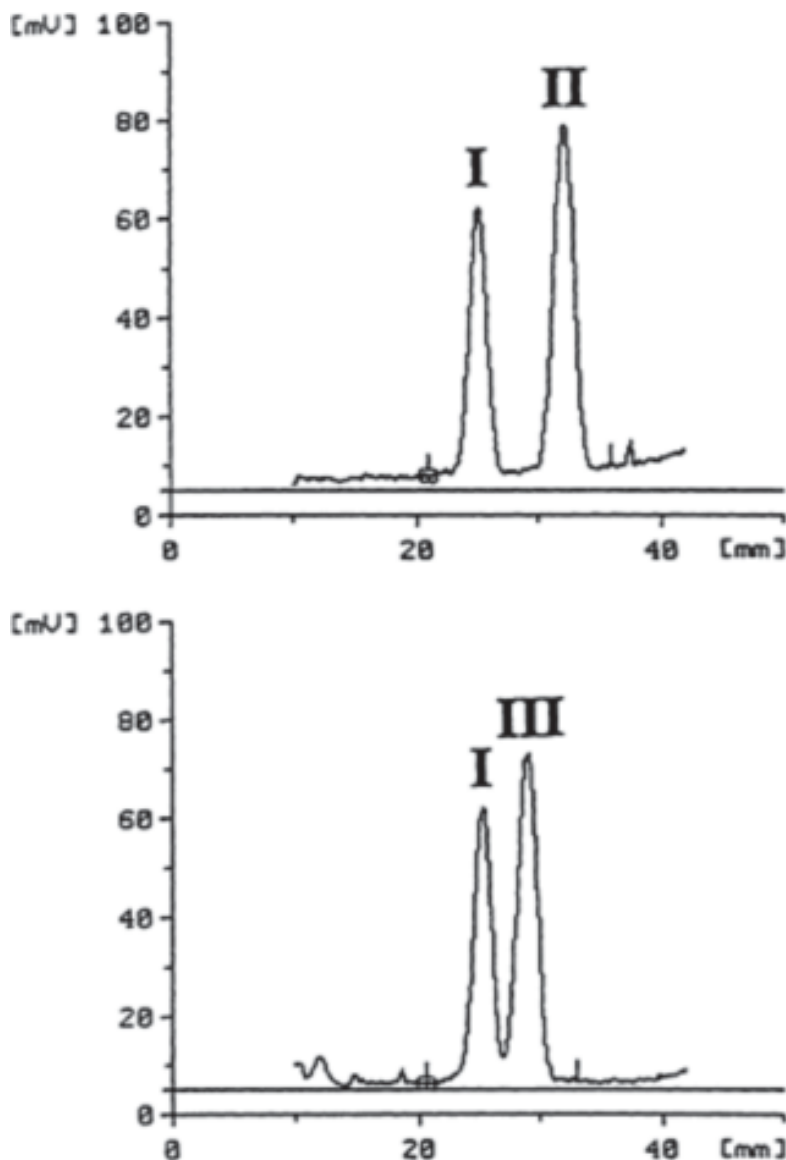


Figure 8 Densitograms obtained from analysis of tablet samples containing two sulfonamide drugs. I, sulfameter;

II, sulfamethoxazole; III, sulfisoxazole.
(From Ref. 99.)

benzene–ethyl ether (30:4:16) as the mobile phase. The spots were examined under UV light after spraying with fluorescamine or Ehrlich reagent.

An HPTLC method was developed for the analysis of sulfadiazine, sulfamerazine, sulfamethazine, sulfadimethoxine, and sulfapyridine in salmon muscle tissue (102). Matrix solid-phase dispersion on C-18 silica gel was used to clean up and isolate the sulfonamides. The HPTLC plate was developed using ethyl acetate–*n*-butanol–methanol–aqueous ammonia (35:45:15:2). The sulfonamide spots were sprayed with fluorescamine.

A quantitative HPTLC method for the detection of sulfonamide residues in animal tissues and milk was established (103). The residues were isolated by liquid extraction followed by SPE for the meat samples and by MSPD for the milk samples. The sulfonamides analyzed were sulfamethazine, sulfadiazine, sulfathiazole, sulfaquinoxaline, sulfadoxine, and sulfadimethoxine. The HPTLC silica plates were developed with a multiple system that consisted of triple development with solvent mixtures of methanol containing 2% ammonia (25%) and dichloromethane

Table 8 Results from Determinations of
Sulfonamides in Pharmaceutical Preparations

Preparation	Amount found (% of labeled amount) ^a	
	HPTLC	Spectrophotometry
Sulfameter (I), 100 mg/tablet	101.7±2.62	102.2±0.7
Sulfamethoxazole (II), 250 mg/tablet	98.6±2.03	100.3±1.1
Sulfameter (I), 100 mg/tablet	101.9±2.07	
Sulfisoxazole (III), 400 mg/tablet	102.1±2.58	

^aAverage of six determinations±relative standard deviation.

Source: Ref. 99.

(30:70, 15:85, and 5:95), respectively, and successive distances of 15, 30, and 45 mm. The plates were scanned at 366 nm after being sprayed with fluorescamine solution.

Some sulfonamides were separated by TLC on silica impregnated with cobalt, copper, nickel, zinc, cadmium, and mercury salts (104). The separation of 10 sulfonamides on poly amide impregnated with metal salts as well as on bare polyamide was presented (105). The plates were developed with solvent mixtures containing various concentrations of ethanol in ethyl acetate or acetone. Scanning was performed by transmission densitometry at 254 nm.

The structures of polyamide layers impregnated with cobalt, copper, and zinc salts were investigated (106). The chromatographic properties of these sorbents were tested on selected sulfonamides.

I. Other Antibiotics

1. Polyethers

Polyether or ionophore antibiotics are chemically characterized by several cyclic ethers, a single terminal carboxylic acid group, and several hydroxyl groups. They are mainly produced by *Streptomyces* species. They are widely used anticoccidiosis agents for poultry. The main members of this class are salinomycin, monensin, lasalocid, and narasin. Lasalocid consists of two cyclic ethers, whereas the rest have six ether rings. Salinomycin and narasin have almost identical structures.

Asukabe et al. (107) established a method for the simultaneous determination of three polyether antibiotics, i.e., salinomycin, monensin, and lasalocid, and two derivatives using silica gel and RP-18 HPTLC. Fluorescent pyrenacyl esters of those antibiotics and of internal standards (18, 19-dihydrosalmomycin and 18,19-dihydro-20-ketosalmomycin) were prepared. Silica gel plates were developed with carbon tetrachloride–ethyl acetate–acetonitrile (50:5:10), and RP-18 plates with dichloromethane–ethyl acetate–acetone–acetonitrile (15:2:1:55). The spots were detected fluorodensitometrically at 360 nm. The same three poly ethers were isolated from poultry tissues and separated by VanderKop and MacNeil (108) by means of TLC bioautography with *Bacillus subtilis*. The tissue extract was spotted on a preheated TLC plate with a concentrating zone and allowed to dry for 0.5 h. The plate was developed with ethyl acetate–acetonitrile (1:1).

Thin-layer chromatography coupled with flame ionization detection was used to separate and determine simultaneously three polyether antibiotics (abierixin, nigericin, and grisorixin) produced by *Streptomyces hygroscopicus* (109). Development on silica gel chromarods with chloroform–methanol–formic acid (97:4:0.6) gave reliable separations of the three antibiotics. The internal standard methyl desoxycholate was suitable for their simultaneous determination.

2. Amphenicols

Chloramphenicol is a broad-spectrum antibiotic produced by *Streptomyces venezuelae*. It was first used in the late 1940s to treat an epidemic of typhus in Bolivia. Thiamphenicol was discovered in the late 1950s, whereas florphenicol is more recent. Chloramphenicol is now banned in the United States and in the European Union because it is believed to cause blood toxicity. Chloramphenicol, florphenicol, and thiamphenicol show strong UV absorption and can be determined by TLC.

A method of determining chloramphenicol in serum has been published (110). An ethyl acetate extract of serum and the chloramphenicol standard were spotted on an HPTLC silica gel plate and developed with heptane–chloroform–methanol (6:12:3). The spots were scanned at 280 nm.

Eyedrops containing chloramphenicol were spotted, after dilution with chloroform, directly on the silica gel sheet together with chloramphenicol standards dissolved in chloroform. The plate was developed with diethyl ether (111).

According to the European Pharmacopoeia, chloramphenicol dissolved in acetone is determined on silica gel plates prewashed with methanol–methylene chloride (8:2) and

developed with water–methanol–chloroform (1:10:90) phase (112). Plates are dried in air and examined at 254 nm. Sterilization of drugs prior to their application can cause formation of by-products other than those arising from conventional degradation mechanisms. Chloramphenicol samples sterilized by γ -irradiation were screened for impurities by thin-layer chromatography according to the European Pharmacopoeia (113). A significant impurity spot was detected that was found not to be a γ -irradiation by-product of chloramphenicol. What the impurity turned out to be was a cyclic ketale, a product of the condensation of chloramphenicol and acetone catalyzed by traces of acid formed during the irradiation process. Therefore, to avoid artifacts, instead of acetone, methanol should be used as the solvent for TLC investigation of the purity of chloramphenicol.

3. Rifamycins

Rifamycins (ansamycins) are structurally similar macrocyclic antibiotics produced by *Streptomyces mediterranei*. All of them possess the characteristic “ansa” structure consisting of aromatic rings spanned by an aliphatic bridge. They are active against gram-positive streptococci and mycobacteria. They are mainly used in treating tuberculosis.

Grassini-Strazza et al. (114) separated four rifamycins (rifamycins S and SV, rifampicin, and 3-formyl-rifamycin) using RP (diphenyl and C-18) plates. A variety of mobile-phase systems were applied, from neat organic solvents (hexane, cyclohexane, chloroform, tetrahydrofuran, acetone, and C₁–C₄ alcohols) to binary nonaqueous solvents (different proportions of hexane–chloroform and hexane–ethanol) and binary aqueous–organic solvents (e.g., methanol–water or acetonitrile–water). Rifamycins are colored compounds and do not require special detection.

4. Nitrofurans

Nitrofuran drugs are synthetic broad-spectrum chemotherapeutic agents that are derivatives of nitrofuran. Their application in the treatment of humans is limited. Nitrofurazone is used externally, furazolidone is used to treat infections of the intestine, and nitrofurantoin for urinary tract infections. Nitrofurans are used as growth promoters and to prevent and treat diseases in poultry and swine.

Abjean (115) described the TLC screening of nitrofurazone, furaltadone, furazolidone, and nitrofurantoin in meat. Extracts were cleaned up on a Sep-Pak silica column, and the standards were spotted on silica gel plates with a concentrating zone. The plate was developed with dioxane–chloroform (1:1), and after drying it was sprayed with pyridine and immediately illuminated with UV light at 366 nm. After a few seconds, nitrofurazone appeared as a yellow spot and three other nitrofurans as yellow-green spots. The positive detection of nitrofuran drug was possible when it was spiked at the 5 ppb level.

J. Miscellaneous Classes of Antibiotics

Scanning densitometry was described for direct quantification of many classes of antibiotics on a hydrocarbon-impregnated silica gel HPTLC plate without solvent elution (116). Standards and samples were dissolved in water and spotted on the plate. Each

sample remained as a single spot centered at the point of application, thereby facilitating direct quantification by densitometry at different wavelengths. The detection level was 0.1 ng. The quantity of sample applied and the areas and peak heights of the spots formed on the plate were directly related. The antibiotics applied were gentamycin, erythromycin, tobramycin, amikacin, ciprofloxacin, norfloxacin, ofloxacin, cefotetan, aztreonam, clavalinic acid, imipenem, vancomycin, rifampin, trimethoprim, chloramphenicol, clindamycin, nitrocefin, tetracycline, and sulfamethazole.

Multiclass qualitative detection of chloramphenicol, nitrofurans, and sulfonamide residues in animal muscle was described (117). The drugs were extracted from animal tissue with ethyl acetate and purified by silica SPE. The extract was spotted and chromatographed on the HPTLC silica gel plate. Nitrofurans were visualized first by their specific UV photochemical reaction with pyridine. Chloramphenicol was then reduced to its amino derivative, and the derivative and sulfonamides were sprayed with fluorescamine and examined under UV light. Detected residue levels were 10, 5, and 100 ppb for chloramphenicol, nitrofurans, and sulfonamides, respectively.

A simple classification method for 24 antibiotics using a TLC bioautographic procedure was described (118). The antibiotics were divided into four groups (β -lactams, aminoglycosides, macrolides, and tetracyclines) by developing silica gel plates with an ammonium chloride solution in a graded concentration range (0.5%, 1%, 2%, 3%, 5%, 10%, and 20%). For bioautographic detection *Bacillus subtilis* and *Micrococcus luteus* were used.

A TLC method was described for identification and quantification of oxytetracycline, tiamulin, lincomycin, and spectinomycin in veterinary preparations (119). Silica gel plates were developed with two mobile phases: 10% aq. citric acid–*n*-hexane–ethanol (80:1:1) and *n*-butanol–ethanol–chloroform–25% ammonia (4:5:2:5). R_f values, spot colors, and direct UV and densitometric measurements were used for identification. The results of quantitative analysis were close to the declared contents of the constituents. The recoveries ranged from 100.01% to 102.54%.

Markakis presented two papers on TLC separation and detection combined with microbiological quantification by the agar diffusion method of various antibiotics. The first paper (120) reported the determination of oxytetracycline and chlortetracycline in the presence of 11 other drugs (nitrofurans, macrolides, sulfonamides, coccidiostatics). The second paper (121) reported the determination of erythromycin and tylosin in the presence of 11 other drugs (nitrofurans, tetracyclines, sulfonamides, coccidiostatics).

A routine method was described (122) for detecting the following antimicrobial agents in feeds: avilamycin, avoparcin, Zn-bacitracin, erythromycin, flavomycin, furazolidone, lasalocid, monensin, narasin, penicillin, salinomycin, spiramycin, tetracyclines, tylosin, and virginiamycin. The method uses the agar diffusion of buffered samples, neutral extraction of polyether antibiotics followed by thin-layer chromatography, and acid extraction for other antibiotics followed by TLC. Identification after TLC was achieved by bioautography.

Vega et al. (82) described HPTLC analysis of various antibiotics in fish feed: (a) oxolinic acid on silica impregnated with 0.1 M K_2HPO_4 with chloroform–acetone (9:1), (b) erythromycin on silica with ethyl acetate–acetic acid–water (6:2:2), and (c) oxytetracycline on silica impregnated with EDTA with dichloromethane–methanol–water (59:35:6).

A TLC method was described (123) for detecting tetracycline and amino glycopeptide antibiotics on silica gel plates developed with ethanol–acetic acid–water (10:6:6) and butanol–formic acid–water (6:5:7). Detection was done by exposing the obtained spots to iodine vapor.

A TLC screening method was established for determining flumequine and doxycycline in milk; these two antibiotics are often used in veterinary treatment (124). Samples of milk spiked with the antibiotics were applied onto the concentrating zone of a silica gel plate with fluorescent indicator and predeveloped with hexane to remove lipids. The plate was then developed with the mobile phase. Several mobile phases were tested; the most effective was 0.05 M citric acid–methanol–2-propanol (1:3:1). Spots were detected under UV light at 254 nm for flumequine and at 366 nm for doxycycline. Calibration curves of the antibiotics were obtained by scanning in the reflection mode at 325 nm for flumequine standards and at 360 nm for doxycycline standards. The recoveries were close to 100%. A similar TLC method but combined with bioautography was also described by Choma et al. (125).

III. TLC OF ANTIBIOTICS—OTHER APPLICATIONS

Apart from antibiotic analysis focused on the separation of antibiotics of one or different classes, there are many other examples of TLC applications. Some of them are described in this section.

A. Purification of Newly Discovered Antibiotics

Antimicrobial substances produced by *Bacillus subtilis* BS 107 were isolated from culture filtrate by precipitation and extraction. They were then purified by TLC on silica gel plates developed with ethanol–water mixture (2:1) (126). The AH7 antibiotic produced by *Streptosporangium roseum* strain 214 was extracted with chloroform from the filtrate culture and purified using TLC (127). A mixture of polypeptides was isolated from the culture broths of the mold *Stibella flavipes*. Using preparative TLC, three groups of peptides—stilboflavins A, B, and C—could be separated (128).

B. Biomolecular-Chemical Screening

Chemical screening can be regarded as a systematic approach in the search for new biologically active compounds in extracts from natural sources (e.g., microorganisms or plants) (129). The chromatographic parameters of microbial metabolites separated on TLC plates as well as their chemical reactivity toward staining reagents allow an almost complete picture of a secondary metabolite pattern (fingerprint) to be obtained (130). In contrast to biological screening, chemical screening is not correlated with any biological effect. Therefore, a new screening strategy, called biomolecular-chemical screening, was developed that combines the chemical screening strategy with binding behavior toward DNA (131). Pure secondary metabolites were analyzed for DNA-binding properties on silica gel RP-18 W F₂₅₄ plates in a solvent consisting of methanol–1 M aq. ammonium acetate (4:1) (131, 132). Crude extracts were analyzed by two-dimensional TLC. In the first dimension the metabolites of the extract were separated using methanol–0.5 M aq.

ammonium acetate (1:3). Interactions with DNA were analyzed in the second dimension using methanol–1 M aq. ammonium acetate (4:1). DNA was spotted in a thin straight line above the separated extract before the second chromatographic step (131, 133). Detection was done by means of UV extinction at 254 and 366 nm as well as by colorization with staining reagents. Changes in R_f values indicated an interaction between ligand and DNA and were expressed by the R_{f2}/R_{f1} ratio, in which R_{f1} represents the R_f value without DNA and R_{f2} represents the R_f value with DNA.

C. Examining the Stability and Breakdown Products of Antibiotics in Solutions and Dosage Forms

The stability of tetracycline in methanol solution was investigated by ultraviolet-visible (UV-Vis) spectroscopy, HPLC, and TLC (134). HPTLC analysis was done using a scientifically operated charge-coupled device (CCD) detector (135). Fluorescence detection mode was used with a 360 nm excitation source and a 550 nm bandpass filter. HPTLC plates impregnated with saturated Na_2EDTA solution were developed with methanol–dichloromethane–water (30:64:6). After separation, the plates were dried and dipped in a solution of paraffin in hexane. Compared with the standards (tetracycline, anhydrotetracycline, 4-*epi*-anhydrotetracycline, 4-*epi*-tetracycline, and chlortetracycline), more than 10 fluorescent compounds were detected from the degraded sample, in which a very small amount of tetracycline remained.

Epicillin breakdown products were demonstrated by thin-layer chromatography (136). The results were correlated with iodometric and spectrophotometric data.

Chloramphenicol monosuccinate and gentamycin as well as their degradation products were determined by TLC for quality control both of freshly produced drugs and of those stored long-term (137). Chromatography of gentamycin was done on silica with chloroform–methanol–conc. aq. ammonia (1:1:1). Chloramphenicol was analyzed on silica with *n*-butyl acetate–acetic acid (96:4).

D. Chiral Separations Using the Macrocyclic Antibiotics

The macrocyclic antibiotics (ansamycins, glycopeptides, and polypeptides) have recently gained popularity as chiral selectors in capillary electrophoresis, high-performance liquid chromatography, and thin-layer chromatography (138). Vancomycin was used as a chiral mobile-phase additive for the TLC resolution of AQC-derivatized* amino acids, racemic drugs, and dansyl amino acids (139).

The enantiomeric resolution of 10 dansyl D,L-amino acids was achieved on TLC silica plates impregnated with erythromycin as the chiral selector (140). The mobile phases were mixtures of 0.5 M aqueous NaCl, acetonitrile, and methanol prepared in various proportions. Spots were detected with 254 nm UV light. Dansyl D,L-amino acids were also separated by normal-phase TLC on silica gel impregnated with vancomycin (141). The mobile phases were acetonitrile–0.5 M NaCl (10:4 or 14:3). Spots were detected by use of UV light at 254 nm.

E. Thermodynamic Study of the Retention Behavior of Antibiotics

The influence of temperature and mobile-phase composition on retention of cyclodextrins and two macrocyclic antibiotics, rifamicin B and rifampicin, was examined by RP-TLC, using wide-range (0–100%) binary mixtures of methanol-water (142). R_f values of the solute molecules were measured at temperatures ranging from 5°C to 60°C. From linear Van't Hoff plots, thermodynamic parameters, such as the change of enthalpy (ΔH°) and the change of entropy (ΔS°), were estimated.

F. Interactions of Antibiotics with Biological Matrices

The interaction of drugs with human serum albumin (HSA) modifies the biological efficacy and stability of the drugs. The interaction of 13 antibiotics with HSA was studied by charge-transfer RP-TLC in neutral, acidic, basic, and ionic environments, and the relative strength of interaction was calculated (143). The pH and the presence of mono- and divalent cations markedly influenced the strength of interactions.

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16

Carbohydrates

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I. INTRODUCTION

A. Carbohydrates

Their importance as nutrients has historically been the focus of research on carbohydrates. The importance of carbohydrate moieties as components of glycoconjugates—glycoproteins, glycolipids, mucins, and structural polymers—has become increasingly apparent. Examples include carbohydrate moieties as critical determinants of protein transport within cells and of cell homing within tissues of the body. The carbohydrate components of glycoproteins influence protein function, conformation, stability, and half-life in vitro and in vivo and function as important components of receptors in signal transduction pathways. Glycolipids and carbohydrate matrix components are important in a variety of biological systems such as the nervous system with regard to myelin formation and directed neuronal growth and in B cells and other types of cells as regulatory components of signal transduction pathways (epidermal growth factor, interferon- α , apoptosis, and antiviral pathways), cell adhesion pathways (selectin systems, CD19–CD77 interaction), and potentially other immune functions such as antigen presentation.

Carbohydrates are widely distributed in organisms. They usually have the general formula $C_n(H_2O)_n$, where five units (pentoses) or six units (hexoses) form the basic cyclic structures. This large family of natural products includes monomers of basic structures called simple sugars or monosaccharides, their dimers or disaccharides, polymers of a few basic structures known as oligosaccharides, and complex polymers called polysaccharides. Most simple sugars are either polyhydroxyaldehydes (aldoses, e.g., glucose) or polyhydroxyketones (ketoses, e.g., fructose). However, there are some that have at least one—OH group replaced by some other substituent. The two most common groups that replace the—OH group in these monosaccharides are—H and—NH₂. Deoxy sugars (2-deoxy-D-ribose, etc.) have a CH₂ group in place of the α -CH(OH) group. Amino sugars (D-glucosamine, D-galactosamine) have an α -CH(NH₂) group in place of the α -CH(OH) group.

Even though monosaccharides exist largely as cyclic hemiacetals or hemiketals, the presence of carbonyl-containing compounds necessitates consideration of both acyclic and cyclic structures in sugar reactions. Common monosaccharides can be reduced to alkanepolyols (D-manitol, sorbitol) or oxidized to aldaric, aldonic, or uronic acids.

Because carbohydrates are present in various forms and there are many isomers and analogs, separation of these compounds often involves more difficult problems than the separation of other very abundant natural products. Difficulties are also encountered in detection owing to the lack of chromophores or fluorophores.

B. Analytical Methods

Simple sugars, disaccharides, and some oligosaccharides are frequently analyzed in various industries concerned with the preparation and stabilization of food. Their quantity in food and beverages is often a good indicator of product quality, contamination, or adulteration. Carbohydrate detection is also important for monitoring a variety of fermentation and other biotechnological processes. It is often used in the research of natural products and in research and applications involving synthesis of sugars or glycoconjugates. In addition, analysis of carbohydrate and glycoconjugate composition is important in a number of other fields, including many areas of biology, biochemistry, medicine, and pharmacy. TLC has applications in diseases associated with deficient degradation of oligosaccharides of glycolipids in which carbohydrates may accumulate in tissues or in body fluids such as urine. Other applications involve research into understanding the importance of naturally occurring free oligosaccharides in body fluids such as colostrum and milk. Unusual expression of glycolipids, glycoproteins such as mucins and other complex glycoconjugates is associated with some types of cancer (1, 2). TLC has also been used in studies to determine the expression of carbohydrates and glycoconjugates in parasites and their utilization of host carbohydrates and glycoconjugates (3–5). TLC following acid hydrolysis was recently used to assay the sialic acid content of *Aspergillus conidia* as a potential virulence factor (6).

Although some assays for carbohydrates can be performed by nonchromatographic means, such assays have inherent limitations. For example, clinical analyses of carbohydrates in blood and other body fluids are performed mainly by enzymatic or microenzymatic methods, which are fast, specific, and very sensitive. However, these methods are limited to the analysis of a few sugars. The best choice for analysis of complex mixtures of sugars is chromatographic separation. Analytical separations of monosaccharides and oligosaccharides have, for many years, been achieved by thin-layer chromatography. A number of other chromatographic methods are also available. These include paper chromatography; liquid chromatography on ion-exchange, amino, and silica gel columns; gas-liquid chromatography on various phases (7–9); capillary supercritical fluid chromatography; polyacrylamide gel electrophoresis; capillary electrophoresis; and capillary electrokinetic chromatography (10–13).

Each method has its applications, but thin-layer chromatography has a number of advantages over the other methods. It is simple to run and affordable, it can be applied to the separation of a number of molecules with the choice of an appropriate sorbent-solvent combination, and it is relatively fast and sensitive. Thin-layer chromatography usually requires no special pretreatment or derivatization of the samples. The planar

chromatographic system enables simultaneous analysis of a variety of experimental samples as well as reference standards. Comigration of spots with carbohydrate standards coupled with appropriate detection reagents allows for presumptive identification of carbohydrates or glycoconjugates in the samples. Detection of separated sugars is relatively simple and typically involves the use of postchromatographic derivatization methods. Postchromatographic visualization of sugars is usually based on the formation of colored or fluorescent products observed as discrete spots (18, 19). The plate can be documented by photography or by scanning the plate by use of a digital video scanner or appropriate CCD video camera. This unique feature of planar techniques enables easy capture and storage of the migration and detection information about the samples (17). Quantitative evaluation can be done by densitometry or image processing scanners in transmission or reflection mode. The detection limits for some monosaccharides are below 10 ng per spot (15, 18–20).

Thin-layer chromatography of carbohydrates has not undergone as many modifications in recent years as the column-based methods. Perhaps this is because planar chromatography is such an established technique in carbohydrate chemistry with little room for improvement, with the exception of new automated developing techniques (14–17), sensitivity and specificity of detection reagents, and image processing techniques for documentation and evaluation of chromatograms (10b) (Fig. 1). However, TLC is often coupled with more sophisticated state-of-the-art analytical systems. Frequently, isolation of samples by preparative TLC precedes further analysis by gas chromatography or mass spectrometry. In some instances, mass spectrometry can be applied to individual bands of isolated oligosaccharides or glycolipids directly on a thin-layer chromato-

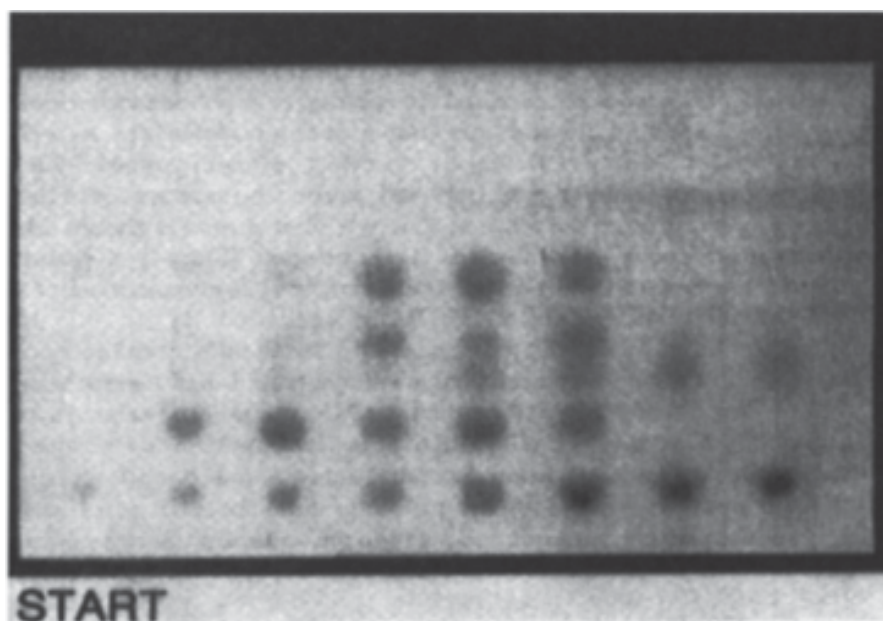


Figure 1 Sugars in dairy products (yogurts). Documentation of a developed TLC plate with an image

analyzing system. Conditions: Silica gel HPTLC plates; acetonitrile–phosphate buffer pH 5.9 (85:15) solvent system containing 0.05% NST; two developments; detection by aniline 2%–diphenylamine 2%–phosphoric acid (85%) 15% in methanol (ADP reagent), activation 5 min at 120°C. Sugars (in order of migration): Lac, Sue, Gal, Glc, Fru. Video system: Javelin JA3622X; PC/FS200; thermal printer Toshiba.

gram. Laboratory synthesis of neoglycolipids using oligosaccharides released from complex carbohydrates has important applications in determining some carbohydrate structures by such techniques (2, 21a). Examples of the above-mentioned applications are discussed in subsequent sections, as appropriate.

II. SAMPLE PREPARATION PRIOR TO TLC ANALYSIS

Sample preparation is the most critical aspect of chromatographic analysis. Improper treatment of the sample before analysis is one of the most frequent causes of erroneous or misleading results in the chromatographic separation. Samples for carbohydrate analysis can be derived from many different sources, and there is no universal method for sample preparation. Sometimes samples can be applied to a TLC plate directly, but biological or environmental samples are often complex, dilute, or incompatible with the chromatographic system and do not permit direct application. In most instances, some purification and/or concentration of the sample components of interest is needed before analysis. Biological material frequently requires a digestion step for release of carbohydrate moieties from glycoconjugates or the hydrolysis of complex sugars to more easily identifiable monosaccharides. These additional procedures may affect the performance of the analytical method. Therefore, they should be evaluated for each application and optimized as appropriate (21b). Additional descriptions of sampling and cleanup procedures of specific samples such as clinical and biological specimens are as referenced in the original articles (22–24).

A. Solutions

Solutions of appropriate concentrations can be spotted on TLC plates without any pretreatment except for filtration through a 0.45–0.8 μm pore-diameter filter to remove particulates. However, filtered samples may still contain large amounts of interfering compounds such as salts, urea, lipids, or proteins that influence the application and separation. In some cases, these compounds can be removed simply by adding washed

resin of an appropriate chromatographic packing material to a sample in a flask, shaking the mixture for 10 min, and filtering the sample or passing it through a column of the mixed bed resin using an appropriate eluent. More commonly, sample preparation involves passing samples, using an appropriate water-based eluent, through disposable, ready-to-use columns packed with various resins (C-18, amino, ion-exchange, size exclusion, or other resins as appropriate). For example, free carbohydrates in aqueous solution can be separated from gangliosides and other polar glycolipids by passing samples repeatedly through C-18 cartridges or minicolumns. Carbohydrates pass through the cartridges or columns. Lipids are retained by the C-18 gel but can easily be obtained, if desired, by elution with methanol. To evaluate the performance of a given cleanup procedure, five to 10 different standard mixtures of sugars should be submitted to the identical procedure. In most instances, this type of solution pretreatment is sufficient for application and separation on TLC plates.

Extraction and sample preparation methods may result in some dilution of the carbohydrates. Bandwise application of samples at the origin using spray-on techniques and modern instrumental applicators enables application of relatively large volumes of solutions (up to approximately 100 μL).

More typically, especially for spotwise application on HPTLC plates, Hamilton syringes or micropipets are used to apply much smaller volumes of sample. To obtain sufficient concentrations, samples may have to be concentrated. However, if the concentration is too high, sample solutions will have relatively high viscosity and surface tension, which prevents the filling and emptying of syringes or micropipets by capillary forces. Therefore, it may be necessary to adjust sample concentration by dilution with methanol until a suitable combination of concentration and viscosity is obtained. Solid samples should be dissolved in distilled water (pH 5.5), dilute acid, or alcohol and water mixtures and treated as solutions (with proper pretreatment, as appropriate).

B. Plant Material

Analysis of free sugars in fruits and vegetables is usually done by extraction of fresh plant tissue with ethanol or methanol or with mixtures of methanol and water. This is followed by concentration of the extract, which removes all the alcohol, and filtration through Celite or centrifugation of the concentrated aqueous extract. The clear solution can be spotted directly on the TLC plate. In some instances it is necessary to dry a sample of a fresh material to constant weight at elevated temperature. The appropriate amount of dry material, usually 1–5 g, is then blended with aqueous ethanol (about 10 mL/g), using an appropriate homogenizer, for 3–5 min at room temperature. The slurry is centrifuged and the clear supernatant decanted. The residue is treated as before, supernatants are combined, the solvent is evaporated, and the dry residue is dissolved in an exact volume of water or aqueous methanol. It may be useful to include, at some stage of the sample preparation, a washing of the extract with light petroleum to remove lipids. However, this is not always necessary. Fruit acids can also influence the separation. They can be removed with disposable solid-phase extraction columns packed with silica gel or ion-exchange resins or by using the following classical procedure: addition of 10% aqueous solution of lead acetate to the clean supernatant to precipitate the fruit acids, centrifugation, removal of the excess Pb ions in the clear solution by H_2S , neutralization

of the clear solution with an anion exchanger, and sample cleanup using gel permeation chromatography.

C. Samples with Traces of Sugars

Sometimes samples contain only traces of the sugars of interest or small amounts of disaccharides or oligosaccharides together with the main sugars such as glucose, fructose, and sucrose. The separation and detection of such sugars in complex mixtures by TLC is a difficult and timeconsuming task. Purification and enrichment of samples for TLC or for any other chromatographic technique can be accomplished by gel permeation chromatography or by passing the sample through a prepared disposable aminopropyl or polyamide column for sample preparation. These columns can also be used for removing proteins, lipids, and salts. For such precleaning, samples can be accepted in virtually any form, provided they are stable and not complexed. It is desirable, however, to bring the pH to 5–8 before purification.

D. Sugars Isolated from Polysaccharides and Conjugated Carbohydrates

A wide variety of naturally occurring compounds contain sugars. Monosaccharides are linked, by glycosidic bonds, to another sugar component or to a moiety of another origin, called an aglycone. Individual sugar components of conjugated sugars can be analyzed after appropriate enzymatic or chemical hydrolysis. Acid hydrolysis is most frequently used. It is suitable for cleavage of polysaccharides and almost all types of conjugated compounds such as glycolipids, glycoproteins, and various glycosides. Various acid hydrolytic conditions may be chosen for different types of compounds depending on factors such as the monosaccharide composition of the individual compound, the ring form of the component sugars, and the configuration of the glycosidic bonds. It is known that furanoside linkages are much more labile than pyranoside linkages, that α -glycosidic bonds are usually more labile than β -glycosidic bonds, and that pentaglycans, in the pyranoside form, are more readily hydrolyzed than pyranoside hexoglycans. The presence of uronic acid groups and amino sugars also increases the resistance to acid hydrolysis. Hydrolysis is usually done with hydrochloric acid, sulfuric acid, or trifluoroacetic acid at concentrations ranging from 0.5 M to up to several moles per liter, and at elevated temperatures. Detailed procedures are described in the literature (25–30).

Thin-layer chromatographic analysis of the hydrolysates requires removal of the mineral acid used for hydrolysis. Ion-exchange resins are widely used, but some classical procedures are also useful. After hydrolysis with 1 M sulfuric acid, the acid may be removed as barium sulfate by adding barium carbonate solution. Small amounts of 1 M hydrochloric acid can be removed in vacuo, and larger amounts of this acid can be conveniently removed from an aqueous hydrolysate by repeated washing with a 10% solution of di-*n*-octylmethylamine in chloroform. The hydrolysates of plant glycosides usually contain interfering compounds such as phenolics. These can be removed by extracting the hydrolysates with diethyl ether or ethyl acetate (26).

TLC is occasionally used to assay the activity of carbohydrate-degrading enzymes. For example, Whitehead et al. (31a) used TLC to assay the activity of an agarase isolated from a marine bacterium.

E. Glycolipid Analysis

Of all the glycoconjugates mentioned above, only glycolipids have chemical properties suitable for TLC analysis as intact molecules without hydrolysis of the sugar moiety. In fact, the amphipathic nature of glycolipids is well suited to TLC, which has many applications in lipid analysis. Lipid fractions may be applied directly to TLC plates, or glycolipids may be purified or enriched prior to analysis (31b, 31c, 31d). Synthesis of neoglycolipids using oligosaccharides isolated from complex carbohydrates is useful in some structural studies involving TLC coupled to mass spectrometry (see Sec. IV.E).

III. SEPARATION

Carbohydrates are weakly acidic (pK_a 12–14), strongly hydrophilic compounds (31e). Their separation by thin-layer chromatography depends on partition and adsorption phenomena, and occasionally, resolution incorporates anion-exchange mechanisms. The high polarity of sugars requires very polar solvents and sorbents with low activity. The essential component of typical solvent systems is water. Because water decreases the layer activity, nonmodified inorganic sorbents such as alumina and silica as well as modified sorbents and organic materials can be used for separation of carbohydrates. Their mobility on polar layers depends primarily on the molecular weight of the carbohydrates and the number of hydroxyl groups. Consequently, the diastereoisomers are often poorly resolved. The water content of the mobile phase as well as polar solvents of low viscosity also have a great influence on mobile-phase velocity. Mobile phases based on mixtures of higher alcohols and water show very slow migration rates.

Most laboratories use commercially available precoated TLC plates. A variety of materials and qualities of layers are available, and there is no need to prepare plates in the laboratory. In addition, there are several ways to adjust the selectivity of commercially available layers by impregnation with a modifier, generally achieved by immersing the layer into a solution of the modifier and allowing the solvent to evaporate. Common impregnating reagents used in the TLC of carbohydrates are phosphates (16, 32–34), borates (35), and boric acid (36, 37). Boric and boronic acids can form reversible complexes and are often used to differentiate isomers with vicinal hydrogen-bonding functional groups (38). Other impregnating reagents include bisulfite, known for its characteristic addition reactions with aldoses and ketoses (24, 37), molybdate, tung-state, and other metal salts (39, 40). The highest separation efficiency can be obtained by using precoated high-performance TLC (HPTLC) plates (41).

The complexity of carbohydrates and the limited separation capacity of TLC can cause the overlap of some spots of the reference standard mixtures. This is not always a disadvantage, because it is uncommon for some sugars to occur together in a sample. For instance, L-fucose arises from animal glycoproteins, L-arabinose from plant polysaccharides, and D-fructose from specific enzyme inversions of D-glucose.

A. Layers

The most frequently used layers for separation of carbohydrates are cellulose, silica, silica 50,000, and amino-bonded silica.

1. Cellulose

Precoated TLC plates with both native and microcrystalline cellulose layers have been used for the separation of carbohydrates and other very polar compounds for several years (8a) (Table 1). An advantage of these low surface area sorbents is that most of the separations previously achieved by paper chromatography can be obtained by TLC using the same solvent systems (8a). It is generally assumed that a partition mechanism is responsible for retention on these materials. Advantages of cellulose plates over paper chromatography include a shorter development time, less spot diffusion, and less background staining with some spray reagents. Cellulose thin-layer plates are sometimes modified by impregnation with buffers or salts. The best results can be obtained on commercially available HPTLC plates coated with a special grade of microcrystalline cellulose (42a). One cellulose HPTLC application involves inositol phosphate analysis (42b).

At present, cellulose layers are often replaced by synthetically prepared wide-pore silica (Si 50,000). This very low surface area material has been recommended for the separation of substances similar to those normally separated on cellulose (42a). Compared to cellulose, the silica gel thin-layer sorbents do not swell in organic solvents, and the plates can be used with aggressive visualizing reagents (18).

2. Silica

Silica gel thin layers are the most commonly used sorbents in TLC of carbohydrates. These layers are suitable for most classes of sugars and sugar derivatives, the major exception being intact complex polysaccharides. Numerous solvent systems provide relatively good separation of monosaccharides, disaccharides, and lower oligosaccharides (Tables 2 and 3); malto-oligosaccharides (14, 16, 43, 44); amino sugars (27, 40) (Table 4); aminoglycoside antibiotics (44, 46); sugar alcohols (48, 49) (Table 4); uronic acids (50, 51); derivatives (52, 53) (Table 4); and cyclodextrins (54). Another advantage of TLC and HPTLC silica gel layers is their chemical stability against almost all solvents, strong acids, and other corrosive reagents used in postchromatographic derivatization procedures. To obtain better selectivity of some sugars of interest, these layers are sometimes modified by pretreatment with a suitable buffer or with inorganic salts. Alternatively, sugars may be derivatized prior to analysis to improve separation or visualization (Table 5).

Separation time required for a single development of silica gel layers varies from a few minutes to a few hours and depends on the solvent system composition, the layer quality (i.e.,

Table 1 Some Solvent Systems Suitable for Separation of Sugars on Cellulose Thin Layers

Solvent system	Carbohydrate(s) analyzed	Sample	Detection	Remarks	Ref.
<i>n</i> -Butanol–acetic acid–water (42:10:7)	Mucopolysaccharides	Urine	Toluidine blue	TLC	86
<i>n</i> -Butanol–ethanol–water (3:2:1)	Fuc, Gal, Glu, Lac, GlcNAc, Mal, Man, Sor, Fru, Xyl, GlcU, LacNac, 3'- and 6'-sialyllactose, maltooligosaccharides DP ₂ –DP ₈	Test mixture, blood, urine, feces, human milk	Silver nitrate, sodium hydroxide, sodium thiosulfate	TLC MD, 3 runs	87
<i>n</i> -Butanol–ethyl methyl ketone–formic acid–water (8:6:3:3)	Sue, Glc, Fru	Infusion solutions	Potassium permanganate–sodium hydroxide	TLC	88
<i>n</i> -Butanol–pyridine–acetic acid–water (10:6:1:3)	Pan, Raf, Mal, Nig, Sue, Glu, Fru, isomaltooligosaccharides DP ₂ –DP ₅	Reaction products of sucrases	Aniline–diphenylamine–phosphoric acid	TLC Avicel SF MD 1st and 2nd runs	89
Phenol–1.5% aq. ammonia (2:1)				3rd run	
<i>n</i> -Butanol–acetic acid–water (3:1:1) Ethyl acetate–pyridine–water (10:4:3)	Monosaccharides of xyloglucan	Test mixture	Aniline–phthalate	TLC, MD, 1st run MD, 2nd run	90
Ethyl acetate–pyridine–water (6:3:1)	Lac, Gal, Glc, Fru	Urine	Aniline–phthalate	TLC	91

Table 2 Acetonitrile and Water Mixtures Reported for Separation of Sugars on Silica Gel Thin Layers

Solvent system	Carbohydrate(s) analyzed	Sample	Detection	Remarks	Ref.
Acetonitrile–water (85:15)	Raf, Mel, Lac, Mal, Sue, Gal, Glu, Fru, Xyl, Rha, dRib	Test mixture	Analine–diphenylamine–phosphoric acid	TLC MD, 3 runs	55, 92, 93
Acetonitrile–water+ammonium carbonate (85:15+1%)	Merck sugar reference standard kit	Test mixture	Thermal	HPTLC	75
Acetonitrile–water (85:15)	Mono- and oligosaccharides trehalose, glucose	Test mixture, hemolymph and digestive gland gonad complex	α -Naphthol–sulfuric acid	HPTLC impreg. 0.1 M sodium bisulfite MD, 3 runs	24
Acetonitrile–0.3% aq. NH ₃ –2 M aq. KCl (85:15:5)	Rha, Xyl, Fru, Glu, Sue, Mal, Lac, maltooligosaccharides DP ₂ –DP ₁₅	Test mixture	Orcinol–sulfuric acid	HPTLC OPTLC	14
Acetonitrile–water+NST (85:15+1.5 mmol/L)	Sue, Glc, Fru	Test mixture, honey	Aniline–diphenylamine–phosphoric acid	HPTLC MD, 2 runs additives	64
Acetonitrile– <i>n</i> -amyl alcohol–water (3:1:1)	GalU, GlcU, Gal, Man, Ara, Xyl, Rha	Cherry cell wall hydrolysates	<i>N</i> -(1-Naphthyl)–ethylenediamine dihydrochloride	TLC, impreg. 0.2 M, pH 6 phosphate buffer	34
Acetonitrile–ethyl acetate– 1-propanol–water (85:20:50: <i>X</i> ; <i>X</i> =50–70 or 90–100)	Glucooligosaccharides DP ₂ –DP ₂₀ (maltodextrins; isomaltodextrins)	Amylopectin hydrolysates	α -Naphthol–sulfuric acid	TLC	44

particle size and pore diameter), and the developing technique. Relatively good resolution of some common sugars can be obtained by fast-migrating solvent systems such as mixtures of acetonitrile and water. Single development of an HPTLC plate is usually finished in less than 10 min. This enables fast routine analyses and also permits the extensive use of the multiple development technique for enhancing the efficiency of separation (55). To minimize the influence of oxidized binders on detection and quantitative evaluation, it is advisable to preclean the commercially available TLC plates by developing them in a mixture of chloroform and methanol (1:1) or in pure methanol, followed by drying and reactivating. Surface-active silica gel thin layers tend to absorb

water molecules from the surrounding atmosphere, leading to a reduction in their activity and in their chromatographic retention capacity. Therefore, it is also advisable to standardize these surface activities to obtain reproducible chromatographic results. This can be done by activating the plate with heat and storing it in a desiccator until it is needed. Carbohydrates can also be on silica gel plates with a sample concentration zone, but such plates are not very effective when solvent systems with a high water content are used. The resolution of sugars on silica TLC plates, although not as robust as with other systems such as HPLC, is sufficient for a number of applications. For example, Cline et al. (3) were able to determine, using TLC, that maltose, not trehalose as had been previously reported, was a host sugar utilized by parasitic flukes (since confirmed by GC-MS).

Silica 50,000 (Si 50,000) is a synthetically prepared inactive silicon dioxide with chromatographic properties comparable to those of the naturally occurring kieselguhrs or diatomaceous earth (a natural product based on the cell walls of diatoms, which consist mainly of silicic acid). Silica 50,000 sorbent has a uniform large pore size of 5000 nm and was originally used as a concentration zone on silica gel 60 TLC plates. Because this wide-pore material has a very low surface area and low activity, it can also be applied as a stationary-phase support for normal-phase partition chromatography. It is very suitable for separation of polar compounds such as carbohydrates, and the time required for analysis is shorter and the resolution achieved better than for analyses based on paper chromatography (42a). Separation of different types of carbohydrates can be attained with water-based solvent systems such as those commonly used with more typical silica gel sorbents.

3. Aminopropyl-Bonded Silica

Amino groups added as aminopropyl groups bonded to silica gel, or simply as amino-silica gel (NH_2 -silica gel), are particularly useful as sorbent modifications for carbohydrate analysis (32, 56). As is the case with other polar bonded phases, such sorbents can be used in either the normal-phase or reversed-phase mode. An anion-exchange mechanism can also influence the separation. Chromatographic properties of amino-silica gel layers are similar to those of nonmodified silica gel, and some identical solvent systems can be used with both sorbents (Table 6). The main advantage of amino-bonded silica is that it affords simple detection of separated sugars by a thermal in situ reaction (56–59). Sugars are readily converted, leading to stable, intensely fluorescing derivatives. The thermal treatment, after development, does not lead to a discoloration of the chromatograms as is often the case with chemical postchromatographic derivatization (56). A disadvantage of the aminopropyl-modified silica gel layer is the tendency for glycosylamine to form between reducing sugars and the amino groups on the stationary phase (32). The separation of sugars on aminopropyl-bonded thin layers is usually done with water-containing solvent systems such as acetonitrile-water mixtures. Due to the basicity of the layer, the pH of the aqueous mobile phase is high, exceeding pH 9 (16). This is a favorable condition for interactions between reducing sugars and the aminopropyl groups of the bonded silica. Sugar residues that are especially apt to interact covalently with the aminopropyl groups are those that contain appreciable levels of the acyclic (aldehydic) forms in tautomeric equilibrium with their ring (furanose and

pyranose) structures. Examples of such sugars are 2-deoxyglucose, xylose, rhamnose, galactose, and mannose. The result of this glycosylamine formation, which is common with sugars containing more than 0.05% of the aldehydic form (in solution), is that these sugars show practically no mobility after being spotted and thus remain in their original positions on the plate. The reaction can also influence spot or band shapes of even those sugars that have very low levels of acyclic forms such as glucose and fructose (32).

Table 3 Some Other Solvent Systems Reported for Separation of Carbohydrates on Silica Gel Thin Layers

Solvent system	Carbohydrate(s) analyzed	Sample	Detection	Remarks	Ref.
<i>n</i> -Butanol–acetic acid–water (14:3:2)	Rha	Urine	4-Hydroxybenzohydrazide	TLC	23
<i>n</i> -Butanol–acetic acid–diethyl ether (27:18:5:3)	Lac, Sue, Gal, Fru, Glc	Urine	Naphthoresorcinol	TLC	86
Water 1-propanol–water (42:6)				MD, 1st syst. MD, 2nd syst.	
<i>n</i> -Butanol–acetic acid–water (2:1:1)	Oligosaccharides, Glc	Urine	Orcinol–sulfuric acid	TLC	22
<i>n</i> -Butanol–acetone–water (3:3:1)	Verbascose, stachylose, Raf, Sue	Plant extract	Aniline–diphenylamine	TLC	94
<i>n</i> -Butanol–ethanol–water (5:3:2)	(¹⁴ C)Glc	Reaction mixture	Autoradiography	Fluorescent labeling PAGE	7
<i>n</i> -Butanol–ethanol–water (4:3:3) (9:6:5) 1-Propanol–acetone–water (5:4:1)	Oligosaccharides up to DP ₁₁	Glycan fractions of glycosyl-phosphatidyl-inositols; dextrane hydrolysates	Orcinol–sulfuric acid; autoradiography;	TLC; sugars reduced with NaB ₃ H ₄ and NaBH ₄	95
<i>n</i> -Butanol-2-propanol-0.5% aq. boric acid soln. (3:5:2)	Raf, Fru, Gal, Glc, Sue, Xyl	Test mixture	Thymol–sulfuric acid	TLC MD, 1st run	67

<i>n</i> -Butanol– acetone– 0.5% aq. boric acid soln. (4:5:1)		MD, 2nd run			
Ethyl acetate– pyridine–acetic acid– water (75:15:10:10)	Xyl, MeGlc, Rha	Urine	4-Aminobenzoic acid	HPTLC; MD, 3 runs	96
Ethyl acetate–2- propanol–water (6:3:1)	Raf, Sue, Gal, Glc, Fru, Ara, Xyl, Rha	Test mixture	Thymol–sulfuric acid	TLC	67
Ethyl acetate–2- propanol–water (50:32:19)	Raf, Cel, Lac, Sue, Gal, Glc, Xyl, Rha, Ara	Microcrystalline cellulose	Oleum vapor	TLC	97
Ethyl acetate– ethanol–acetic acid (60%)–sat. aq. boric acid soln. (5:2:1:1)	Fru, Sue, Glc fructo- oligosaccharides (kes- toses)	Reaction mixture	Thymol–sulfuric acid	TLC	19, 68
1-Propanol– acetone–water (45:30:25) (50:40:10)	Glc, maltooligosaccharides DP ₂ –DP ₁₂	Starch hydrolysate	Aniline– diphenylamine– phosphoric acid	HPTLC MD	43
2-Propanol–acetic acid–0.75% aq. boric acid soln. (40:1:5)	Lac, Fru, Ara, Xyl, Rha	Test mixture	4-Aminohipuric acid	HPTLC	18
Dichlorethane– methanol–acetic acid–water (50:50:25:10)	Lac, Fru	Chocolate	4-Aminobenzoic acid	TLC	18
Chloroform–acetic acid–water (3:6:1) (1:3:1)	Mono- and oligosaccharides	Wort, beer	Sulfuric acid	HPTLC MD 1st and 2nd runs 3rd run	98
Chloroform–ethyl acetate–methanol– water (15:40:22:10)	Glc, Rha, oligosaccharides	Glycoside hydrolysate	MS-FAB	TLC containing 0.6% CMC	99
Chloroform– methanol–water+ CaCl ₂ (120:70:17+0.02%) (50:40:10+0.05%)	Glycosphingolipids (GLSs)	GLSs derived from neutrophils	Immunochemical	HPTLC, fixation	84

Table 4 Some Developing Systems Reported for Separation of Amino Sugars and Sugar Alcohols on Silica Gel Thin Layers

Solvent system	Carbohydrate(s) analyzed	Sample	Detection	Remarks	Ref.
Acetonitrile–acetic acid–ethanol–water (13:1:2:4)	GlcN, GalN, ManN, D-muramic acid	Polysaccharide hydrolysates	Ninhydrin, AgNO ₃	TLC Whatman K-5 MD, 3 runs	27
Methanol–acetone (5:1)	GlcN-HCl, ManN-HCl, GalN-HCl, GlcNAc		Ninhydrin		40
2-Propanol–ethyl acetate–32% aq. ammonia soln. (1:1:1)	GalN, GlcN	Test mixture	4-(Dimethylamino)-benzaldehyde–acetylacetone	HPTLC	19
<i>n</i> -Butanol–pyridine–water (16:5:4) Ethyl acetate–methanol–acetic acid–water (4:1:1:1)	GlcNAc, Fuc, Gal GlcN, GalN, Man	Glycoprotein hydrolysate	<i>o</i> -toluidine	HPTLC; MD, 1st syst. MD, 2nd syst.	30
Ethyl acetate–pyridine–acetic acid–propionic acid–water (50:50:5:5:10)	Ino, SorH, ManH, Ery; mono, di-, and trisaccharides	Test mixture	2',7'-Dichloro-fluorescein–lead tetraacetate	HPTLC	51
Ethyl acetate–acetone–acetic acid–water (10:10:5:1)	Sor, SorH, ManH, mono-, di-, and trisaccharides	Purity test of Sor, SorH, ManH	Lead tetraacetate; (aniline–diphenylamine–phosphoric acid) for reduc. sugars	TLC	48
2-Propanol–ethyl acetate–water (57:7:2)	Sor, SorH, ManH				
1-Propanol–water (9:1)	SorH, ManH, XylH	Test mixture	Silver nitrate–sodium hydroxide	SPTLC Si 50,000	18
2-Pentanol–ethyl acetate–water (83:11:6)	Cyclitols (inositols)	Carob pods	Potassium permanganate–sodium hydroxide	TLC	49

Table 5 Developing Systems Suitable for Separation of Some Sugar Derivatives

Solvent system	Carbohydrate(s) analyzed	Sample	Remarks	Ref.
Chloroform–methanol–water (11:9:2)	Neutral sugars as glycamines	Urine, (glycoproteins) test mixture	Fluorescence measurement	36 76
Chloroform–methanol–aq. 0.4 M Na ₂ B ₄ O ₇ (11:9:1)	Neutral sugars as glycamines	Test mixture	Fluorescence measurement	76
Chloroform–ethanol–0.2 M boric acid (40:16:1)	Gal, Glc, Man, Ara, Xyl as hydrazones	Fish muscle		74
Ethyl acetate–2-propanol–water (11:5:1) (10:5:2)	Glc, mono- and disaccharides as dansylhydrazones	Urine, feces	MD	52
<i>n</i> -Butanol (water sat.)–triethylamine (30:1)	Glc, Gal, Man, Fru, Rib, dRib, Xyl, Ara, as dabsylhydrazones	Test mixture		53
Acetonitrile– <i>n</i> -butanol–hexane (20:2:1)				
Benzene–ethanol–water–aq. ammonia (200:47:15:1) (↑ phase)	Permethylated low molecular carbohydrates	Carbohydrates in seawater		100
Cyclohexane–2-propanol (5:1)	2,3:4,5-bis-O-(1-Methylethylidene) derivatives		MS-FAB analysis	101
1-Propanol–ethyl acetate–water (7:2:1)	Copolymers of maleic anhydride with carbohydrates	Reaction mixture	Thymol–sulfuric acid	102

Table 6 Selected Solvent Systems Reported for Separation of Sugars on Amino-Bonded Silica Gel Thin Layers

Solvent system	Carbohydrate(s) analyzed	Sample	Detection	Remarks	Ref.
Acetonitrile–water (90:10–60:40)	Numerous mono-, di-, and trisaccharides, maltooligosaccharides	Test mixture	Aniline–diphenylamine–sulfuric acid	TLC, impreg. 0.2 M	32
Acetonitrile–water [80:20 (85:15)]	Raf, Lac, Sac, Glu, Fru, Xyl	Test mixture, wine, cola, urine	Thermal, A1 reactor	HPLC (circular)	15

Acetonitrile– water– phosphate buffer pH 5.9 (85:15:10)	Raf, Lac, Sac, Glu, Fru	Test mixture, honey, chocolate, <i>E. coli</i> suspension	Aniline– diphenylamine– phosphoric acid	HPTLC	20, 66
Acetonitrile– water (85:154)	Glc, Fru	Test mixture	Thermal	HPTLC	58
Acetonitrile– 10 mM aq. triethylamine acetate pH7 (3:2)	Oligosaccharides	Mucin-derived Oligosaccharides	4- Methylnaphthalene	TLC	103
Acetonitrile– acetone–water 15 step gradient	Raf, Lac, Cel, Sue, Gal, Glc, Fru Sor, Ara, Xyl, Rib, Rha, dRib maltooligosaccharides	Test mixture Beer	Thermal	AMD; HPTLC impreg. 0.4 M KH ₂ PO ₄	16
Ethyl acetate– pyridine– acetic acid– propionic acid–water (10:10:1:1:2)	Lac, Sac, Gal, Glc, Fru, Ara, Xyl, Rib; ManH, SorH	Test mixture	2,7-Dichloro- fluorescein–lead tetraacetate	HPTLC	56, 59
Ethyl acetate– pyridine– acetic acid– water (12:6:1:2) (2:6:1:3)	Mono-, disaccharides Oligosaccharides	Test mixture (glycoprotein hydrolysates)	Immunochemical	HPTLC HPTLC	56 83
1-Propanol– nitromethane– acetic acid– water (30:30:1:10)	Lac, Sac, Gal, Glc, Fru, Ara, Xyl, Rib	Test mixture, urine, serum	Thermal	No sample preparation	58

Spot tailing can be minimized or prevented by impregnating the amino-bonded layer with a buffer. Impregnation can be done by immersing the plates in a 0.2 M aqueous solution of monosodium dihydrogen phosphate for 15 min. After draining, the plates should be dried in a vacuum oven at 70°C (32). The result of this procedure is neutralization of the aminopropyl groups, which consequently drops the pH of the layer to about pH 6.2. An additional advantage of impregnating the aminopropyl-bonded silica HPTLC plates with monosodium dihydrogen phosphate is that the sugars are visualized more readily after derivatization because the background is cleaner. The impregnation of the layer influences the reaction of some postchromatographic derivatization re-agents. Some reagents for visualizing sugars were found to be ineffective when impregnated aminopropyl-bonded silica plates were used. With regard to detection using the aniline–diphenylamine reagent, all sugars can be detected on these phosphate-impregnated

aminopropyl-bonded plates if phosphoric acid is substituted for sulfuric acid (32). Amino-bonded layers can also be impregnated with other phosphate buffers. A potassium dihydrogen phosphate solution, at concentrations between 0.2 and 1 M, was more satisfactory for multiple developments owing to its lower solubility in the mobile phases (16). Precleaning of amino-modified silica plates can be achieved by successively developing the plates in pyridine–ethyl acetate–water–glacial acetic acid–propionic acid (50:50:10:5:5) and 1-propanol–nitromethane–water–glacial acetic acid (30:30:10:1) (59).

4. Other Layers

Kieselguhr and combinations of silica gel and kieselguhr have also been used for separation of sugars for a number of years (8a). Better results can now be obtained with the newer commercially available plates using sorbents such as Si 50,000. The same is valid for traditional polyamide layers, because one can usually obtain better results with other supports.

Alumina is less suitable for the separation of most carbohydrates. Owing to the presence of oxide ions, the surface of alumina is quite basic (pH approximately 12). Acids with pK_a lower than 13 transfer protons to this surface, producing charged conjugate bases that are strongly absorbed.

Chemically bonded layers, with the exception of the amino-bonded silica gel, are not suitable for carbohydrate analysis. Cyano- and diol-modified silica gel sorbents are not typically used for thin-layer analysis of carbohydrates. These bonded layers exhibit low retention and are generally inferior to the polar amino-modified silica for separation of carbohydrates (60). Retention of carbohydrates is even lower on reversed-phase bonded layers. Although these layers are less suitable for carbohydrate separation, they can be used for separation of some sugar derivatives. Reversed-phase bonded layers have been used for separation of aminoglycoside antibiotics (46), and silanized silica gel has been used for thin-layer electrophoresis (electrophoresis) of some sugars (61, 62).

B. Solvent Systems

Because of the complexity of carbohydrates as a class, there is no universal solvent system optimized to give a complete profile of carbohydrate content in every situation. Various solvent systems using mixtures of water with acetonitrile, alcohols (methanol, ethanol, 1-propanol, 2-propanol, 1-butanol), acetone, acetic acid, ethyl acetate, and pyridine are efficient in the separation of some sugars. The mobility of carbohydrates on polar layers depends primarily on their molecular weight and on the number of hydroxyl groups; disaccharides, for example, show higher retention than monosaccharides. Separation of oligosaccharides and lower polysaccharides can usually be achieved through multiple development of plates with solvent systems containing high proportions of water. For example, mixtures of D-glucose-containing oligo- and polysaccharides (with degrees of polymerization up to 35 glucose units) can be separated on silica gel 60 TLC or Si 50,000 HPTLC plates utilizing such methodology (63) (Tables 2, 3, and 6).

Solvent systems based on a mixture of acetonitrile and water show short developing times (55) and can be used on silica gel, Si 50,000, and amino-bonded layers (20). These developing systems are frequently used and give excellent results, especially when used

in combination with multiple developing techniques. With a simple variation in the water or buffer content of the solvent system, these techniques can be suitable for separation of mono- and disaccharides or for the separation of higher oligosaccharides and maltodextrins. The separation can sometimes be improved by the use of additives such as boric acid or boronic acid (64, 65), buffer solutions (66), or inorganic salts (14).

Some natural sugar derivatives need special separation conditions. Uronic acids, for instance, are best separated by using solvent systems that contain acetic, phosphoric, or hydrochloric acid (50, 51), and some amino sugars and their derivatives can be satisfactorily separated with solvent systems containing ammonia.

Glycolipids are typically resolved using solvent mixtures that contain organic solvents such as chloroform or hexane, alcohols such as methanol or isopropanol, and water. A frequently used separation system for neutral glycolipids is chloroform–methanol–water (65:25:4) and, for the charged gangliosides, chloroform–methanol–water (60:35:8).

C. Mobile-Phase Additives

The selectivity of separation of some closely related carbohydrates can be modified by mobile-phase additives. Typical additives include boric acid, phenylboronic acid, and 2-aminoethyl diphenyl borinate (NST) (64, 65, 67, 68). The reaction of polyhydroxy compounds with boric acid or boronic acids has been used for derivatization and separation of carbohydrates and other compounds containing vicinal diols by using chromatographic and electrophoretic techniques (7a, 11b, 69). The mechanism of reaction is a complex between *cis*-diol moieties and borate or boronate groups. It has been demonstrated that the borate ion, rather than boric acid, is complexed by the polyol (70, 71). The reaction is pH-dependent, and the optimum conditions are usually at pH >8.0. In a pH ranging from 8 to 12, aqueous borate solutions contain tetrahydroxyborate ions and also more highly condensed polyanions such as triborate and tetraborate. Equilibrium between the different species depends on the pH and the total borate concentration. The migration of the resulting complexes of sugars and boric or boronic acids on thin layers is dependent on their polarity. With solvent systems containing boric acid, the migration of some sugars is inhibited, whereas certain sugars have increased R_f values when separated by TLC using mobile phases containing phenylboronic acid (65). Furanoses more readily form complexes or esters than sugars in the pyranose form. Fructose (α -D-fructofuranose) reacts with boric acid or phenylboronic acid at weak acidic pH. This reaction has been exploited to enhance the selectivity of separation of glucose and fructose on silica gel and cellulose thin layers (64, 65, 67, 68) (Figs. 2 and 3). Separation is dependent on the polarity of the additive, its concentration (Fig. 4), pH, and the composition of the buffer. The concentration of the additive also influences the shape of the spots. When additives result in spots that are elongated, for example, separation can be improved by reducing the concentration of additive, by developing the plate at a subambient temperature, or by performing the separation with a more appropriate buffer.

IV. DETECTION

Carbohydrates show very low ultraviolet (UV) absorption. They can be satisfactorily visualized and evaluated on TLC plates only after suitable derivatization. The majority of chemical derivatization procedures are based on the reductive properties of carbohydrates. Reductive animation of sugars in the presence of an acid is a typical example. Methods based on reductive animation require an aldehydic reducing carbon on the saccharide that reacts with the amino group of the chromophore or fluorophore.

A. Prechromatographic Derivatization

Prechromatographic derivatization of carbohydrates is popular in elution chromatographic techniques (7a, 7b), but is rarely used in planar chromatography because of the time-consuming derivatization reactions of individual samples compared to the relatively simple postchromatographic derivatization of the whole plate, the limited number of suitable reagents, and the relatively high detection limits, which are comparable to those obtained by postchromatographic derivatization. A typical example is the derivatization of reducing sugars with dansyl- or dabsylhydrazines to the corresponding fluorescing hydrazones, followed by their separation (72). The lowest limit of visual detection of the chromophoric spot on the plate was in the range of 0.1–1.0 nmol of glucosedabsylhydrazone. This detection limit corresponds to 18–180 ng of glucose. Similar detection limits for some reducing sugars were obtained after derivatization with 4-airnno-4'-dimethylaminobenzene (73) or 4-(4-dimethylaminophenylazo)benzenesulfonylhydrazide (74). Examples of developing systems suitable for TLC analysis of sugar derivatives are given in Table 5.

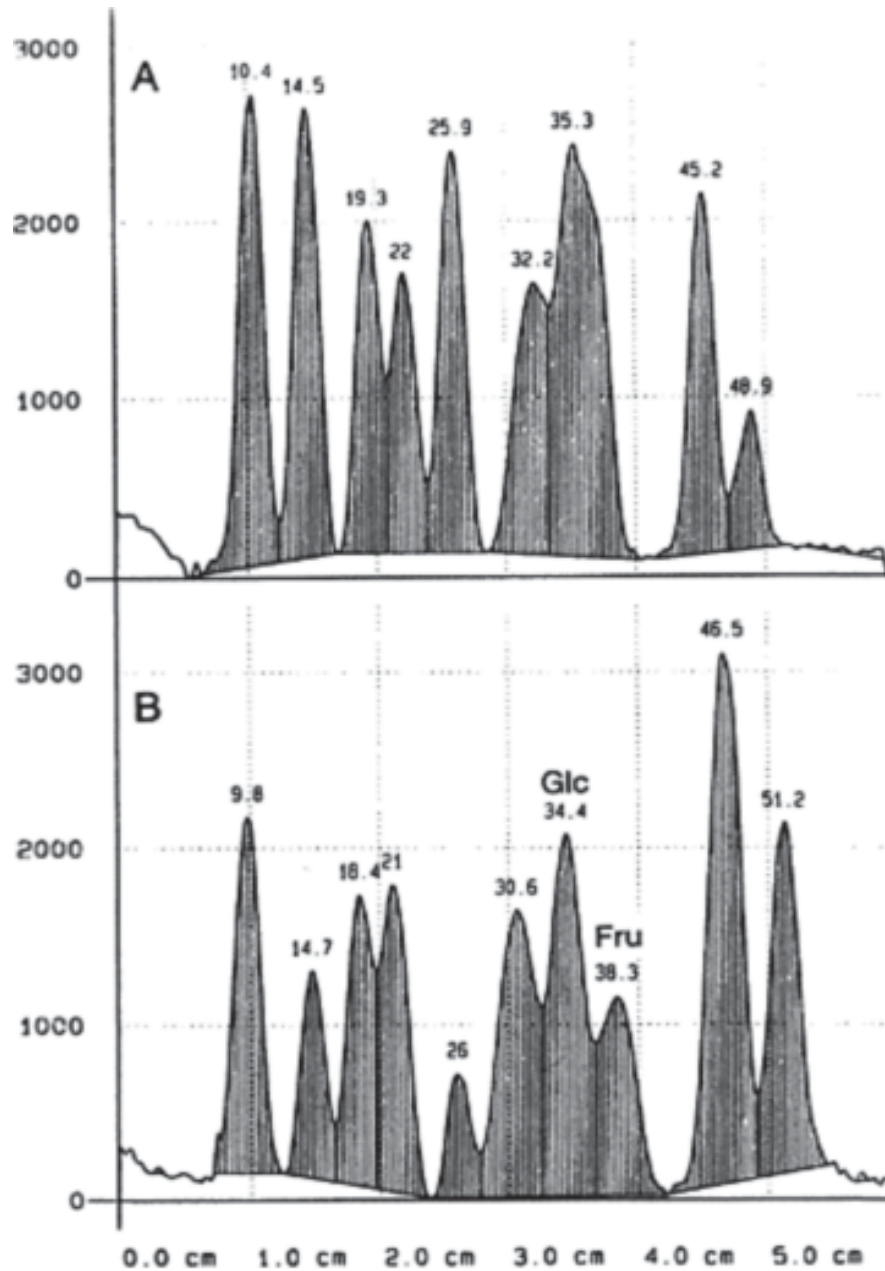


Figure 2 The influence of NST on resolution of glucose and fructose. Conditions: Silica gel HPTLC plates; acetonitrile–phosphate buffer pH 5.9 (85:15) solvent system; single development; detection by ADP reagent, transmission, 560 nm; computer-controlled scanner (Camag); Ifc-AMMP software. Standard mixture (in order of migration) Raf, Mel, Lac,

Mal, Sue, Gal, Glc, Fru, Xyl, Rha. (A)
 Solvent system without additive. (B)
 Equal solvent system+1.5 mmol/L
 (~0.05%) NST.

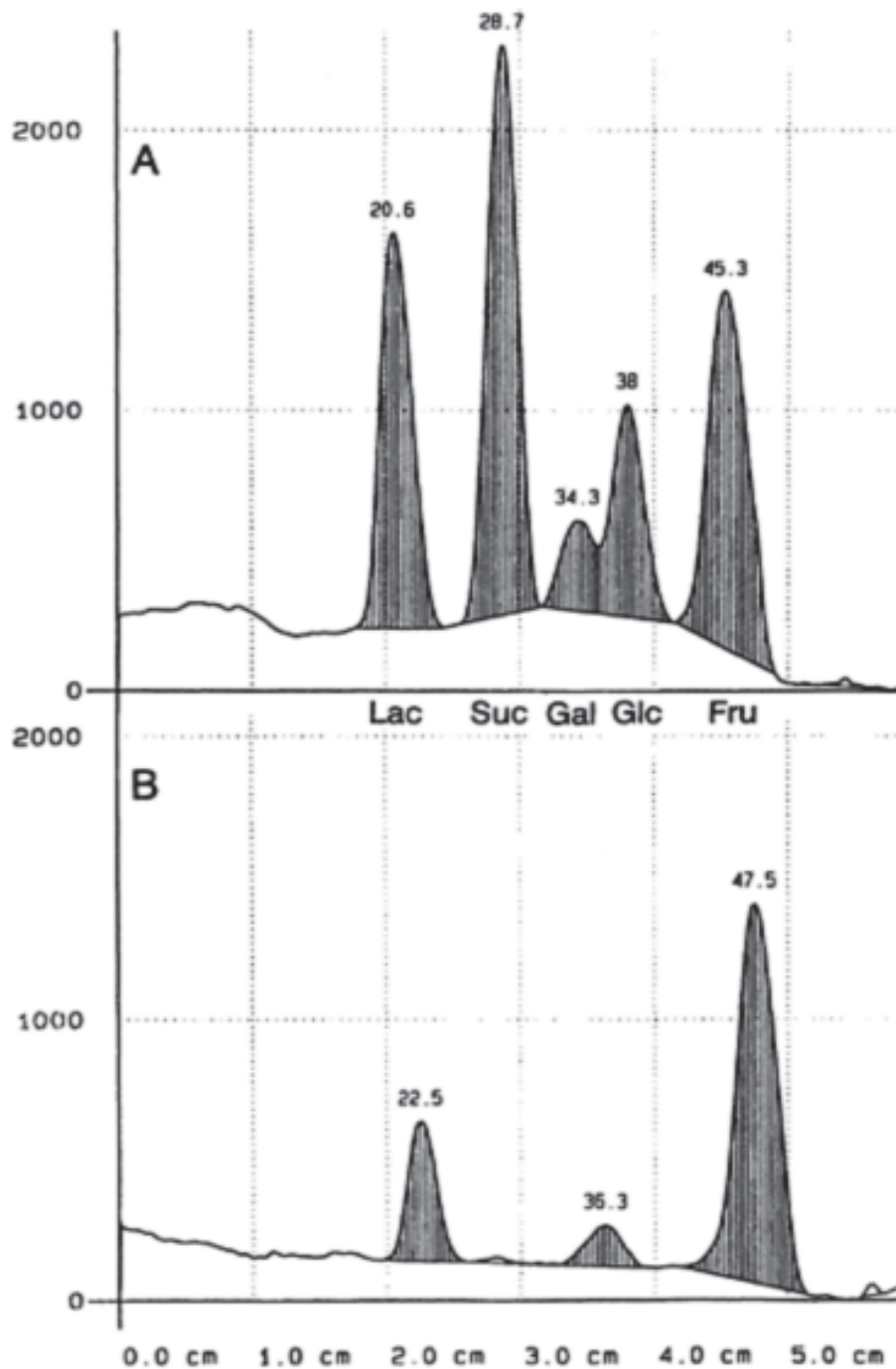


Figure 3 Sugars in a dietetic yogurt.
 Conditions: As in Fig. 1, except for

transmission at 560 nm, computer-controlled scanner (Camag); Ifc-AMMP software. (A) Standard mixture (in order of migration), Lac (0.3%), Sue (0.3%), Gal (0.1%), Glu (0.1%), Fru (0.2%). (B) Yogurt (5% in a mixture of methanol and water (80:20)).

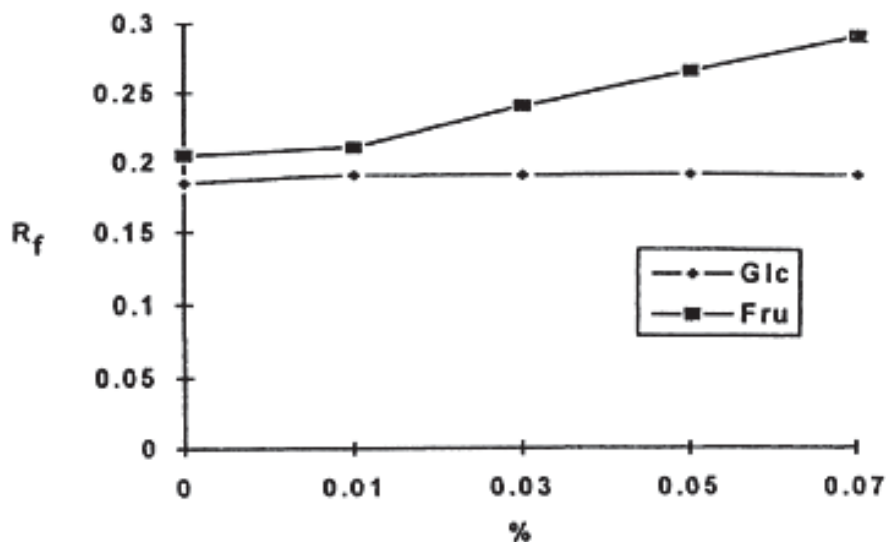


Figure 4 The influence of NST concentration on migration of glucose and fructose. Conditions: Silica gel TLC plates; acetonitrile–phosphate buffer pH 5.9 (85:15) solvent system; single development; detection by ADP, transmission, 560 nm, computer-controlled scanner (Camag); Ifc-AMMP software.

In contrast, derivatization of reducing saccharides with the highly fluorescent fluorophore 2-aminoacridone or with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and separation of the derivatives by polyacrylamide gel electrophoresis enable detection of subpicomolar quantities of the fluorescent saccharides using a cooled charge-coupled device (CCD) imaging system (10b, 11a). Undoubtedly, the development and introduction of new fluorescent labeling reagents to carbohydrate analysis are challenges for thin-layer chromatographers.

B. Postchromatographic Derivatization

The visualization of sugars on TLC plates is often performed with postchromatographic derivatization reagents. Differentiation has to be made between reducing and nonreducing sugars. Detection of nonreducing sugars is usually based on their oxidation with strong mineral acids. Ethanolic solutions of sulfuric acid, sulfuric acid alone or mixed with nitric acid or permanganate are suitable for detecting sugars at the microgram level. These reagents, although suitable for silica, should not be used on organic layers such as cellulose or polyamide.

Derivatization procedures in quantitative thin-layer chromatography include instrumental dipping of the developed and dried plate into the respective derivatization solution and activation by heating. Manual dipping or spraying of the plate with the derivatization reagent, followed by activation, is rarely used in quantitative TLC but is popular in qualitative and semiquantitative analysis. The zones usually become colored and show intense fluorescence. The fluorescence intensity can be stabilized, or enhanced, by dipping the plate into a mixture of paraffin and *n*-hexane (1:3 to 1:1) for 2 s (20, 65). Some of the most frequently used reagents for routine post-chromatographic derivatization of common sugars are presented in Table 7.

Anisaldehyde and α -naphthol are additional carbohydrate spray reagents in common use (24, 77a). Amino sugars are usually detected on ammonia-free layers with ninhydrin or with other reagents specific for amino groups such as fluorescamine, NBD chloride, or OPA-mercaptoethanol (21b). The reducing amino sugars can also be detected with silver nitrate (43) or with other reagents used for common sugars. Sugar alcohols can be detected with reagents suitable for nonreducing sugars such as 2,6-dichlorofluorescein–lead tetraacetate reagent.

Detection of glycolipids on thin-layer chromatograms is usually done using a spray reagent of orcinol in 75% sulfuric acid (31c), because this reagent does not give false-positive reactions with other lipid components. In making the reagent, the source of orcinol is important (Fisher Scientific is a recommended source). Gangliosides, negatively charged glycosphingolipids that

Table 7 Some Commonly Used Reagents for Postchromatographic Derivatization of Carbohydrates

Reagent	Carbohydrate(s)	Detection limit/spot		Layer(s)	Remarks
Aniline (2%)–diphenylamine (2%)–phosphoric acid (15%)	Mono-, di-, and oligosaccharides, maltooligosaccharides	10 ng (fluorescence)	385 nm, 436–546 (>560) nm	Silica gel, NH ₂	10–15 min at 85–120°C, scanning 20 min after derivation
Aniline–phosphoric acid	Mono- and disaccharides	10 ng; Glc, Fru 100 ng; Mal	365 nm 365–>560 nm	Silica gel, NH ₂	Reducing sugars only 45 min at 125–130°C

Aniline-phthalic acid	Mono-, di-, and oligosaccharides, oligouronic acids, methylated sugars	10 μ g	365 nm	Silica gel, alumina, cellulose, polyamide	Reducing sugars only 20–30 min at 80–130°C
4-Aminobenzoic acid	Mono- and disaccharides, uronic acids	50 ng; Lac, Fru (fluor.) 200–300 ng (Vis)	365–>560 nm	Silica gel, cellulose	15 min at 120°C
4-Aminohipuric acid–phthalic acid	Mono- and disaccharides	10 ng; Mal, Glc, Fru	365–>560	Silica gel, cellulose NH ₂ , polyamide	10 min at 140°C
4-Anisidin–phthalic acid	Mono, di-, and oligosaccharide uronic acids	10 ng; pentoses 500 ng; Fru	480 nm	Silica gel, cellulose, NH ₂ , polyamide, RP-bonded layers	10 min at 100–130°C
Anthrone	Ketoses, glycolipids	20–30 ng (absorbance) <10 ng (fluorescence)	435 nm (absorb) 436–>560 nm (fluor.)	Silica gel	8 min at 110°C
2',7'-Dichloro-fluorescein–lead tetraacetate	Mono-, di-, and oligosaccharides, sugar alcohols	<10 ng	313–>390 nm (fluor.)	Silica gel	3–30 min at 100°C
Silver nitrate–sodium hydroxide	Mono-, di-, and oligosaccharides, sugar alcohols	1 ng; ManH, SorH, XylH	530 nm	Silica gel, cellulose, cellulose acetate	1–2 min at 100°C

Source: Ref. 18.

contain sialic acid, can specifically be visualized using a resorcinol-based spray reagent. (Be sure to clamp a glass plate over the sprayed surface of the chromatogram for optimal development.)

C. Visualization by Heating

Most of the common simple sugars are visible on TLC plates under ultraviolet light or in visible light when the developed plate is heated for a few minutes. All aldoses and ketoses from the Merck sugar reference standard kit show intense fluorescence after heating at 160°C for 10 min. The fluorescing spots are visible under UV light at 366 nm. In this condition, no fluorescence is obtained with nonreducing sugars such as alcohols (e.g., mannitol, sorbitol, *meso*-erythritol, *meso*-inositol) and C₁-C₁ linked di- and oligosaccharides (e.g., sucrose, raffinose, and trehalose) (75).

By varying the temperature, it is possible to distinguish different sugars. Di- and trisaccharides are less sensitive and require higher temperatures for activation. Figure 1 shows that detection under visible light is also possible without any derivatization except heating. For each type of sugar, there is a characteristic detection temperature (76, 77b).

The application of a thermal in situ reaction is particularly useful on amino-bonded silica gel layers. Heating the developed plate to approximately 150°C for 3–4 min converts the separated sugars into stable, fluorescing derivatives with a practically unlimited life (56). Longer activation time is necessary for nonreducing di- and trisaccharides. The derivatization expires more quickly but the sensitivity increases when the developed plate is conditioned in the presence of concentrated hydrochloric acid (16). In the presence of acidic moisture, nonreducing di- and trisaccharides are most likely cleaved into more reactive monosaccharide components that undergo a Maillard reaction (16, 56).

D. Nondestructive Detection Methods

Nondestructive detection methods have been used primarily for qualitative determination of sugars, but recent developments in near-infrared (NIR) imaging detector arrays should enable the development of NIR video densitometers (78–80). Compared to derivatization reagents, detection limits achieved by this hyphenated technique are relatively high and exceed 1 μg per spot of selected sugar (80).

Visualization of sugars by iodine vapor has been popular in paper chromatography. Although this method is not very sensitive, it is nondestructive because of the short exposure time required and because the adsorbed iodine evaporates when the plate is exposed to the air (19).

Separated zones of sugars can be detected on silica gel TLC plates by immersing the developed and dried aluminum-backed silica gel plates into hexane (81). After 5 min, the silica layer appears slightly transparent whereas the sugar spots remain opaque.

More sensitive nondestructive methods for determination of selected sugars after separation include biological tests such as enzymatic reactions (82) and immunochemical detection (83). Immunostaining is particularly popular in molecular biochemistry and clinical chemistry research because of the possibility of direct detection of conjugated carbohydrates such as glycolipids (84). TLC-overlay procedures allow for the specific detection of carbohydrate moieties of glycosphingolipids using antibody binding, lectin binding, or binding to toxins such as cholera toxin or Shiga toxin (verotoxin) (85a, 85b, 85c). This detection method is analogous to Western blotting of proteins except that glycolipids are not typically blotted to a membrane but remain on the TLC plate during the entire procedure. An example of TLC overlay used in detection of glycolipid receptors (globotriaosyl ceramide) for Shiga toxin (verotoxin) in murine tissue lipid extracts is illustrated in Fig. 5. Such glycolipid immunostaining is highly specific for carbohydrate conformations; for example, Shiga toxin recognizes only glycosphingolipids that contain terminal galactose $\alpha 1$ -4 galactose residues (104). Neoglycolipids synthesized by using carbohydrates isolated from more complex glycoconjugates (105) have also been used in overlay procedures in circumstances where carbohydrate moieties are specifically investigated as potential receptors for bacteria and toxins (106).

Silica-based TLC plates must be chosen that can withstand prolonged exposure to aqueous solution. Spraying typical silica-based plates with polyisobutylmethacrylate has been reported to

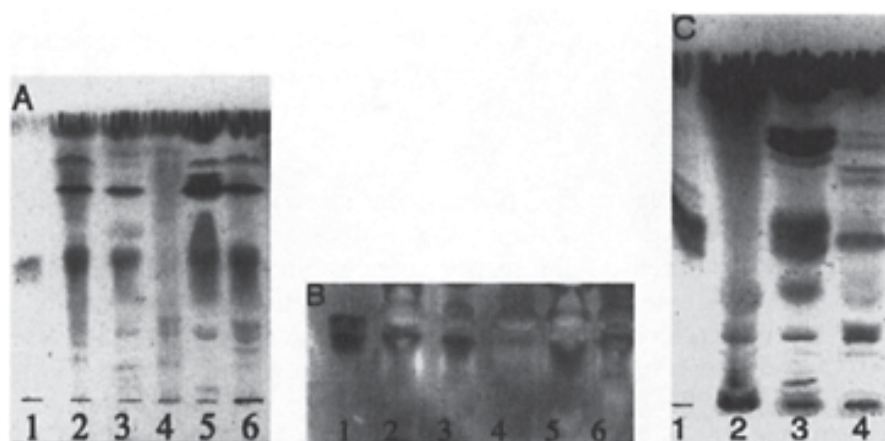


Figure 5 TLC-based analyses of lipid extracts of murine tissue to identify tissues that contain high levels of globotriaosyl ceramide (Gb3), a glycolipid receptor for Shiga toxin (verotoxin). (A) Thin-layer chromatogram of total lipid extracts from mouse (3-week-old) tissues as visualized with an orcinol spray reagent specific for carbohydrate residues. Chromatograms were developed in chloroform–ethanol–water (65:25:4). Lanes contained the following glycolipid standards and tissue extracts: 1, Gb3 standard; 2, heart; 3, testes; 4, lung; 5, brain; 6, kidney. (B) Thin-layer chromatogram overlay assay for identification of mouse tissues containing the Shiga toxin/verotoxin receptor glycolipid Gb3. Thin-layer chromatograms were developed as in (A) and allowed to air dry. Chromatograms were blocked with gelatin and incubated sequentially with Shiga toxin (verotoxin-1),

antitoxin monoclonal antibody, anti-mouse antibody conjugated to horseradish peroxidase, and chloronaphthol in TBS solution. Tissues containing Gb3 were identified by lanes containing purple bands comigrating with the Gb3 standard. (C) Thin-layer chromatogram of saponified lipid extracts of some mouse tissues developed and visualized as in (A): 1, Gb3 standard; 2, testes; 3 brain; 4, kidney. Note that orcinol staining of glycolipid bands is much clearer [compare lanes 3 and 4 with lanes 5 and 6 of (A)] when phospholipids have been removed following saponification with sodium hydroxide.

result in artifacts such as false-positive band development in overlays (107). In a similar procedure involving detection of glycosphingolipids blotted from a high-performance thin-layer chromatogram to a polyvinylidene difluoride membrane, the detection limit was confirmed to be more sensitive than detection on an HPTLC plate by typical chemical visualization and immunological staining procedures (85d).

E. TLC Coupled with Other Chromatographic and Spectrometric Analysis

A number of investigations involving carbohydrate or glycoconjugate analysis by gas chromatography or mass spectrometry have used TLC for the initial purification or separation of samples. Initially, such purification or separation from carbohydrate-containing mixtures took the form of preparative TLC for purification of carbohydrate or glycolipid bands. Some recently developed mass Spectrometric techniques, however, have the capacity to analyze samples separated by TLC directly from the thin-layer chromatograms. Some examples of such TLC use are given below.

Urashima et al. (108, 109) used a combination of gel filtration chromatography, preparative TLC, and ¹H-NMR to purify and determine the composition of oligosaccharides from milk. DeJong et al. (110) used TLC with silica gel 60 plates to detect oligosaccharides in urine as a measure of the severity of Gaucher's disease and preparative TLC prior to analysis of the sugar composition of urine by gas chromatography. Kimber et al. (77a) used HPTLC to compare the glycosphingolipid composition of untreated and retinoic acid-treated embryonic stem cells and preparative HPTLC to purify glycolipids prior to fast atom bombardment mass spectrometry. Obermeier et al. (111) analyzed oligosaccharides in milk and urine by a combination of

gel filtration chromatography, HPTLC, isotope ratio mass spectrometry (IRMS), and high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Hanisch et al. (2) used thin-layer chromatography coupled with liquid secondary ion mass spectrometry (TLCLSIMS) as part of an analytical protocol to determine the core structures of O-linked glycans isolated from mucins. In this procedure, glycan alditols derived from mucins were used to synthesize neoglycolipids, which can be separated by TLC and analyzed by IRMS directly on the thin-layer chromatogram (21a).

V. CONCLUSION

Thin-layer chromatography is a well-established method in carbohydrate analysis. It is suitable for analysis of monosaccharides and short-chain sugars, oligosaccharides, and carbohydrate polymers. Glycolipids can be analyzed as intact glycoconjugates by TLC or by TLC-immuno-overlay procedures, whereas carbohydrate moieties of other glycoconjugates must be enzymatically cleaved or digested by acid hydrolysis prior to TLC analysis. Although there may be little room for improvement on the basic uses of TLC, some unique advantages of planar chromatography, including the potential for interfacing with modern image processing systems, have not yet been fully exploited. The ability of analytical and preparative TLC to interface with more recently developed chromatographic and mass spectrometric analytical techniques ensures that TLC will have a place in carbohydrate analysis in the future.

ABBREVIATIONS

Abbreviations used in the tables and figures are as follows: Ara=arabinose, Rib=ribose, Gal = galactose, Glc=glucose, Xyl=xylose, Man=mannose, Fuc=fucose, Fru=fructose, Sor = sorbose, MeGlc=3-O-methylglucose, dGlc=2-deoxyglucose, dRib=2-deoxyribose, Sue=sucrose, Mal=maltose, Lac=lactose, Pan=panose, Nig=nigerose, Raf=raffinose, Mel = melezitose, AraH=arabinitol, XylH=xylitol, ManH=mannitol, SorH=sorbitol, Ino=inositol, Ery=erythritol, GalN=galactosamine, GlcN=glucosamine, GlcNAc=N-acetylglucosamine, ManNAc=N-acetylmannosamine, LacNAc=N-acetyllactosamine, GlcU=glucuronic acid, GalU = galacturonic acid, DP=degree of polymerization, GSL=glycosphingolipid, TLC=thin-layer chromatography (plate), HPTLC=high-performance thin-layer chromatography (plate), MD = multiple development, AMD=automated multiple development, HPPLC=high-pressure planar liquid chromatography, OPLC=overpressured planar liquid chromatography, PAGE=polyacrylamide gel electrophoresis, NST=2-aminoethyldiphenylborinate, ADP=aniline-diphenylamine-phosphoric acid reagent, aq.=aqueous, sat=saturated, sol=solution.

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17

Enantiomer Separations

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I. INTRODUCTION

Because of different biological activities on enantiomers of active ingredients, the preparation of highly enantio-pure compounds is of utmost importance (1–10). Frequently, only one of the two antipodes is pharmaceutically active, while the other may be at best inactive or even toxic.

Only about 20% of the optically active pharmaceuticals are sold as pure enantiomers (11). This has resulted in an increasing interest in stereoselective syntheses based on chiral intermediates. The production of these so-called auxiliaries ultimately requires enantiomerically pure natural substances, with optically active amino acids playing an important part as a chiral pool. Consequently, efficient analytical procedures for control of optical purity are needed to supplement modern procedures for asymmetric synthesis.

Polarimetry is used in many laboratories for control of optical purity. However, this method suffers from some well-known specific drawbacks. Furthermore, calculation of the enantiomeric excess from optical rotation is often impossible because the specific rotation of the pure enantiomer is not known precisely, or calculated enantiomeric excess values may be incorrect owing to impurities. For these reasons direct chromatographic analytical procedures are preferred.

Because simple separation techniques are not known, gas chromatographic (8, 12–16) and high-pressure liquid chromatographic (8, 17–26) and capillary electrophoresis (27) procedures are generally used for direct determination of enantiomeric composition. These systems require costly equipment; sometimes sample derivatization is necessary; and for routine applications, standardized stationary phases must be commercially available.

Application of thin-layer chromatography (TLC) separation techniques is desirable, especially with large test series. Furthermore, TLC allows simple reaction control of a synthesis—on the spot—by laboratory personnel.

The present review discusses the versatile applicability and separation mechanisms of thin-layer chromatographic enantiomeric separations. R_f values and separation conditions for numerous classes of racemic compounds are summarized in tabular form. More detailed descriptions are given for practical applications—separations of underivatized samples—on commercially available, ready-to-use plates, focusing on the thin-layer chromatographic racemate separation based on ligand exchange that was introduced in 1983 by Günther et al. (28) and on the use of a densitomer for determination of antipode distributions at trace level.

This chapter does not discuss the numerous and interesting diastereomeric separations by paper and thin-layer chromatography. We refer to the literature on amino acids (29–35), peptides, diketopiperazines (36–45), and other classes of compounds (46–55). In this context the work of Palamareva and coworkers (52–55) should be mentioned. They investigated the thin-layer chromatographic behavior of diastereomeric aliphatic compounds $\text{Ar-CH(X)-CH(Y)-Ar'}$ on silica. This group studied the behavior of different diastereomeric compounds of type $\text{RO}_2\text{C-CH(Br)-CHCO}_2\text{R}$ on alumina and silica (56) and the chromatographic separation of esters of *Z*- and *E*-2,3-diphenylcopropenoic acids (57). They used 20 computer-selected mobile phases on the basis of the Snyder theory. The application of the Snyder theory to the diastereoisomers was summarized.

Interesting separations were shown by Lippmann and Mann (58). Thin-layer and high-performance liquid chromatographic procedures for the analytical identification as well as column chromatographic methods for preparative separation of diastereomeric resorcinol-based calyx [4]arenes and for cavitands derived from these metacyclophanes were developed.

II. CHROMATOGRAPHIC METHODS OF CONFIGURATIONS. ANALYSIS

As in liquid chromatography, in thin-layer chromatography one may use one of three basic techniques for separation of enantiomeric compounds:

1. Direct separation by using chiral stationary phases, effected by the formation of diastereomeric association complexes
2. Separation on ordinary stationary phases by means of chiral additives in the eluent, which form diastereomeric complexes with the substrate
3. Separation on achiral stationary phases via diastereomeric derivatives formed by reaction of the sample with a chiral reagent

III. TLC ENANTIOMERIC SEPARATIONS BASED ON ADSORBENTS WITH CHIRAL CAVITIES

A. Resolution Mechanism

A different fit of the two enantiomers into the asymmetrical cavities—the key–lock principle—of these polymers effects separation of the antipodes. For optimal

enantioselectivity, the secondary structure of the chiral spatially fixed matrix is decisive. This type of separation is usually called inclusion chromatography.

B. Paper Chromatographic Separations

The results of paper chromatographic investigations have strongly contributed to the present understanding of chiral separation mechanisms. As early as 1951, Kotake et al. (60) studied the influence of chiral eluents on the resolution of racemic amino acids. They obtained complete separation for tyrosine-3-sulfonic acid and partial separation for tyrosine and glutamic acid. Their conclusion, "It is most reasonable to consider that these resolutions are due at least in part to the asymmetric character of cellulose," stimulated numerous interesting publications about paper chromatographic separations (61–88). Obviously stimulated by the vain attempt to separate 3,4-dihydroxyphenylalanine though similar compounds could be separated, Dalgliesh (65) postulated in 1952 the "three-point attachment model"—i.e., for successful racemate separations, at least three simultaneous interactions (π - π interactions, dipole-dipole interactions, hydrogen bondings, steric repulsion) are required. In this context we wish to mention the work of Weichert (87), who studied, among other things, the influence of the hydrogen atom at the α -carbon of different amino acids on the antipode separation. As a result of steric constraints, neither the respective racemates nor the *N*-acetylated, *N*-methylated amino acids and amino acid esters could be separated by paper chromatography. Due to the extremely long developing times, however, this separation technique is seldom used in laboratories today.

Table 1 summarizes selected applications, including separation parameters.

Paper chromatography was used for the separation of diphenylmethyl alcohols (DPMA) of type ArCH(OH)ArR by Fu et al. (89). Aqueous hexadecyltrimethylammonium, bromide (0.05

Table 1 Selected Examples of Published Paper Chromatographic Enantiomeric Separations

Compounds separated	R_f value	Eluent	Remarks	Ref.	Year
<i>d,l</i> -Glutamic acid	0.10 (<i>d</i>)/0.12 (<i>l</i>)	1-Amine–water, 4:1	Paper: No. 2, Toyo	60	1951
<i>d,l</i> -Tyrosine	0.78 (<i>d</i>)/0.75 (<i>l</i>)	1-Amine–acetic acid– water– <i>n</i> -butanol, 1:1:1:1			
<i>d,l</i> -Tyrosine-3-sulfonic acid	0.79 (<i>d</i>)/0.63 (<i>l</i>)	<i>d,l</i> -Amine–acetic acid– water– <i>n</i> -butanol, 1:1:1:1	Development time: 24– 48 h		
<i>d,l</i> - β -Naphtholbenzylamine	0.45 (<i>d</i>)/0.17	Aq. solution of D- tartaric acid	Paper: No. 1, Whatman, USA	61	1951

(l)					
			Development time: 5–15 h		
			Visualization: Wood's light		
D,L-Kynurenine	0.58 (D)/0.61 (L)	Lutidine	Paper: No. 131, Toyo	63	1951
D,L-Tyrosine-3-sulfonic acid	0.59 (D)/0.50 (L)	Lutidine and other mixtures			
D,L-Kynurenine	See Ref. 64.	Methanol– <i>n</i> -butanol–benzene–water, 4:2:2:2	Paper: No. 1, Whatman, USA	64	1951
			Development time: 10–12 h		
			Visualization: fluorescence		
D,L-2,5-Dihydroxyphenyl-alanine	0.89 Ratio of R_f	<i>n</i> -Butanol–acetic acid–water, 4:1:5	Paper: No. 4, Whatman, USA	65	1952
			Development time: 40 h		
D,L-2,3-Dihydroxyphenyl-alanine	0.91 Ratio of R_f		Visualization: ninhydrin		
D,L-3,4-Dihydroxy-2-methylphenylalanine	0.89 Ratio of R_f				
D,L-Tryptophan	No R_f values	<i>n</i> -Butanol–acetic acid–water, 4:1:5	Paper: No. 1, Whatman, USA	66	1953
			Development time: 72 h		
			Visualization: ninhydrin		
(±)-Galocatechin	See Ref. 68.	Two-way chromatograms: 1st solvent, water; 2nd solvent, butanol–acetic acid mixtures	Paper: No. 1, Whatman, USA	68	1953
			Visualization: ferric alum (0.2%, w/v) and ammoniacal AgNO ₃ (equal volumes of 0.1 N AgNO ₃ and 5 N (NH ₃ aq.))		

D,L-2,3-Dihydroxyphenyl-alanine	0.30 (L)/0.32 (D)	<i>n</i> -Butanol–water–acetic acid and other mixtures	Paper: No. 1, Whatman, USA	69	1954
			Visualization: ninhydrin		
D,L-2,4-Dihydroxyphenyl-alanine	0.29 (L)/0.31 (D)				
D,L-2,5-Dihydroxyphenyl-alanine	0.27 (L)/0.29 (D)				
Compounds separated	R_f value	Eluent	Remarks	Ref.	Year
D,L-3,5-Dihydroxyphenylalanine	0.26 (L)/0.27 (D)				
D,L-Kynurenine	0.63 (L)/0.57 (D)	Water and other mixtures	Paper: No. 4, Whatman, USA	70	1954
D,L-Tryptophan	0.53 (L)/0.55 (D)				
D,L- α - ϵ -Diaminopimelic acid	No R_f values	Methanol–water–pyridine, 77:20:10	Paper: No. 1, Whatman, USA Development time: 26 h Visualization: ninhydrin	72	1955
D,L- α - ϵ -Diaminopimelic acid	No R_f values	Methanol–water–5 N HCl pyridine, 80:17.5:2.5:10	Paper: No. 1, Whatman, USA Visualization: ninhydrin	73	1955
D,L-Histidine	Migration distance 26.3 cm (D), 29.7 cm (L)	Methanol–water, 3:1	Paper: No. 4, Whatman, USA Development time: 23.5 h	74	1955
D,L-Cystathionine	No R_f values	Methanol–water–5 N HCl–pyridine, 80:17.5:2.5:10	Paper: No. 1, Whatman, USA Visualization: ninhydrin	75	1956
D,L-Histidine D,L-Tryptophan	No R_f	Methanol–water, 9:1	Paper: 2043b, Schleicher & Schüll, Dassel, FRG Development time:	76	1957

			17–47 h		
			Visualization: ninhydrin		
(±)-Catechin	0.38 (+)/0.32 (–)	Water	Paper: 2043b, Schleicher & Schüll, Dassel, FRG	78	1961
(±)-Epicatechin	0.29 (+)/0.34 (–)				
Aporphine derivatives	See Ref. 80.	<i>n</i> -Butanol–1% formic acid, 1:2	Paper: 2043b, Schleicher & Schüll, Dassel, FRG, impregnated with 0.5 m KH ₂ PO ₄	80	1962
D,L-Cystine	See Ref. 82.	Methanol–water mixtures; influence of pH, ionic strength, temperature, etc., is explained	Paper: 2043b, Schleicher & Schüll, Dassel, FRG	82	1963
D,L-Tryptophan			Development time: >16 h		
D,L-Histidine			Visualization: ninhydrin resp. fluorescence		
D,L-Kynurenine					
D,L-Rhodommatin	0.42 (D)/0.52 (L)	Collidine– lutidine–water, 1:1:2	Paper: 2043b, Schleicher & Schüll, Dassel, FRG	83	1963
			Development time: 24–32 h		
D,L-Tryptophan	No <i>R_f</i> values	Butanol–pyridine– water, 1:1:1	Paper: No. 1, Whatman, USA	84	1965
D,L-5-Hydroxytryptophan			Visualization: Ehrlich's reagent		
D,L-6-Hydroxytryptophan					
8-Hydroxyaudanosolin and aporphine derivatives	See Ref. 85.	<i>n</i> -Butanol–1% formic acid, 1:1	Paper: 2043b, Schleicher & Schüll, Dassel, FRG, impregnated with 0.5 m KH ₂ PO ₄	85	1966
			Visualization: diazot. sulfanilic acid resp. modified Dragendorff reagent		
D,L-3-(4'-Amino-1'- naphthyl)-alanine	0.43–0.51	<i>n</i> -Butanol– pyridine–water, 1:1:1	Paper: No. 1, Whatman, USA	86	1966
			Development time: 12 h		

D,L-3-(3'-Amino-1'-naphthyl)-alanine	0.36–0.55		Visualization: ninhydrin	
D,L-3,4-Dihydroxyphenylalanine and aliphatic isocyclic and aliphatic heterocyclic amino acids	Migration distance 23 cm (L), 25 cm (D) See Ref. 87.	Methanol–water, 3:1, and other mixtures	Review: 12 different chromatographic papers Development time: 24 h (3, 4–dihydroxyphenylalanine) Visualization: ninhydrin	87 1969
D,L-Aspartic acid	0.52 (D)/0.78 (L)	Pyridine–water–amyl alcohol–Na ₂ HPO ₄ ·7H ₂ O (0.5% solution), 7:7:2:6	Paper: No. 1, Whatman, USA, im pregnated with sodium alginate-silica gel, 2:1 (w/w)	88 1974
D,L-Methionine	0.30 (D)/0.75 (L)			
D,L-Serine and other D,L-amino acids	0.55 (D)/0.00 (L)		Development time: 24 h Visualization: ninhydrin	

mol/L) as the mobile phase showed excellent results for a variety of ortho-, meta-, and para-substituted diphenylmethyl alcohols.

IV. ENANTIOMERIC SEPARATIONS ON CELLULOSE THIN-LAYER PLATES

A. Resolution Mechanism

Cellulose is a linear macromolecule composed of optically active D-glucose units with its chains arranged on a partially crystalline fiber structure with helical cavities. Separation of enantiomers is made possible by differences in the way they fit into the lamellar chiral layer structure of the support.

B. Survey of Applications of Racemic Separations

Thin-layer chromatography on cellulose can be considered a continuation of classical paper chromatography. The first investigations during the mid-1970s concentrated on a transfer of paper chromatographic racemate separations to cellulose layers with the aim of shortening development times and improving separation efficiencies. Thus, Contractor and Wragg (84) needed only 1 h for the resolution of racemic tryptophan and hydroxy analogs on cellulose CC 41 with a solvent system already known from the paper

chromatographic separation of these compounds. Antipode separation of kynurenine and derivatives can be achieved on Avicel SF-coated glass plates in about 2.3 h (90).

The research listed in Table 2 (91–108) deals mainly with separation problems concerning amino acids, amino acid derivatives, and dipeptides, focusing on the influence of the structure of the chiral support and the eluent temperature on the separation behavior of the racemates. Separation of the aromatic amino acids phenylalanine, β -2-thienylalanine, 4-fluorophenylalanine, and tyrosine could not be achieved on microcrystalline or amorphous cellulose; tryptophan isomers, however, could be reproducibly resolved on microcrystalline cellulose layers (93). Lowering the eluent temperature from 30°C to 0°C enhances enantiomeric resolution. However, developing times of 10 ± 1 h (0°C), 7.5 ± 0.5 h (10°C), 5 ± 0.5 h (20°C), and 3.5 h (30°C) have to be tolerated; hydrophobic eluent combinations further enhance separation, because they improve formation of the helical cellulose conformation (97). Separation of racemic 3,4-dihydroxyphenylalanine, tryptophan, and 5-hydroxytryptophan can be achieved in only 2 h on a cellulose HPTLC plate (99); these experiments are described in detail in Section IV.C.

Lederer and coworkers (100, 101, 104–108) investigated the influence of various salt concentrations in the mobile phase on the separation of tryptophan, methyltryptophan, and fluorotryptophan. In these experiments lithium chloride, sodium chloride, and ammonium sulfate solutions were used for the separation on native and microcrystalline cellulose, and also separations with aqueous copper sulfate and sodium chloride solutions containing α -CD were also described.

Roomi and Tsao (110) described a method for the separation of isomers of ascorbic acid and their oxidation product dehydroascorbic acid on sodium borate-impregnated silica gel and cellulose plates. Studies of additives such as metaphosphoric acid were done. The mobile phase was acetonitrile–acetone–water–acetic acid (80:5:15:2). This procedure was adopted to separate and identify ascorbic acid and its oxidation product in food products, pharmaceutical preparations, and biological tissues and fluids.

Mixtures of microcrystalline cellulose and cellulose derivatives were used by Suedee and Heard (111). For the mixtures they used triphenylcarbamates and tested the separation of *rac*-propranolol and *rac*-bupranolol. The best resolution of propranolol was obtained on cellulose tris(3, 5-dimethylphenylcarbamate) and that of bupranolol, on cellulose tris(3, 4-dichlorophenylcarbamate) with the mobile phase hexane–propan-2-ol (80:20).

Cyclohexylcarbamates of cellulose and amylose were prepared by Okamoto and coworkers (112) and were tested as the chiral stationary phase for thin-layer chromatography, showing good separation factors for various compounds such as 1-(9-anthryl)-2,2,2-trifluoroethanol, Tröger's base, and benzoïn.

Table 2 Enantiomeric Separations on Cellulose Thin-Layer Plates

Compounds separated	R_f values	Eluent	Remarks	Ref.	Year
D,L-Tryptophan	0.52 (L)/0.62 (D)	Butanol–pyridine– water, 1:1:1	Plates: Homemade (20×20 cm), cellulose powder CC41, Whatman, USA	84	1965
D,L-5- Hydroxytryptophan	0.47 (L)/0.54 (D)				
D,L-6- Hydroxytryptophan	0.36 (L)/0.41 (D)		Development time: 1 h		
			Visualization: Ehrlich's reagent		
D,L-Tryptophan	0.53 (L)/0.50 (D)	Methanol–butanol– benzene– water, 2:1:1:1, and other mixtures	Plate: Homemade (20×20 cm), microcrystalline cellulose, Avicel SF, Funakoshi, Japan	90	1967
D,L-5- Hydroxytryptophan	0.31 (L)/0.25 (D)				
D,L-Kynurenine	0.61 (L)/0.54 (D)				
D,L-3- Hydroxykynurenine	0.53 (L)/0.47 (D)		Development time: 2.3 h		
D,L-5- Hydroxykynurenine	0.26 (L)/0.20 (D)		Visualization: UV (3650 Å)		
D,L-3- Methoxykynurenine	0.62 (L)/0.55 (D)				
D,L- α - Acetylkynurenine	0.82 (L)/0.74 (D)				
d,l- Trisethylenediamine– cobalt(III) complex	0.46 (d)/0.25 (1)	Aqueous mixtures of Na,K- <i>d</i> -tartrate and AlCl ₃	Plates: Homemade (20×5 cm), microcrystalline cellulose blended with <i>d</i> -quartz or Na,K- <i>d</i> - tartrate	91	1973

			Visualization: Na ₂ S solution		
D,L-Diaminopimelic acid	0.25 (D,D)/0.37 (L,L)	Methanol–water–acetic acid, 40:10:2	Plates: Cellulose powder, Merck, FRG	92	1975
			Visualization: Ninhydrin		
D,L-Diaminoadipic acid	0.23 (D,D)/0.28 (L,L)				
D,L-Tryptophan	See Ref. 93.	Methyl ethyl ketone–2-methylpyridine–formic acid–water, 31:47:2:20, and other mixtures	Plates: Cellulose	93	1976
D,L-Fluorotryptophan derivatives			(a) Microcrystalline 4056, Merck, FRG		
D,L-Methyltryptophan derivatives			(b) MN 300, M-N, FRG		
			(c) MN 300 HR, M-N, FRG		
			Visualization: Ninhydrin		
D,L-4-Aminophenylalanine	0.40 (L)/0.45 (D)	Methanol–water, 3:1	Plates: DC-Alufolien cellulose	94	1977
D,L-Phenylalanine–4-sulfonic acid	0.71 (L)/0.74 (D)	<i>n</i> -Butanol–acetic acid–water, 1:1:1	F ₂₅₄ , Merck, Germany Development time: 1.8–4.5 h Visualization: Ninhydrin		
Compounds separated	<i>R_f</i> values	Eluent	Remarks	Ref.	Year
D,L- <i>p</i> -Tyrosine	0.81 (L)/0.45 (D)	Methanol–water, 7:3			
D,L-3,4-Dihydroxyphenylalanine	0.53 (L)/0.57 (D)	Methanol–water, 3:2			
D,L- <i>p</i> -Tyrosine-3-sulfonic acid	0.63 (L)/0.73 (D)	Methanol–water, 7:3			
D,L-Tryptophan	0.46 (L)/0.52 (D)	Methanol–water, 3:2			

D,L-5-Hydroxytryptophan	0.34 (L)/0.41 (D)	Methanol–water, 3:2			
D,L-Kynurenine and other compounds	0.47 (L)/0.38 (D)	Methanol–water, 3:2			
D,L-Tryptophan	See Ref. 95.	Ethanol–pyridine–water, 1:1:1 or 2:2:3	Plates: 10×20 cm cellulose, Avicel SF, Lot 8390, Funakoshi, Japan	95	1980
D,L-Histidine					
D,L-Phenylalanine			Development time: 6 h (25°C)		
D,L-Tyrosine			Visualization: ninhydrin		
D,L-3,4-Dihydroxyphenylalanine					
D,L-Tryptophan	0.40 (L)/0.49 (D)	Ethanol–pyridine–water, 2:3:1 or 1:1:1; other mixtures: <i>n</i> -butanol–pyridine–water, 1:1:1, ethanol–water–sat. phenol–ammonium hydroxide, 40:60:1	Plates: 10×20 cm cellulose, Avicel SF, Lot 8390, Funakoshi, Japan	96	1982
D,L-Histidine	0.13 (L)/0.11 (D)				
D,L-Phenylalanine	0.55 (L)/0.59 (D)		Development time: 11.5 h (0°C)		
D,L-Tyrosine	0.53 (L)/0.60 (D)		Visualization: ninhydrin		
D,L-3,4-Dihydroxyphenylalanine	0.43 (L)/0.50 (D)				
D,L-Cystine	0.08 (L)/0.06 (D)				
D,L-Threonine	0.56 (L)/0.51 (D)				
D,L-Asparagine and further D,L-amino acids	0.22 (L)/0.22 (D)				
D,L-Phenylalanine	See Ref. 97.	Pyridine–ethanol–water, 4:1:1	Plates: cellulose, Avicel SF, Lot 8390 (10×20 cm), Funakoshi, Japan	97	1986

D,L-Tyrosine					
D,L-3,4-Dihydroxyphenyl-alanine and other D,L-amino acids			Development time: 12 h (0°C)		
			Visualization: ninhydrin		
Dipeptides (L,L/D,D and D,L/L,D pairs)	See Ref. 98.	Pyridine–water, 2:1 or 4:1	Plates: microcrystalline cellulose, Merck, FRG	98	1986
Trp-Trp			Development time: 10 h (–10°C)		
Ala-Ala			Visualization: ninhydrin		
Phe-Phe					
Tyr-Tyr					
Lys-Ala					
Asp-Ala					
D,L-DOPA	0.58 (D)/0.53 (L)	Methanol–water, 3:2	Plates: 10×20 cm cellulose, HPTLC, Merck, FRG	99	1988
D,L-Tryptophan	0.51 (D)/0.44 (L)				
D,L-5-Hydroxytryptophan	0.40 (D)/0.32 (L)		Development time: 2 h		
			Visualization: ninhydrin		
D,L-Tryptophan	See Ref. 100, 101, 104–107.	Water; various salt solutions, e.g., LiCl, NaCl, and (NH ₄)SO ₄ solutions	Plates: microcrystalline cellulose, HPTLC, TLC, Merck, native and microcrystalline cellulose Polygram [®] CEL 300 and CEL 400, TLC, Macherey-Nagel,	100, 101, 104–107	1989–1993
D,L-Methyltryptophan					
D,L-Fluorotryptophan					
			Visualization: ninhydrin		
D,L-Tryptophan	See Ref. 108.	CuSO₄ and NaCl solutions containing α-CD	Plates: microcrystalline cellulose, HPTLC, TLC, Merck, Germany native cellulose Polygram [®] CEL 300, TLC, Macherey-Nagel, Germany	108	1994
D,L-Methyltryptophan					
D,L-Fluorotryptophan			Visualization: ninhydrin		

C. Applications

1. Separation Parameters for the Racemic Compounds Cited in Ref. 99

a. Chromatographic Conditions

Method: Ascending, one-dimensional development in a TLC chamber with chamber saturation.

Plates: Cellulose precoated HPTLC plates (Cat. No. 5786, Merck), 10×20 cm; layer thickness 0.1 mm, without fluorescent indicator.

Eluent: Methanol–water, 3:2.

Sample volume: 1 μ L of a 0.05% methanolic solution (1:1) applied as a 10 mm streak.

Length of run: 17 cm.

Time of run: 2 h.

Detection: The dried plates were immersed for 3 s in a 0.3% ninhydrin solution in acetone (Tauchfix, Baron) and then dried in a cabinet for about 4 min at 105°C. Blue-violet derivatives formed on the white background.

b. Spectroscopy

Apparatus: Chromatogram spectrometer CD 60 (Desaga, Heidelberg, Germany).

Measuring principle: Monochromator-TLC plate (reflectance).

Light source: Tungsten lamp. Wavelength 565 nm. Slit: 6×0.2 mm.

Scanning: 0.05 mm. Results: See Figs. 1 and 2.

V. ENANTIOMERIC SEPARATIONS ON MICROCRYSTALLINE TRIACETYLCELLULOSE THIN-LAYER PLATES

A. Resolution Mechanism

The resolving capability of this polysaccharide derivative is based on its morphological structure. Peracetylation of the cellulose has to be performed such that the conformation and relative position of the carbohydrate bands in their crystalline domains remain intact. In this state cellulose triacetate includes enantioselectivity; i.e., antipode separations are possible (59).

B. Survey of Applications of Racemic Separations

In 1973 Hesse and Hagel (113) for the first time described the thin-layer chromatographic racemate separation of Troeger's base on cellulose triacetate. Systemic investigations of this chiral support by Faupel (114) resulted in commercialization of a microcrystalline

triacetylcellulose plate by Antec, Bannwil. These plates are stable with aqueous eluent systems and resistant to dilute acids

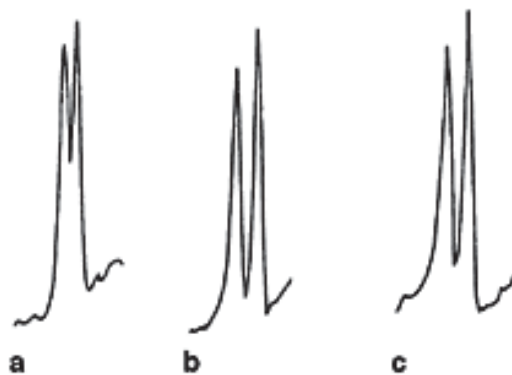


Figure 1 Remission-location curves:
(a) D,L-Dopa; (b) D,L-tryptophan; (c)
D,L-5-hydroxytryptophan.

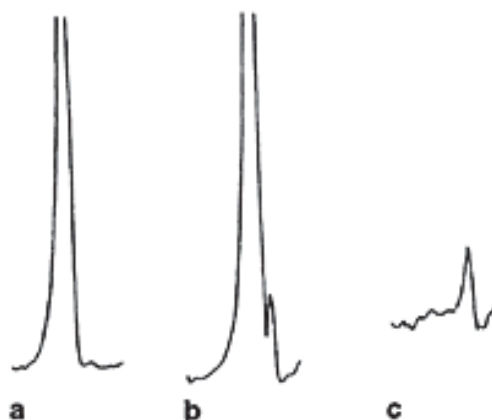


Figure 2 Remission-location curves:
(a) L-tryptophan; (b) L-tryptophan
spiked with 5% D-tryptophan; (c) 5%
D-tryptophan (applied volume 2 μ L).

and bases. They are stable in alcoholic and phenolic eluents but are attacked by glacial acetic acid and ketonic solvents. Enantiomeric separation of racemic oxindanac was first described by Faupel. Other separation examples were published (115) that used this racemate as “pilot substance” and transferred the separation conditions (114). These are described in detail in Section V.C and listed in Table 3. Günther and Merget (116) were successful in separating the pesticide ($\pm$)-2-(4-chloro-6-methylarnino-[1,3,5]-triazin-2-ylamino)-2-methylbutyronitrile on a microcrystalline tiacetylcellulose plate OPTI-T.A.C. (116).

Further separations of microcrystalline triacetylcellulose plates were done by Lepri et al. (117–122) and Wang (123). Their results are listed in Table 3. Tribenzoylcellulose

cellulose (CTB) plates were prepared from mixtures of CTB and silica gel in various proportions (124). Hexane–propan-2-ol was used as the mobile phase.

C. Applications

1. Separation Parameters for the Substances Cited in Ref. 115

a. Chromatographic Conditions

Method: Ascending, one-dimensional development in a TLC chamber without chamber saturation

Plates: OPTI-T.A.C. TLC L.254 (Cat. No. 4006, Antec, Bennwil)

Eluent: Ethanol–water, 80:20 (for oxindanac, 85:15)

Sample volume:

Oxindanac: 5 μ L of a 0.2% methanolic solution applied as a 15 mm streak

2-Phenylcyclohexanone: 10 μ L of a 1% methanolic solution applied as a 15 mm streak
(R,S)-2,2,2-Trifluoro-1-(9-anthryl)-ethanol: 1 μ L of a 0.2% methanolic solution applied as a 10 mm streak

Troeger's base: 2 μ L of a methanolic solution applied as a 15 mm streak

Length of run: 10 cm

Time of run: 1.3 h

Detection: UV (254 or 366 nm)

b. Spectroscopy

Apparatus: Chromatogram spectrometer CD 60 (Desaga, Heidelberg, Germany)

Measuring principle: Monochromator-TLC plate (fluorescence or reflectance)

Light source: Deuterium lamp or mercury lamp [for 2,2,2-trifluoro-1-(9-anthryl)ethanol]

Wavelength: λ =254 nm or λ_{exc} =366 nm, λ_{em} =420 nm (cutoff filter) (for anthryl derivative)

Slit: 6×0.2 mm

Scanning: 0.05 mm

Results: See Fig. 3.

Table 3 Enantiomeric Separations on Triacetylcellulose and Tribenzoylcellulose Thin-Layer Plates

Compounds separated	R_f value	Eluent	Remarks	Ref.	Year
(±)-Tröger's base	0.19 (+)/0.41 (-)	Ethanol	Plates: homemade (20×20 cm)	113	1973
(±)-Oxindanac benzyl ester	See Ref. 114 (3D plot).	Ethanol–water, 85:15	Plates: 20×20 cm OPTI-T.A.C. F 254, Antec, Switzerland Development time: 1.3 h Visualization: UV (254 nm)	114	1987
(±)-Oxindanac benzyl ester	0.23 (±)/0.33 (-)	Ethanol–water, 85:15	Plates: 20×20 cm, OPTI-T.A.C. F 254, Antec, Switzerland Development time: 1.3 h	115	1988
(±)-2-Phenylcyclohexanone	0.39/0.57	Ethanol–water, 80:20	Visualization: UV (254 nm) resp. fluorescence (γ_{exc} 366 nm, γ_{cm} >420 nm)		
(±)-Tröger's base	0.40 (+)/0.64 (-)	Ethanol–water, 80:20			
<i>R,S</i> -2,2,2-Trifluoro-1-(9-anthryl) ethanol	0.34 (<i>R</i>)/0.51 (<i>S</i>)	Ethanol–water, 80:20			
(±)-2-(4-Chloro-6-methylamino-[1,3,5]triazin-2-ylamino)-2-methyl butyronitrile	0.35/0.41	Ethanol–H ₂ O, 1:1	Plates: 20×20 cm, OPTI-T.A.C. F254, Antec, Switzerland Visualization: ninhydrin	116	1993
(±)1,1'-Binaphthyl-2,2'-diamine	Separation factor α =1.35	Ethanol–water, 80:20	Plates: 20×20 cm precoated acetylated cellulose Cel 300-10/AC-20; Macherey-Nagel, FRG	117	1994
(±)7,8,9,10-Tetrahydrobenzo[a]pyren-7-ol	α =1.63				
(±) γ -(2-Naphthyl) γ butyroacetone	α =1.49				
(±)1-(2-Naphthyl)ethanol	α =1.10				
Tröger's base	α =1.80	Ethanol–	See Ref. 123.	123	1997

		water, 80:20			
Methaqualone	$\alpha=1.44$	Ethanol– buffer (pH 10), 2:1			
Cholmezanone	$\alpha=1.52$	Ethanol			
Chloroquine	$\alpha=3.60$	Ethanol– water, 6:1			
Alfamethrin	$\alpha=1.37$	Alcohol– water (see Ref. 119.)	CTA–silica gel GF mixture, 3:1	119	1997
Fenprothrin	$\alpha=1.20$				
Fenoxaprop-ethyl	$\alpha=1.52$				
Taxifolin	$\alpha=1.17$				
Hesperetin	$\alpha=1.24$				
Naringenin	$\alpha=1.30$				
Flavonone	$\alpha=1.12$				
6-Methoxyflavanone	$\alpha=1.17$				
6-Hydroxyflavanone	$\alpha=1.14$				
(4 <i>S</i> ,5 <i>R</i>)/(4 <i>S</i> ,5 <i>R</i>)-4-Methyl-5-phenyl- 2-oxazolidone	$\alpha=1.58$				
(4 <i>R</i> ,5 <i>S</i>)/(4 <i>S</i> , <i>R</i>)-1,5-Dimethyl-4- phenyl-2imidazolidone	$\alpha=2.06$				
<i>trans</i> -4-Chlorostilbene oxide	$\alpha=2.00$				
2-Phenylcycloheptanone	$\alpha=2.20$				
1-(9-Fluorenyl)ethanol	$\alpha=2.24$				
<i>N</i> -Benzylproline ethyl ester	$\alpha=1.20$				
γ -Trityloximethyl)- γ -butyroacetone	$\alpha=1.08$				
2,3- <i>O</i> -Isopropylidene 1,1,4,4- tetraphenylerythritol	$\alpha=1.14$				
2-Methyl-1-indanone	$\alpha=1.33$				
3-Methyl-1-indanone	$\alpha=1.28$				
Tröger's base	$\alpha=2.64$				
<i>N</i> -tBOC-3-(2-naphthyl)-Ala	$\alpha=1.16$				
1-Acenaphthenol	$\alpha=1.28$	2-Propanol– water, 80:20	CTA–silica gel GF mixture, 3:1	122	2000

Phenoxymethyl oxirane	$\alpha=1.45$	Ethanol–water, 80:20		
4-Chloromethoxymethyl oxirane	$\alpha=1.17$			
2-Methylphenoxymethyl oxirane	$\alpha=1.13$			
Trityloxymethyl oxirane	$\alpha=1.14$			
α -Tetralol	$\alpha=1.54$	<i>n</i> -Hexane-2- propanol, 80:20	Tribenzoyl cellulose–silica, 2:1	124 2001
1-Indanol	$\alpha=1.52$		Tribenzoyl cellulose–silica, 2:1	
1-Acenaphthenol	$\alpha=1.27$		Tribenzoyl cellulose–silica, 2:1	
1-Phenyl-1,2-ethanediol	$\alpha=1.32$		Tribenzoyl cellulose–silica, 2:1	
<i>trans</i> -2-Phenyl-1-cyclohexanol	$\alpha=1.11$		Tribenzoyl cellulose–silica, 2:1	
1-Phenylethanol	$\alpha=1.10$		Tribenzoyl cellulose–silica, 1:1	
2-Phenyl-3-butyryl-2-ol	$\alpha=1.18$		Tribenzoyl cellulose–silica, 1:1	
Tröger's base	$\alpha=1.80$		Tribenzoyl cellulose, silica, 3:1	

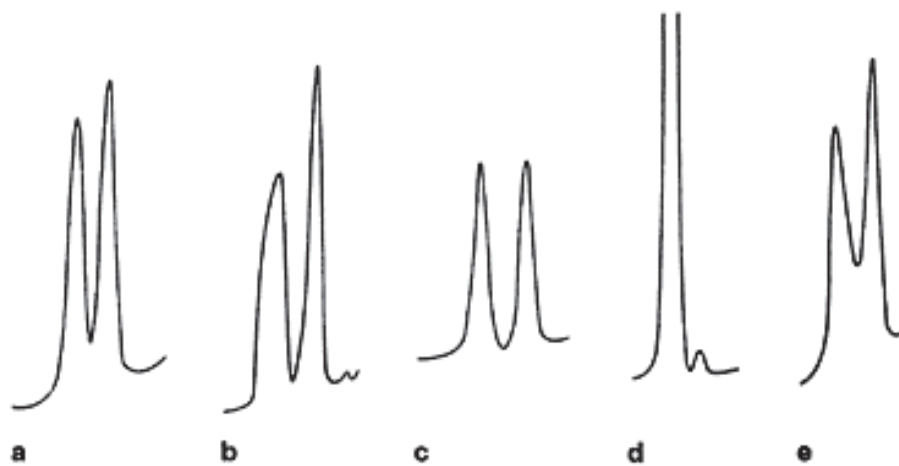


Figure 3 Remission-location curves:
 (a) D,L-Oxindanac benzyl ester; (b)
 D,L-2-phenylcyclohexanone; (c) (*R,S*)-
 2,2,2-trifluoro-1-(9-anthryl)-ethanol;
 (d) (*R*)-2,2,2-trifluoro-1-(9-anthryl)-

ethanol spiked with 1% (*S*)-
enantiomer; (e) Tröger's base.

VI. ENANTIOMERIC SEPARATION ON POLYMORPHIC FORMS OF CHITIN

α -Chitin is a polysaccharide that contains amino sugar units. Chitin is related to cellulose and is built from *N*-acetyl-D-glycosamine monomers. The hydroxy group in position 2 of D-glucose is substituted by an acetamido group. In natural form, chitin is about 90% acetylated. This sorbent is often used for the chromatographic separation of metal cations. Transition metal ions (e.g., Cu^{2+}) can be bound strongly to this type of polysaccharide. Therefore this material can be used for ligand exchange chromatography (LEG). This fact is used in this kind of enantiomeric separation method. The α -chitin is impregnated with Cu^{2+} ions by shaking it with an aqueous solution of the salt for 12 h. Then enantiomers can be separated by interacting with Cu^{2+} ions bound to the matrix.

Malinowska and Rozylo (125, 126) used this method for the enantiomeric separation of amino acids with various solvent systems, e.g., methanol, ethanol, or ternary mobile phases of methanol–water–acetonitrile mixtures. Besides many interesting separations, their results show some absurdities, because the nonenantiomeric glycine was also separated into two compounds! Also, the authors mentioned that separated amino acid enantiomers were detected in two spots with different colors. Normally separated enantiomers will show identical colors when detected on the plate. Nevertheless, chitin seems to be a very interesting sorbent for enantiomeric separation.

VII. THIN-LAYER CHROMATOGRAPHY BASED ON THE MOLECULAR IMPRINTING TECHNIQUE

Molecular imprinting is a method for the synthesis of polymers with predetermined selectivity for various compounds. This technique uses noncovalent prearrangement of functional monomers in the presence of the print molecules prior to the polymerization for the creation of highly specific binding sites. After polymerization the print molecules are washed out of the macroporous polymer matrix. The result is a polymer with recognition sites due to the shape of the print molecules. The proper arrangement of the functional groups in the polymer have the affinity for the print molecules. This fact leads to the restriction that the structure of the enantiomers, to be separated, must be very similar to those of the print molecules.

Andersson and coworkers (128) prepared a polymer using ethylene glycol dimethacrylate as a cross-linker methacrylic acid as monomer, and D- or L-phenylalanine anilide as the print molecule. The chromatographic data on the separation of D- and L-phenylalanine anilide is shown in Table 4.

Table 4 TLC Separation of D- or L-Phenylalanine Anilide on TLC Plates Containing a Stationary Phase Based on Imprinted Polymer

Print molecule	% Acetic acid ^a	R_f (L)	R_f (D)	α
L-Phenylalanine anilide	0	0.10	0.20	2.0
	5	0.10	0.35	3.5
	10	0.30	0.45	1.5
	15	0.45	0.60	1.3
D-Phenylalanine anilide	5	0.35	0.10	3.5
D,L-(<i>rac</i>)-Phenylalanine anilide	5	0.25	0.25	1.0
None	0	0.30	0.30	1.0
	5	0.65	0.65	1.0
	10	0.90	0.90	1.0
	15	1.00	0.95	1.1

^aMobile phase; acetic acid concentration in acetonitrile.

Suedee et al. used synthetic polymers imprinted with quinine as chiral stationary phases in thin-layer chromatography for the separation of ephedrine and norephedrine (129, 130), pseudo-ephedrine, isoproterenol, salbutamol, nandolol, pindolol, propranolol, and oxprenolol (131) and obtained good separation factors.

VIII. THIN-LAYER CHROMATOGRAPHY BASED ON SILICA GEL BOUND TO OPTICALLY ACTIVE POLY(METH)ACRYLIC ACID AMIDES

Mack and Kinkel (132) described optically active poly(meth)acrylic acid amide bound to silica gel layers with a binding system consisting of a mixture of carboxyl group-containing poly vinyl and acrylic acid polymers. They formed the sorbent by in situ polymerization of the optically active methacrylic acid amides in the presence of diol-, cyano-, or ammo-modified silica gel. Typical solvent systems for enantiomeric separations on such layers are various *n*-hexane–dioxane mixtures. Until recently layers of this kind could not be commercialized, so Chiralplate[®] and CHIR[®] plates (28), based on ligand exchange chromatography, are the only ready-to-use plates available on the market.

IX. SEPARATION OF ENANTIOMERS USING CHIRAL β -CYCLODEXTRIN

A. Resolution Mechanism

β -Cyclodextrin (β -CD) is a chiral, toroidal molecule consisting of seven glucose units connected via α -1,4 linkages. The enantiomers are selectively retained because they fit differently into the cavity of the oligomer.

B. Survey of Applications of Racemic Separations

Alak and Armstrong (133–136) investigated the influence of different silicas and binders on the separation behavior of β -cyclodextrin TLC plates. Besides nine racemates, three diastereomeric compounds and six structural isomers were separated. Wilson (137) impregnated silica plates with a 1% solution of β -CD in ethanol–dimethylsulfoxide (80:20 by volume); racemic mandelic acid was barely separated, and the antipode separation of β -blockers was not possible.

Bhushan and Martens (138) presented a paper concerned with methods of impregnation of thin-layer materials with a variety of reagents and the role of impregnation in enantiomeric separation. Armstrong et al. (139) were the first to describe applications of β -cyclodextrin as a chiral eluent additive for separations on reversed-phase TLC plates. The success of separation was strongly dependent on the type and quantity of modifier applied but above all on the concentration of β -CD. The low solubility of β -CD in water (0.017 M, 25°C) can be improved by addition of urea; sodium chloride stabilizes the binder of the RP plates. Compared to β -CD bonded phases, a reversed retention behavior was noticed, the D-enantiomer eluting above the L-isomer. The separation of steroid epimers and other diastereomeric classes of compounds is also possible with this technique. Hydroxypropyl and hydroxyethyl β -cyclodextrins are also suitable as chiral mobile-phase additives for thin-layer chromatographic enantiomer separations (140). Their better solubility in water and aqueous-organic eluents (compared to β -CD) enhances enantioselectivity; 0.6 M substituted β -CD has proven especially active for separation. Duncan and Armstrong (136) also described the separation of amino acids and alkaloids on different types of reversed-phase plates using the mobile phase acetonitrile–water containing maltosyl- β -cyclodextrin. The preferred TLC plate was the ethyl-modified one because a greater number of compounds were separated using this type of plate.

Lepri et al. (141–143) and Duncan and Armstrong (144) investigated the chromatographic behavior of dansyl-, dinitrophenyl-, and β -naphthyl-substituted amino acids and alkaloids on layers of partially C-18 modified silica with aqueous-organic solutions containing β -cyclodextrin as chiral agent. Also, the influence of the concentration of urea in the eluent was studied.

All applications including separation parameters are summarized in Table 5.

As mentioned before, cyclodextrins (CDs) are often used as mobile-phase additives (146–153), and interesting results using microcrystalline cellulose as thin layers (146, 147) have been obtained.

Bhushan and coworkers (154, 155) achieved the resolution of ($\pm$)-atenolol, ($\pm$)-propranolol, and ($\pm$)-metoprolol into their enantiomers on silica gel plates impregnated with optically pure L-lysine (0.5%) and L-arginine (0.5%) as chiral selector. They also performed good separations of 2-arylpropionic acids on (–)-brucine-impregnated silica gel plates (156). Also, ammonium-D-10-camphorsulfonates were used for enantiomeric separations. Huang et al. (157, 158) showed separations of propranolol, propafenone, pindolol, and atenolol with good separation factors. Methylene chloride–methanol in various ratios with 8.8 mM ammonium-D-10-camphorsulfonate as chiral ion-interaction agent was used as the mobile phase.

Another strategy is to use cyclodextrin as chiral information in the separation system with β -CD bonded stationary phases. Deng and coworkers (159–161) prepared a phenylcarbamate-substituted β -CD bonded stationary phase and separated a large number of binaphthalene derivatives on this layer using petroleum ether–ethyl acetate–methanol mixtures as the mobile phase.

X. DIRECT SEPARATION OF ENANTIOMERS ON TLC PLATES COATED WITH CHIRAL COMPOUNDS

Standardized commercial TLC plates are essential for routine handling of large sample volumes. “Homemade” layers usually do not meet the quality requirements of modern analysis. However, they often contribute substantially to the understanding of chiral separation principles (162–185). It is not the purpose of this chapter to present a detailed description of layer preparations; we refer to the separation examples listed in Table 6. In this context the published works of Lepri et al. (176–182) and Armstrong and Zhou (1985) are worth mentioning.

Lepri and coworkers investigated the chromatographic behavior of racemic dinitropyridyl, dinitrophenyl, dinitrobenzoyl, and 9-fluorenylmethoxycarbonyl amino acids, tryptophanamides, lactic acid derivatives, and unusual enantiomers such as binaphthols on reversed-phase TLC plates developed with aqueous-organic mobile phase containing bovine serum albumin (BSA) as chiral agent. More than 75 racemates were separated in these experiments with planar chromatography using BSA in the mobile phase. BSA showed enantioselectivity toward racemates with structures

Table 5 TLC Separation of Enantiomers Using Chiral β -Cyclodextrin (β -CD)

Compounds separated	R_f values	Eluent	Remarks	Ref.	Year
D,L-Dansyl-leucine	0.49 (D)/0.66 (L)	Methanol–1% triethylammonium acetate (pH 4.1), 40:60	Plates: homemade, 5×20 cm, β -CD bonded through a spacer to Macherey-Nagel silica gel plus Astec “All solvent binder”	133	1986
D,L-Dansyl-methionine	0.28 (D)/0.43 (L)	Methanol–1% triethylammonium acetate (pH 4.1), 25:75			
D,L-Dansyl-alanine	0.25 (D)/0.33 (L)	Methanol–1% triethylammonium acetate (pH 4.1), 25:75	Visualization: fluorescence, nin hydrin, visible		
D,L-Dansyl-valine	0.31 (D)/0.42 (L)	Methanol–1% triethylammonium acetate (pH 4.1), 25:75			
D,L-Alanine- β -naphthylamide	0.16 (D)/0.25 (L)	Methanol–1% triethylammonium acetate (pH 4.1), 30:70			
D,L-Methionine- β -naphthylamide	0.16 (D)/0.24 (L)	Methanol–1% triethylammonium acetate (pH 4.1), 30:70			
($\pm$)-1-Ferrocenyl-1-methoxyethane	0.31 (–)/0.42 (+)	Methanol–1% triethylammonium acetate (pH 4.1), 90:10			
($\pm$)-1-Ferrocenyl-2-methoxyethane	0.33 (–)/0.39 (+)	Methanol–1% triethylammonium acetate (pH 4.1), 90:10			
($\pm$)-S-(1-Ferrocenyethyl)-thioglycolic acid	0.37 (–)/0.44 (+)	Methanol–1% triethylammonium acetate (pH 4.1), 90:10			
D,L-Mandelic acid	See Ref. 137.	Methanol–water, 1:1	Plates: silica gel doubly coated with β -CD Visualization: UV (254 nm)	137	1986
Dansyl-D,L-leucine	0.30 (L)/0.35 (D)	Acetonitrile–0.151 M β -CD, 30:70	Reversed-phase plates: 5×20 cm and 20×20 cm, KC18F, Whatman, USA	139	1988
Dansyl-D,L-valine	0.36 (L)/0.43 (D)	Acetonitrile–0.151 M β -CD, 30:70	Chiral eluent additive: β -CD. Adv. Sep. Technol., USA		
Dansyl-D,L-	0.34	Acetonitrile–0.151 M			

methionine	(L)/0.38 (D)	β -CD, 30:70	
Dansyl-D,L-glutamic acid	0.65 (L)/0.72 (D)	Methanol–0.163 M β -CD, 35:65	Solutions also contain urea and NaCl
Dansyl-D,L- α -amino- <i>n</i> -butyric acid	0.42 (L)/0.47 (D)	Acetonitrile–0.151 M β -CD, 30:70	Development time: 6–8 h Visualization: UV (254 nm), resp. TLC scanner ^a (254, 280, and 230 nm)
Dansyl-D,L-norvaline	0.32 (L)/0.34 (D)	Acetonitrile–0.151 M β -CD, 30:70	

Compounds separated	R_f values	Eluent	Remarks	Ref.	Year
Dansyl-D,L-norleucine	0.24 (L)/0.28 (D)	Acetonitrile–0.151 M β -CD, 30:70			
Dansyl-D,L-phenylalanine	0.35 (L)/0.39 (D)	Acetonitrile–0.151 M β -CD, 30:70			
Dansyl-D,L-serine	0.41 (L)/0.47 (D)	Acetonitrile–0.133 M β -CD, 20:80			
Dansyl-D,L-aspartic acid	0.64 (L)/0.70 (D)	Acetonitrile–0.133 M β -CD, 25:75			
Dansyl-D,L-tryptophan	0.43 (L)/0.45 (D)	Acetonitrile–0.231 M β -CD, 35:65			
Dansyl-D,L-threonine	0.42 (L)/0.51 (D)	Methanol–0.151 M β -CD, 30:70			
Mephentyoin	0.32/0.38	Methanol–0.308 M β -CD, 35:65			
($\pm$)- <i>S</i> -(1-Ferrocenyl-2-methylpropyl)thioethanol	0.42/0.51	Acetonitrile–0.125 M β -CD, 15:85			
($\pm$)- <i>S</i> -(1-Ferrocenylethyl)-thiophenol	0.38/0.42	Acetonitrile–0.151 M β -CD, 30:70			
<i>N'</i> -Benzylornicotine	0.29/0.34	Methanol–0.200 M β -CD, 60:40 (1% aqueous triethylammonium acetate, pH 7.1)			
<i>N'</i> -(2-Naphthylmethyl)-nornicotine	0.190/0.24	Methanol–0.200 M β -CD, 60:40 (1% aqueous			

			triethylammonium acetate, pH 7.1)		
(±)-2-Chloro-2-phenylacetyl chloride	0.02/0.07	Acetonitrile–0.151 M β - CD, 30:70			
D,L-Alanine-2-naphthyl-amide HCl	0.59/0.66	Methanol–0.163 M β -CD, 35:65			
(1 <i>R</i> ,2 <i>S</i> ,5 <i>R</i>)-(–)Menthyl-(<i>S</i>)- and (1 <i>S</i> ,2 <i>R</i> ,5 <i>S</i>)-(+)-menthyl-(<i>R</i>)- <i>p</i> - toluenesulfinate	0.06/0.08	Acetonitrile–0.151 M β - CD, 30:70			
(±)2,2'-Binaphthyl-diyl- <i>N</i> - benzylmonoaza-16-crown-5	0.05/0.08	Methanol–0.265 M β -CD, 60:40			
Dansyl-D,L-leucine	3.6 (R_s)	0.4 M HP- β -CD, acetonitrile– water, 30:70	Reversed-phase plates: 5×20 cm and 20×20 cm, KC18F, Whatman, USA Chiral eluent additive: hydroxy-propyl- β - cyclodextrin (HP- β -CD), Consortium für Elektro- chemische Industrie, Germany	140	1988
Dansyl-D,L-valine	1.3 (R_s)	0.4 M HP- β -CD, acetonitrile–water, 30:70			
Dansyl-D,L- methionine	1.6 (R_s)	0.4 M HP- β -CD, acetonitrile– water, 30:70			
Dansyl-D,L- threonine	0.9 (R_s)				
Dansyl-D,L- phenylalanine	1.3 (R_s)	0.4 M HP- β -CD, acetonitrile– water, 35:65	Visualization: UV (254 nm)		
Dansyl-D,L- norleucine	1.8 (R_s)	0.4 M HP- β -CD, acetonitrile– water, 30:70			
D,L-Methionine- β - naphthylamide	1.8 (R_s)	0.3 M HP- β -CD, acetonitrile– water, 35:65			
Mephénytoin	2.0 (R_s)	0.4 M HP- β -CD, acetonitrile– water, 30:70			
5-(4- Methylphenyl)-5- phenylhydantoin	0.8 (R_s)	0.3 M HP- β -CD, acetonitrile– water, 35:65			
<i>R,S</i> -Benzyl-2- oxazolidinone	0.9 (R_s)	0.3 M HP- β -CD, acetonitrile– water, 35:65			
D,L-Alanine- β - naphthylamide	0.71/0.66	0.4 M maltosyl- β - CD 0.6 M NaCl	Reversed-phase plates: 5×20 cm, KC2F, Whatman, USA	136	1990

D,L-Methionine- β -naphthylamide	0.39/0.34	acetonitrile–water, 30:70			
<i>N'</i> -(2-Naphthylmethyl)-nornicotine	0.05/0.30		Maltosyl- β -CD, Sigma, USA Visualization: UV (254 nm)		
Dansyl amino acids	See Ref. 141.	0.1 M β -CD, saturated aqueous solution of urea containing 3.5% NaCl	Reversed-phase plates: 10×10 cm, Nano-SIL C18–50/UV, Macherey-Nagel, Germany	141	1990
DNP-D,L-amino acids		0.1 M β -CD, aqueous solution of urea (20%) containing 3% NaCl–acetonitrile, 80:20	5×20 cm, KC18F, Whatman, USA Visualization: UV (254 nm)		
MTH-D,L-Phenylalanine	0.33/0.40	0.1 M β -CD aqueous solution of urea (20%) containing 3% NaCl–acetonitrile, 80:20	Reversed-phase plates: 10×10 cm, Nano-SIL C18–50/UV, Macherey-Nagel, FRG Visualization: UV (254 nm)	142	1990
MTH-D,L-Tyrosine					

Compounds separated	R_f values	Eluent	Remarks	Ref.	Year
D,L-Methionine-2-naphthylamide	See Ref. 143.	0.15 M β -CD, aqueous solution of urea (26%) containing 2.5% NaCl–acetonitrile, 80:20	Reversed-phase plates: 10X10 cm, Nano-SIL C18-50/UV, Macherey-Nagel, Germany β -CD, Sigma, USA Visualization: UV (254 nm)	143	1991
D,L-Leucine-2-naphthylamide					
D,L-Leucine- <i>p</i> -nitroanilide					
Dansyl-D,L-alanine	0.47 (D)/0.40 (L)	Methanol–0.2 M β -CD, 35:65	Reversed-phase plates: 5×20 cm, KC18F, Whatman, USA	148	1993
Dansyl-D,L- <i>allo</i> -isoleucine	0.38 (D)/0.30 (L)	Acetonitrile–0.2 M β -CD, 32:68			
Dansyl-D,L-isoleucine	0.40 (D)/0.33 (L)		β -CD, Aldrich, USA		
Dansyl-D,L-asparagine	0.69 (D)/0.60 (L)	Acetonitrile–0.2 M β -CD, 20:80	Visualization: UV (254 nm)		

	(L)				
Dansyl-D,L-arginine	0.65 (D)/0.55 (L)				
Dansyl-D,L-citrulline	0.63 (D)/0.54 (L)				
Dansyl-D,L-glutamine	0.66 (D)/0.57 (L)				
Dansyl-D,L-histidine	0.64 (D)/0.58 (L)				
Dansyl-D,L-cystine	0.42 (D)/0.37 (L)	Methanol-0.2 M β - CD, 55:45			
Dansyl-D,L-lysine	0.39 (D)/0.35 (L)	Methanol-saturated β - CD, 60:40			
Dansyl-D,L-ornithine	0.40 (D)/0.35 (L)				
Dansyl-D,L-tyrosine	0.26 (D)/0.23 (L)				
Dansyl-N-methyl-D,L- valine	0.28 (D)/0.24 (L)	Methanol-0.2 M β - CD, 50:50			
Dansyl-D,L-proline	0.41 (D)/0.39 (L)				
Tyrosine	α =1.21	Methanol-formic acid-0.2 M	Homemade cellulose plates with MN300 cellulose, Macherey- Nagel, FRG	148	1996
3,4- Dihydroxyphenylalanine (Dopa)	α =1.15	α -CD solution of urea, 7:1:2			
<i>p</i> -Hydroxyphenylglycine	α =1.40				
Thyronine	α =1.14				
<i>p</i> -Aminophenylalanine	α =1.12				
Epinephrine	α =1.12				
Isopropylepinephrine	α =1.00				

Phenylalanine	$\alpha=1.00$				
Tryptophan	$\alpha=1.00$				
Arginine	$\alpha=1.44$	Acetonitrile–water, 1:2.5 1.5:2	Silica plastic foils: Kieselgel 60F, Merck, FRG	149	1995
Citrulline	$\alpha=1.20$				
Glutamine	$\alpha=1.35$	1.5:2	Chiral mobile-phase additive was 2- <i>O</i> -([<i>R</i>]-2-hydroxy- propyl]- β -CD		
Histidine	$\alpha=1.43$	1:2.5			
Lysine	$\alpha=1.56$	1:2.5			
Valine	$\alpha=1.30$	1.5:2			
Alanine	0.47/0.53	Methanol–0.2 M β - CD, 2:3	C18-silica plates LKC18, What-man, USA	153	2000
α -Amino- <i>n</i> -butyric acid	0.39/0.46				
Norvaline	0.26/0.32				
Norleucine	0.18/0.23				
α -Aminocaproic acid	0.12/0.17				
Phenylglycine	0.21/0.24				
Phenylalanine	0.22/0.27				
Homophenylalanine	0.21/0.28				
Isoleucine	0.25/0.32				
<i>allo</i> -Isoleucine	0.22/0.30				
Leucine	0.24/0.33				
<i>tert</i> -Leucine	0.35/0.47				
Valine	0.32/0.41				
Allylglycine	0.31/0.37				
<i>N</i> -Methylleucine	0.24/0.23	Acetonitrile–0.2 M β -CD, 35:65			
<i>N</i> -Methylvaline	0.31/0.29				
Serine	0.59/0.67	Methanol–0.2 M β - CD, 3:7			
<i>O</i> -Methylserine	0.45/0.50				
Tyrosine	0.51/0.57	Methanol–0.175 M β -CD, 2:3			
<i>O</i> -Methyltyrosine	0.26/0.30				

(±)-Atenolol	3 (-)/10 (+)	Acetonitrile– methanol, 16:4	Silica gel plates containing 10% L-lysine	155	1998
(±)-Propranolol	4 (-)/13 (+)	15:2			
(±)-Metoprolol	11 (-)/15 (+)	15:4			
(±)-Atenolol	8 (-)/12 (+)	15:5	Silica gel plates containing 10% L-arginine		
(±)-Propranolol	7 (-)/26 (+)	15:3			
(±)-Metoprolol	6 (-)/17 (+)	15:3			
Propranolol	α =1.31	Dichloromethane– methanol, 50:50	Homemade silica plates: silica gel GF, Qingdao Haiyong Chemical Factory, China	157	1997
Propafenone	α =1.22	50:50			
Pindolol	α =1.30	60:40			
Atenolol	α =2.00	70:30 (all with 6.8 mM CSA)			

^aCSA=ammonium-D-10-camphorsulfonate.

Table 6 Direct Enantiomeric Separations on Chiral TLC Phases and Resolution Techniques Using a Chiral Eluent Additive

Compounds separated	R_f value	Separation technique	Ref.	Year
(±)-Ephedrine	0.34 (-)/0.55 (+)	Plates: mixture of silica gel G and D-galacturonic acid bound to the plate (10×20 cm) Eluent: isopropanol–1 M aq. D-galacturonic acid, 94:3	162	1967
(±)-Ephedrine	0.30 (-)/0.45 (+)	Plates: 10×20 cm, aluminum oxide G, Merck, Germany Eluent: isopropanol–1 M aq. D-galacturonic acid, 94:3		
(±)-Ephedrine	0.28 (-)/0.48 (+)	Plates: 10×20 cm, silica gel G, Merck, Germany Eluent: isopropanol–1 M aq. D-galacturonic acid		

		94:3		
		Visualization: iodine, potassium iodide–potassium iodo-platinate		
<i>R,S</i> -2,2,2-Trifluoro-1-(9-anthryl)-ethanol	0.59 (–)/0.49 (+)	Plates: slides coated with γ -aminopropyl silanized silica gel (Zorbax, DuPont, USA), then impregnated with (<i>R</i>)- <i>N</i> -(3, 5-dinitrobenzoyl)phenylglycine	163	1983
		Eluent: hexane–isopropanol, 9.5:1		
<i>N</i> -3,5-Dinitrobenzoyl-D,L-valine-methyl ester	0.51 (D)/0.38 (L)	Plates: <i>N</i> -(1 <i>R</i> ,3 <i>R</i>)- <i>trans</i> -chrysanthemoyl-L-valine chemically bonded onto γ -aminopropyl silanized silica gel	164	1985
		Eluent: hexane–1,2-dichloroethane–ethanol, 50:20:1		
		Visualization: UV (254 nm)		
PTH-D,L-methionine	0.16 (D)/0.83 (L)	Plates: slurry of silica gel (Merck, FRG) and (+)-tartaric acid spread on the plate (20×20 cm)	165	1987
PTH-D,L-phenylalanine	0.15 (D)/0.85 (L)			
PTH-D,L-tryptophan	—/0.95 (L)	Eluent: chloroform–ethyl acetate–water, 28:1:1		
PTH-D,L-valine	0.21 (D)/0.80 (L)	Development time: 0.5 h		
PTH-D,L-isoleucine	0.15 (D)/0.92 (L)	Visualization: iodine vapor		
PTH-D,L-tyrosine	0.16 (D)/0.95 (L)			
PTH-D,L-threonine	0.30 (D)/0.85 (L)			
PTH-D,L-alanine	0.12 (D)/0.55 (L)			
PTH-D,L-serine	0.10 (D)/0.84 (L)			

D,L-Methionine	0.18 (D)/0.29 (L)	Plates: slurry of silica gel (Merck, Germany) and (–)-brucine brought to pH 7.1 with 0.1 N NaOH and spread on the plate (20×20 cm)	166	1987
D,L-Phenylalanine	0.27 (D)/0.40 (L)			
D,L-Tryptophan	0.17 (D)/0.31 (L)			
D,L-Tyrosine	0.22 (D)/0.29 (L)	Eluent: butanol–acetic acid–chloroform, 3:1:4		
D,L-Threonine	0.16 (D)/0.29 (L)	Development time: 0.5 h		
D,L-Alanine	0.18 (D)/0.53 (L)	Visualization: ninhydrin		
D,L-Serine	0.12 (D)/0.50 (L)			
D,L-Valine	—/0.25 (L)			
D,L-Isoleucine	0.16 (D)/0.35 (L)			
(±)-Hexobarbital	0.65/0.70	Plates: ionic and covalent bonded <i>N</i> -(3, 5-dinitrobenzoyl)- <i>R</i> -(–)- α -phenylglycine or <i>N</i> -(3,5-dinitrobenzoyl)-L leucine on precoated HPTLC NH ₂ F ₂₅₄ (Merck, Germany)	167, 168	1989
(±)-Oxazepam	0.20/0.23			
(±)-Lorazepam	0.20/0.23			
(±)-Propranolol	0.40/0.43			
(±)-Atenolol	0.11/0.14	β -Amino alcohols were dissolved in dichloromethane and shaken with 1-isocyanatonaphthalene		
(±)-Metoprolol	0.15/0.33	Eluent: mixtures of hexane–2-propanol (see Ref. 167, 168.)		
3,5-Dinitroanilyl-ibuprofen	0.45 (<i>R</i>)/0.28 (<i>S</i>)		169	1989
3,5-Dinitroanilyl-naproxen	0.24 (<i>R</i>)/0.15 (<i>S</i>)			

3,5-Dinitroanilyl-fenoprofen	0.33/0.23			
3,5-Dinitroanilyl-flurbuprofen	0.33/0.23			
3,5-Dinitroanilyl-benoxaprofen	0.30/0.20			
3,5-Dinitrobenzoyl- α -methyl-benzylamine	0.33 (<i>S</i>)/0.25 (<i>R</i>)			
3,5-Dinitrobenzoyl-tocoinide	0.37/0.31			
Alprenolol	See Ref. 170.	Plates: Diol F254 HPTLC plates, Merck, Germany	170	1989
Propranolol		Eluent: dichloromethane containing 0.4 mM ethanolamine and 5 mM <i>N</i> -carbobenzyglycyl-L-proline Visualization: UV (280 or 300 nm)		
Phenylpropanolamine	0.06/0.26	Plates: HPTLC silica gel plates HP-KF, Whatman, USA	172	1990
Octopamine	0.15/0.33	Eluents: methylene chloride-methanol, 75:25+different amounts of <i>N</i> -benzoxycarbonyl-alanyl-L-proline (ZAP), <i>N</i> -benzoxycarbonyl-isoleucyl-L-proline (ZIP), <i>N</i> -benzoxycarbonyl-L-proline (ZP), <i>N</i> -benzoxycarbonyl-glycyl-L-proline (ZGP), (<i>IR</i>)-(-)-ammonium-10-camphorsulfonate (CSA) or/and triethylamine (TEA) (see Ref. 172.)		
Pindolol	0.07/0.12			
Norphenylephedrine	0.05/0.26			
Propranolol	0.08/0.20			
Isoproterenol	0.14/0.38			
Metoprolol	0.11/0.17			

Compounds separated	R_f value	Separation technique	Ref.	Year
Timolol	0.26/0.51	HPTLC Diol F254 plates, Alltech, USA Eluents: methylene chloride-isopropanol, 95:5+different amounts of <i>N</i> -benzoxycarbonyl-alanyl-L-proline (ZAP), <i>N</i> -benzoxycarbonyl-isoleucyl-L-proline (ZIP), <i>N</i> -benzoxycarbonyl-L-proline (ZP), <i>N</i> -benzoxycarbonyl-glycyl-L-proline (ZGP), (<i>IR</i>)-(-)-ammonium-10-camphosulfonate (CSA) or/and triethylamine (TEA) (see Ref. 172.) Visualization: UV (254 nm) Development time: 30–45 min. (3.5 cm)		

(<i>R,S</i>)-3,5-Dinitro- <i>N</i> -(1-phenylethyl)benzamide	0.47 (<i>S</i>)/0.31 (<i>R</i>)	Plates: HPTLC plate NH ₂ F254s, Merck, Germany chemically bonded with (<i>R</i>)-1-(α -naphthyl)ethylaminocarbonyl-(<i>R</i>)-valine in presence of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline or carbonyl-diimidazol	173	1990
(<i>R,S</i>)-3,5-Dinitrophenyl-1-phenyl-ethylcarbaminoacid ester	0.50/0.36			
(<i>R,S</i>)-Ibuprofen-3,5-dinitroanilide	0.52/0.41	Eluent: hexane–dichloromethane–ethanol, different ratios (see Ref. 173.) Visualization: UV (254 and 366 nm)		
($\pm$)-2,2,2-Trifluoro-(9-anthryl)-ethanol (<i>R,S</i>)-($\pm$)-1,1'-bi-2-naphthol	See Ref. 174.	Plates: HPTLC plate NH ₂ (Merck, FRG) impregnated with a 0.05 M solution of <i>N</i> -(3, 5-dinitrobenzyl)-L-leucine Eluent: hexane–isopropanol, 80:20 Development distance: 8 cm	174	1991
($\pm$)-2,2'-Dihydroxy-1,1'-binaphthyl	08.3 (+)/0.88 (-)	Plates: HPTLC plate NH ₂ F254, Merck, FRG impregnated with <i>R</i> -(-)-4-trichloromethyl-2-oxetanone Eluent: trichloromethane Development distance: 7.5 cm	175	1991
Dinitrophenyl-D,L-norleucine	0.25/0.63	Plates: RP-18W/UV ₂₅₄ , 10×10 cm, Macherey-Nagel, Germany	176	1992
Dinitrophenyl-D,L-leucine	0.45/0.70			
Dinitrophenyl-D,L-methionine	0.31/0.56	Eluent: water containing 2% isopropanol and various percentages BSA (see Ref. 176.)		
Dinitrophenyl-D,L-phenylalanine	0.28/0.61			
2,4-Dinitrophenyl-D,L-norleucine	0.31/0.63	Development time: 1–2 h		
2,4-Dinitrophenyl-D,L-leucine	0.28/0.54	Visualization: UV (254 nm)		
2,4-Dinitrophenyl-D,L-norvaline	0.40/0.89			
2,4-Dinitrophenyl-D,L-methionine	0.28/0.61			
2,4-Dinitrophenyl-D,L-methionine sulfone	0.44/0.50			
2,4-Dinitrophenyl-D,L-methionine sulfoxide	0.27/0.34			

3,5-Dinitrobenzoyl-D,L-leucine	0.34/0.51		
3,5-Dinitrobenzoyl-D,L- α -phenyl-glycine	0.30/0.67		
<i>N</i> - α -(9-fluorenylmethoxycarbonyl)		Plates: Nano-SIL C 18–50 UV ₂₅₄ , 10×10 cm, Macherey-Nagel, Germany	177 1992
-(D,L)-valine	0.36 (L)/0.43 (D)		
-(D,L)-phenylalanine	0.41 (L)/0.50 (D)	Eluent: 0.1 M acetate buffer (pH 4.86) containing iso-propanol (12–36%) and BSA (5–6%) (See Ref. 177)	
-(D,L)-leucine	0.29 (L)/0.36 (D)		
-(D,L)-norleucine	0.18 (L)/0.27 (D)	Development distance: 7 cm	
-(D,L)-proline	0.77 (L)/0.47 (D)	Development time: 1–1.5 h	
-(D,L)-norvaline	0.19 (L)/0.27 (D)	Visualization: UV (366 nm)	
-(D,L)-tryptophan	0.41 (L)/0.20 (D)		
-(D,L)-alanine	0.29 (L)/0.37 (D)		
-(D,L)- β -cyclohexylalanine	0.17 (L)/0.30 (D)		
-(D,L)-methionine	0.28 (L)/0.20 (D)		
Tryptophan	See Ref. 178.	Plates: Nano-SIL C 18–50 UV ₂₅₄ , 10×10 cm, Macherey-Nagel, Germany	178 1992
Tryptophanamide			
α -Methyltryptophan		Eluent: 0.05 M sodium tetraborate containing 12% iso-propanol and 6% BSA, pH 9.30–9.92	
1-Methyltryptophan			
4-Methyltryptophan		Development distance: 8 cm	

		Development time: 1 h 50 min.		
5-Methyltryptophan		or		
6-Methyltryptophan		Homemade layer microcrystalline cellulose, Merck, Germany		
7-Methyltryptophan		Eluent: 1 M aqueous sodium chloride		
5-Hydroxytryptophan		Development distance: 15 cm		
5-Methoxytryptophan		Visualization: Van Urk's reagent		
Glycyltryptophan				
PTH-D,L-proline	See Ref. 179.	Plates: RP-18W/UV ₂₅₄ , 10×10 cm, Macherey-Nagel, FRG	179	1992
PTH-D,L-tyrosine				

Compounds separated	<i>R_f</i> value	Separation technique	Ref.	Year
PTH-D,L-isoleucine		Eluent: 0.5 M acetic acid containing 2% isopropanol and 7% BSA, pH 3.50		
PTH-D,L-methionine				
PTH-D,L-tryptophan		Development distance: 6.5 cm		
PTH-D,L-valine		Development time: 2h		
D,L-Kynurenine	See Ref. 179.	Eluent: 6% BSA in 0.05 M sodium tetraborate containing 6% isopropanol, pH 9.30	179	
D,L-3-(1-Naphthyl)alanine		Eluent: 6% BSA in 0.05 M sodium bicarbonate+0.05 sodium carbonate containing 6% isopropanol, pH 9.80		
<i>O</i> -Hippuryl-D,L-β-phenyllactic acid				
<i>N</i> -α-Benzoyl-D,L-arginine-7-amido-4-methylcoumarin				
(±)-2,2,2-Trifluoro-1-(9-anthryl)-ethanol		Eluent: 6% BSA in 0.05 M sodium tetraborate containing 20% isopropanol, pH 9.75		
Dansyl-D,L-norvaline	0.25 (L)/0.73 (D)	Plates: RP-18W/UV ₂₅₄ , 10×10 cm, Macherey-Nagel, Germany	180	1993
Dansyl-D,L-asparagine	0.79 (L)/0.68 (D)			
Dansyl-D,L-glutamic acid	0.65 (L)/0.45 (D)	Eluent: 5% BSA in water containing 2% isopropanol, pH 4.72		

Dansyl-D,L-valine	0.20 (L)/0.33 (D)	
Dansyl-D,L-tryptophan	0.62 (L)/0.73 (D)	
Dansyl-D,L-threonine	0.34 (L)/0.43 (D)	Eluent: 6% BSA in water containing 2% isopropanol, pH 4.72
Dansyl-D,L-phenylalanine	0.24 (L)/0.45 (D)	
Dansyl-D,L-methionine	0.32 (L)/0.50 (D)	
Dansyl-D,L-norleucine	0.38 (L)/0.50 (D)	
Dansyl-D,L-threonine	0.32 (L)/0.25 (D)	Eluent: 7% BSA in water containing 2% isopropanol, pH 3.40
Dansyl-D,L-serine	0.46 (L)/0.39 (D)	
Development distance: 7 cm		
<i>N</i> -Acetyl-5-methyl-D,L-tryptophan	0.33/0.76	Plates: RP-18W/UV ₂₅₄ , 10×10 cm, Nano-SIL 181 1993 C18-50 UV ₂₅₄ , 10×10 cm, Macherey-Nagel, FRG
<i>N</i> -Benzyloxycarbonyl-D,L-tryptophan	0.44 (D)/0.88 (L)	
<i>N</i> - <i>tert</i> -Butyloxycarbonyl-D,L-tryptophan	0.16 (L)/0.23 (D)	Eluent: BSA (3–8%) in different buffer systems (phosphate, acetate, or sodium bicarbonate–sodium carbonate buffers) containing 2% (for RP-18W/UV ₂₅₄) or 6% (for Nano-SIL C18-50 UV ₂₅₄) isopropanol
<i>N</i> -Phthalyl-glycyl-D,L-tryptophan	0.19/0.33	
<i>N</i> - <i>tert</i> -Butyloxycarbonyl- <i>p</i> -nitro-D,L-phenylalanine	0.20 (D)/0.30 (L)	
<i>N</i> - <i>o</i> -Nitrophenylsulfenyl-D,L-norvaline	0.48/0.59	

<i>N</i> - <i>o</i> -Nitrophenylsulfenyl-D,L-norleucine	0.44/0.61		
2,4-Dinitrophenyl-D,L-pipecolic acid	0.45/0.56		
2,4-Dinitrophenyl-D,L-ethionine sulfone	0.26/0.35		
Dinitropyridyl-D,L-alanine	0.47/0.53		
Dinitropyridyl-D,L-norvaline	0.21/0.31		
4-Fluoro-D,L-tryptophan	0.51/0.66		
5-Fluoro-D,L-tryptophan	0.43/0.63		
6-Fluoro-D,L-tryptophan	0.42/0.54		
(<i>R,S</i>)-Metoprolol	See Ref. 183.	Plates: Diol F254 HPTLC plates, Merck, Germany	183 1993
(<i>R,S</i>)-Propranolol		Eluent: dichloromethane containing 5 mM <i>N</i> -benzoxycarbonyl-glycyl-L-proline (ZGP)	
(<i>R,S</i>)-Alprenolol		Visualization: UV (280 or 300 nm)	
(±)-Hyoscyamine	0.35 (+)/0.50 (-)	Plates: 20×20 cm homemade plates with silica gel G, Merck, Germany, impregnated with L-aspartic acid	184 1993
(±)-Colchicine	0.65 (+)/0.70 (-)	Eluent: <i>n</i> -butanol–chloroform–acetic acid–water, 3:6:4:1, 0°C Development distance: 10 cm Development time: 3.5 h Visualization: iodine vapor	
2,4-Dinitrophenyl-D,L-ethionine	0.45/0.61	Plates: RP-18W/UV ₂₅₄ , 10×10 cm, Macherey-Nagel, Germany	182 1994
2,4-Dinitrophenyl-D,L-citrulline	0.34/0.41	Eluent: 0.1 M acetate buffer containing 6% BSA and 2% isopropanol	
D,L-Amethopterin	0.09/0.19	Eluent: 0.1 M acetic acid containing 8% BSA and 2% isopropanol	
(±)-Warfarin	0.18/0.25	Eluent: 0.5 M sodium acetate containing 8% BSA and 2% isopropanol	
(±)-Chlorwarfarin	0.11/0.06	Development distance: 6–7 cm	

Coumachlor	0.14/0.20	Plates: 5×20 cm chemically bonded diphenyl-F reversed-phase plates, Whatman, USA	185	1994
Indoprofen	0.58/0.63			

Compounds separated	R_f value	Separation technique	Ref.	Year
Warfarin	0.04/0.06	Eluent: acetonitrile–0.6 M NaCl–1% triethylammonium acetate buffer (pH 4.1), various ratios containing 0.025–0.08 M vancomycin (macrocyclic antibiotic)		
Bendroflumethiazide	0.02/0.06			
AQC- α -aminophenylacetic acid ^a	0.13 (L)/0.16 (D)	(See Ref. 185.)		
AQC-3-amino-3-phenylpropionic acid	0.11/0.19	Development distance: 19 cm		
AQC-3-aminopiperidine dihydrochloride	0.24/0.28	Development time: 1–3 h Visualization: fluorescence (254 and 365 nm)		
AQC- α -amino-2-thiopheneacetic acid	0.16/0.19			
AQC-ethionine	0.14/0.17			
AQC- <i>allo</i> -isoleucine	0.14 (L)/0.21 (D)			
AQC-methionine	0.19 (L)/0.23 (D)			
AQC-norleucine	0.13 (L)/0.16 (D)			
AQC-norvaline	0.21 (L)/0.25 (D)			
AQC-valine	0.23 (L)/0.27 (D)			
Dansyl- α -amino- <i>n</i> -butyric acid	0.09 (L)/0.21 (D)			
Dansyl-glutamic acid	0.21 (L)/0.22 (D)			
Dansyl-leucine	0.03 (L)/0.09 (D)			

Dansyl-methionine	0.05 (L)/0.12 (D)	
Dansyl-norleucine	0.04 (L)/0.16 (D)	
Dansyl-norvaline	0.05 (L)/0.12 (D)	
Dansyl-phenylalanine	0.03 (L)/0.05 (D)	
Dansyl-serine	0.16 (L)/0.24 (D)	
Dansyl-threonine	0.13 (L)/0.17 (D)	
Dansyl-tryptophan	0.01 (L)/0.03 (D)	
Dansyl-valine	0.06 (L)/0.10 (D)	
AQC-Leu-Leu ^a	0.03 (D,L) 0.04 (L,L) 0.10 (L,D) 0.24 (D,D)	
Dansyl-DL-amino acids of α -Amino- <i>n</i> -butyric acid	87 (D)/73 (L)	Plates: homemade silica gel (30 g silica gel+60 mL distilled water, containing 0.34 mM vancomycin hydrochloride) 186 2000
Leucine	87 (D)/73 (L)	
Norleucine	87 (D)/73 (L)	Eluent: acetonitrile–0.5 M NaCl (aq), 10:4
Norvaline	87 (D)/73 (L)	

Tryptophan	87 (D)/73 (L)	
Valine	87 (D)/73 (L)	
Phenylalanine	79 (D)/68 (L)	14:3
Eluent: acetonitrile–0.5 M aq. NaCl–2-propanol		
Serine	94 (D)/82 (L)	10:4:1
Threonine	88 (D)/75 (L)	15:3:1
Plates: homemade silica gel (50 g silica gel+100 mL distilled water, containing 0.05 g erythromycin)		
Eluent: 0.5 M aq. NaCl–acetonitrile–methanol		
Serine	68 (D)/64 (L)	10:4:1
Glutamic acid	36 (D)/30 (L)	15:1:1
	56 (D)/45 (L)	22:1:0.5
	65 (D)/56 (L)	22:1:0
	59 (D)/52 (L)	26:1:0
	65 (D)/50 (L)	10:4:1
Phenylalanine	27 (D)/20 (L)	10:4:1
Valine	30	10:4:1

	(D)/22 (L)	
Leucine	32 (D)/24 (L)	10:4:1
Tryptophan	47 (D)/38 (L)	10:4:1
Methionine	63 (D)/56 (L)	10:4:1
	57 (D)/50 (L)	10:4:1
Aspartic acid	63 (D)/50 (L)	10:4:1
α -Amino- <i>n</i> -butyric acid	51 (D)/42 (L)	10:4:1
Norleucine	71 (D)/63 (L)	10:4:1

^aAQC is the fluorescence-tagging agent 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate.

completely different from those of amino acids, their derivatives, and similar compounds such as hydroxy acids.

Armstrong and Zhou (185) focused on the use of the macrocyclic antibiotic vancomycin as a chiral mobile-phase additive. In this work the separations of carbamates, derivatized amino acids, racemic drugs, and dansyl amino acids were performed on diphenyl-modified stationary phases with the eluent system acetonitrile–0.6 M NaCl–1% triethylammonium acetate buffer (pH 4.1).

Another group (186) used the macrocyclic antibiotic vancomycin as a chiral selector on silica gel layers. The mobile phase enabling successful resolutions of the most racemic dansyl amino acids was acetonitrile–0.5 M aq. NaCl (5:2 and 14:3). The same group prepared a chiral stationary phase using a slurry of silica gel in 0.05% erythromycin solution that was spread on glass plates (187). Spots of the dansyl derivatives of DL- and L-amino acids were applied and detected under 254 nm radiation. The best mobile phase was 0.5 M NaCl–acetonitrile (1.5–25:1), in some instances with a small addition of methanol. Results were reported with a development distance of 10 cm. Separation factors ranged from 1.06 to 1.36 with the D form having the higher mobility.

XI. ENANTIOMER SEPARATION USING DIASTEREOMERIC DERIVATIVES

With the increasing number of commercially available, extremely pure chiral auxiliaries, thin-layer chromatographic purity control via formation of diastereomers has gained increasing importance. In contrast to direct enantiomer separations, antipode separation via diastereomers is not usually achieved with chiral adsorbents; however, enhanced “diastereomer selectivity” is also noted for asymmetrical supports. The type of chiral reagent for formation of the diastereomer depends upon, among other parameters, the structure—mono- or bifunctional—of the compound to be derivatized (see Table 7).

The published work (188–200) focuses on reactions of racemic compounds with $\text{NH}_2(\text{NH})$ —, OH —, and COOH —functionalities with the auxiliaries known from liquid chromatography, especially with commercial ready-to-use reagents.

XII. THIN-LAYER ENANTIOMERIC RESOLUTION VIA LIGAND EXCHANGE

A. Resolution Mechanism

Recent experimental results have confirmed the principle of chiral interaction (three-point rule) postulated in 1952 by Dalglish (65). Additionally, the results prove that the separation models developed for ligand exchange by high-performance liquid chromatography (21, 204, 205) are also valid for TLC; the diastereomeric complexes formed with the metal ion (e.g., Cu^{2+}) and the chiral adsorbent have different stabilities for the different antipodes, and thus chromatographic separation is achieved.

B. Survey of Applications of Racemic Separations

Thin-layer chromatographic enantiomeric separations based on ligand exchange were published independently by Günther et al. (206) and Weinstein (207) in 1984. Though very similar in their technique, the procedures differ in their choice of chiral selector and consequently in their range of applicability. Using commercially available reversed-phase TLC plates, Weinstein (207) impregnated the layers with the optically active copper complex of *N,N*-di-*n*-propyl-L-alanine after preconditioning the ready-to-use plate with buffer A (0.3 M sodium acetate in 40% acetonitrile and 60% water, adjusted to pH 7 with acetic acid). With the exception of proline, all proteinogenic amino acids are resolved—as dansyl derivatives—into L- and D-enantiomers. Section IX.C.1 presents a detailed description of this procedure for some selected separation examples. Another paper from this group (208) describes a two-dimensional reversed-phase thin-layer chromato-

Table 7 Enantiomeric Separations on Thin-Layer Plates Through Diastereomer Formation by Chiral Reagents

Compounds separated	R_f value	Eluent ^a	Chiral reagent	Remarks	Ref.	Year
D,L-Dopa-carboxyl- ¹⁴ C	0.38 (D)/0.56 (L)	A	(L)-Leucine <i>N</i> -carboxyanhydride	Plates: silica gel G, radiochromatographic method Development time: 1 h (developed twice) Visualization: ninhydrin	188	1972
<i>d,l</i> -Amphetamine	0.49 (D)/0.55 (L)	B	<i>N</i> -Trifluoroacetyl-L-prolyl chloride	Plates: silica gel, Merck, Germany	189	1976
<i>d,l</i> -Amphetamine	0.43 (D)/0.47 (L)	C	<i>N</i> -Benzyloxycarbonyl-L-prolyl chloride	Visualization: sulfuric acid– formaldehyde (10:1)		
D,L-Methamphetamine	0.57 (L)/0.61 (D)	D	<i>N</i> -Benzyloxycarbonyl-L-prolyl chloride			
D,L-Alanine methyl ester	0.33 (L)/0.43 (D)	E	Protected muramic acid	Plates: DC-Plastik-folien, Kieselgel 60	190	1979
D,L-Leucine methyl ester	0.47 (L)/0.52 (D)	E	Protected muramic acid	Radiochromatographic method		
D,L-Phenylalanine methyl ester	0.46 (L)/0.54 (D)	E	Protected muramic acid	Development time: 24 h Visualization: 25% ammonium bisulfate solution		
<i>R,S</i> -Cyclophosphamide	0.28 (<i>S</i>)/0.33 (<i>R</i>)	F	(–)-1-Phenethyl alcohol (Norse Labs., USA)	Plates: silica gel G, 5×20 cm, Merck, Germany Visualization: iodine vapor or 4-(4-nitrobenzyl)pyridine	191	1979
<i>R,S</i> -Bunitrolol	0.42 (<i>R</i>)/0.37 (<i>S</i>)	G	<i>R</i> -(–)-1-(1-Naphthyl)ethyl isocyanate (E.öa)	Plates: HPTLC silica gel, Merck, Germany	192	1984

			Chemie, Steinheim, Germany)	Visualization: UV		
<i>R,S</i> -Metoprolol	0.32 (<i>R</i>)/0.27 (<i>S</i>)	G	<i>R</i> -(-)-1-(1-Naphthyl)ethyl isocyanate (Ega Chemie, Steinheim, Germany)			
<i>R,S</i> -Alprenolol	0.41 (<i>R</i>)/0.33 (<i>S</i>)	G	<i>R</i> -(-)-1-(1-Naphthyl)ethyl isocyanate (Ega Chemie, Steinheim, Germany)			
<i>R,S</i> -Propranolol	0.51 (<i>R</i>)/0.42 (<i>S</i>)	G	<i>R</i> -(-)-1-(1-Naphthyl)ethyl isocyanate (Ega Chemie, Steinheim, Germany)			
<i>R,S</i> -Oxprenolol	0.37 (<i>R</i>)/0.32 (<i>S</i>)	<i>G</i>	<i>R</i> -(-)-1-(1-Naphthyl)ethyl isocyanate (Ega Chemie, Steinheim, Germany)			
Compounds separated	<i>R_f</i> value	Eluent ³	Chiral reagent	Remarks	Ref.	Year
<i>R,S</i> -Pindolol	0.38 (<i>R</i>)/0.33 (<i>S</i>)	G	<i>R</i> -(-)-1-(1-Naphthyl)ethyl iso-cyanate (Ega Chemie, Steinheim, Germany)			
<i>R,S</i> -Amphetamine	0.21 (<i>R</i>)/0.14 (<i>S</i>)	H	(<i>S</i>)-(+)-Benoxaprofen chloride (benoxaprofen, Eli Lilly, Bad Homburg, Germany)	Plates: silica gel 60, Merck, Germany	193	1984
<i>R,S</i> -Methamphetamine	0.33 (<i>R</i>)/0.27 (<i>S</i>)	H				
<i>R,S</i> - α -Methylbenzylamine	0.28 (<i>R</i>)/0.16 (<i>S</i>)	H		Visualization: fluorescence (KM3, Zeiss, Germany)		
<i>R,S</i> -Tranilcypromine	0.16 (<i>R</i>)/0.21 (<i>S</i>)	H				
<i>R,S</i> -Ketoprofen	0.38 (<i>R</i>)/0.48 (<i>S</i>)	I	(<i>R</i>)-(+)-1-Phenylethylamine hydrochloride	Plates: HPTLC silica gel 60 F 254	194	1986

<i>R,S</i> -Suprofen	0.36 (<i>R</i>)/0.41 (<i>S</i>)	I		Merck Germany		
<i>R,S</i> -Indoprofen	0.35 (<i>R</i>)/0.39 (<i>S</i>)	I		Visualization: UV (254 nm)		
D,L-2-Hydroxypalmitic acid	0.30/0.34	J	(-)- α -Methoxy- α -trifluoromethylphenylacetic acid chloride (acid: Fluka, Buchs, Switzerland)	Plates: silica gel (Merck, Germany) coated with phenyl methyl vinyl chlorosilane	195	1986
<i>R,S</i> -3-Bromo-2-methylpropionic acid	0.34 (<i>S</i>)/0.45 (<i>R</i>)	K	(1 <i>R</i> , 2 <i>R</i>)-(-)-1-(4-Nitrophenyl)2-amino-1,3-propanediol (levobase: EGIS, Budapest, Hungary)	Plates: silica gel F 254, 10×20 cm, Merck, Germany	196	1987
(1- <i>R,S</i>)-(2- <i>R,S</i>)- <i>cis</i> -Permethrinic acid	0.57 (<i>S,S</i>)/0.61 (<i>R,R</i>)	K		Visualization: UV (254 nm)		
<i>R,S</i> -Lactic acid	0.43 (<i>S</i>)/0.48 (<i>R</i>)	K				
<i>R,S</i> -Mandelic acid	0.26 (<i>S</i>)/0.33 (<i>R</i>)	K				
<i>R,S</i> -Naproxen	0.63 (<i>S</i>)/0.53 (<i>R</i>)	K				
<i>R,S</i> -Fenoprofen and related compounds	0.54/0.65	K				
DL-Alanine	0.17 (ΔR_f L max.)	L	1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent: Pierce, USA)	Plates: C18 (catalog No. 4803800), Whatman, USA	197	1987
DL-Arginine	0.06 (ΔR_f L max.)	L				
DL-Asparagine	0.13 (ΔR_f L max.)	L		Visualization: UV		
DL-Aspartic acid	0.22 (ΔR_f L max.)	L				
DL-Citrulline	0.12 (ΔR_f L max.)	L				

DL-Cystine	0.08 (ΔR_f L max.)				
DL-Ethionine	0.20 (ΔR_f L max.)				
DL-Glutamic acid	0.11 (ΔR_f L max.)				
DL-Histidine	0.08 (ΔR_f L max.)				
DL-Isoleucine	0.21 (ΔR_f L max.)				
DL-Leucine	0.20 (ΔR_f L max.)				
DL-Lysine	0.15 (ΔR_f L max.)				
DL-Methionine	0.20 (ΔR_f L max.)				
DL-Norvaline	0.20 (ΔR_f L max.)				
DL-Norleucine	0.20 (ΔR_f L max.)				
DL-Phenylalanine	0.18 (ΔR_f L max.)				
DL-Proline	0.13 (ΔR_f L max.)				
DL-Serine	0.11 (ΔR_f L max.)				
DL-Threonine	0.21 (ΔR_f L max.)				
DL-Tryptophan	0.15 (ΔR_f L max.)				
DL-Tyrosine	0.22 (ΔR_f L max.)				
DL-Valine	0.21 (ΔR_f L max.)				
<i>R,S</i> -Ethyl-4-(dimethylamino)-3-hydroxybutanoate (carnitine precursor)	0.55 (<i>R</i>)/0.79 (<i>S</i>)	M	(<i>S</i>)-(+)- α -Methoxyphenyl-acetic acid	Plates: silica gel, 5×10 cm, Fisher, USA	198 1987
<i>R,S</i> -Metoprolol	0.24 (<i>R</i>)/0.28 (<i>S</i>)	N	(<i>S</i>)-(+)-Benoxaprofen chloride	Plates: silica gel 60, 20×20 cm, Merck, Germany	199 1987

<i>R,S</i> -Oxprenolol	0.32 (<i>R</i>)/0.38 (<i>S</i>)	N		Visualization: fluorescence (KM 3, Zeiss, Germany)		
<i>R,S</i> -Propranolol	0.32 (<i>R</i>)/0.39 (<i>S</i>)	N				
<i>R,S</i> -Methionine methyl ester	0.56 (<i>R</i>)/0.60 (<i>S</i>)	0	(<i>S</i>)-(+)-Naproxen chloride	Plates: silica gel, 20×20 cm, Merck, FTG	200	1988
<i>R,S</i> -Asparagine methyl ester	0.46 (<i>R</i>)/0.53 (<i>S</i>)	0	[(<i>S</i>)-(+)-Naproxen: Sigma, München, Germany]			
<i>R,S</i> -Leucine methyl ester	0.79 (<i>R</i>)/0.85 (<i>S</i>)	O		Development time: 0.8 h		
<i>R,S</i> -Proline methyl ester	0.57 (<i>R</i>)/0.64 (<i>S</i>)	O		Visualization: UV		
<i>R,S</i> -Norvaline methyl ester	0.63 (<i>R</i>)/0.73 (<i>S</i>)	O				
<i>R,S</i> -Valine methyl ester	0.63 (<i>R</i>)/0.71 (<i>S</i>)	O				
<i>R,S</i> -Phenylalanine methyl ester	0.66 (<i>R</i>)/0.69 (<i>S</i>)	O				
<i>R,S</i> - α -Aminobutyric acid methyl ester	0.49 (<i>R</i>)/0.57 (<i>S</i>)	O				
<i>R,S</i> -Tyrosine methyl ester	0.12 (<i>R</i>)/0.17 (<i>S</i>)	O				
<i>R,S</i> -Norleucine methyl ester	0.69 (<i>R</i>)/0.75 (<i>S</i>)	O				
Pindolol	See Ref. 202.	P		HPTLC C8 F, Merck, FRG	202	1997

^aA, upper phase of ethyl acetate–formic acid–water, 60:5:35; B, chloroform–methanol, 197:3; C, *n*-hexane–chloroform–methanol, 10:9:1; D, *n*-hexane–ethyl acetate–acetonitrile–diisopropyl ether, 2:2:2:1; E, benzene–acetone, 3:1; F, chloroform–methanol, 9:1; G, benzene–ether–acetone, 88:10:5; H, toluene–dichloromethane–tetrahydrofuran, 5:1:1, ammonia atmosphere; I, benzene–methanol, 93:7; J, heptane–methyl formate–diethyl ether–acetic acid, 50:40:10:2; K, chloroform–ethanol–acetic acid 9:1:0.5; L, methanol–0.3 M aqueous sodium acetate adjusted to pH 4.1:1. M

ether; N, toluene–acetone, 100:10, ammonia atmosphere; O, toluene–dichloromethane–tetrahydrofuran, 5:1:2, ammonia atmosphere; P, water–2-propanol, 70:30 (24 mL eluent contains 0.3 g NaCl).

graphic procedure for simultaneous separation of racemic dansyl amino acids mixtures. In the first direction the dansyl amino acids were separated on RP-18 TLC plates with eluents without chiral additives using, e.g., a convex gradient with increasing acetonitrile content (20–30%) in 0.3 M sodium acetate (pH 6.3). In the second direction the plate was treated with that above-mentioned chiral selector and then again developed with aqueous acetonitrile–sodium acetate buffer. The separation was further improved by using a temperature gradient (6.2°C/cm). The influence of the temperature on enantiomeric separation behavior is detailed in Ref. 209. Chiral diaminodiamide copper(II) complexes (Fig. 4) are also suitable as chiral selectors for thin-layer chromatographic enantiomeric separations of racemic dansyl amino acids (210). In these ligands two L-amino acids are joined via an amide bond by ethylene and trimethylene bridges and are endowed with varying degrees of lipophilicity and bulkiness, depending on the nature of the amino acid side chain. The coating procedure in general corresponds to that of Weinstein (207). The authors also work with one- or two-dimensional techniques with or without chiral additive in the eluent (acetonitrile–water, 33:67, adjusted to pH 6.8 with acetic acid).

Based on the work of Davankov and coworkers (20, 204), who modified commercial HPLC columns for distribution chromatography with alkyl derivatives of L-amino acids such as *n*-decyl-L-histidine or *n*-hexadecyl-L-proline, Günther et al. (206) used (2*S*, 4*R*, 2'*RS*)-*N*-(2'-hydroxydodecyl)-4-hydroxyproline (Fig. 5a), which is easier to prepare, as a chiral selector (211). The following impregnation procedure proved to be most efficient. A glass plate coated with hydrophobic silica gel (RP-18 TLC) was dipped into a 0.25% copper(II) acetate solution (methanol–water, 1:9) and dried. Then the plate was immersed in a 0.8% methanolic solution of the chiral selector for 1 min. After air drying, the plate was ready for enantiomeric separations. Unlike the procedures described above, in this case antipode separation of amino acids was possible without derivatization. Because the commercially available chiral TLC/HPTLC plates are based on this ligand-exchange chromatographic technique, a detailed description of chromatographic conditions is given in Section XII.C.2.

Efforts were made to illuminate the structure of the complex of the 4-hydroxyproline selector and to find new selectors for the enantiomeric separation based on ligand-exchange chromatography. Martens and coworkers (212) tried to do X-ray investigations of the 4-hydroxyproline–copper(II) complex, but it was not possible to get a crystalline complex of this selector. Therefore they synthesized a model compound with a methyl group instead of the C₁₀H₂₁ group. With this short alkyl group–modified selector, the chelate complex crystallized in the orthorhombic crystal system, and X-ray data are available. The same group also mentioned that the configuration in the 2'-position of the side chain of the 4-hydroxyproline selector has no influence on the stereoselectivity of its copper complex in the enantiomeric separation of amino acids (215). Recently, new experiments on the crystallization and structure determination of the copper(II) complex were successful (216). The results show that coordination at the copper center of the selector complex is fundamentally different from that of the short alkyl chain model compound mentioned previously.

New selectors for the separation of enantiomers based on LEC were synthesized (Figs. 5b– 5d). Iminocarboxylic acid (Fig. 5b) was used for the enantiomeric separation of 5,5-dimethyl-3-thiazoline-4-acetic acid with the eluent system acetonitrile–methanol–water (3:5:5) (214), whereas

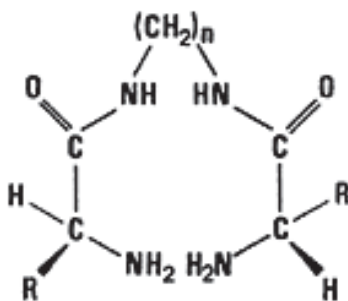


Figure 4 Ligands AA-NN-*n*: *n*=2, 3; R=C₆H₅CH₂ (Phe), (CH₃)₂CH (Val), CH₃ (Ala).

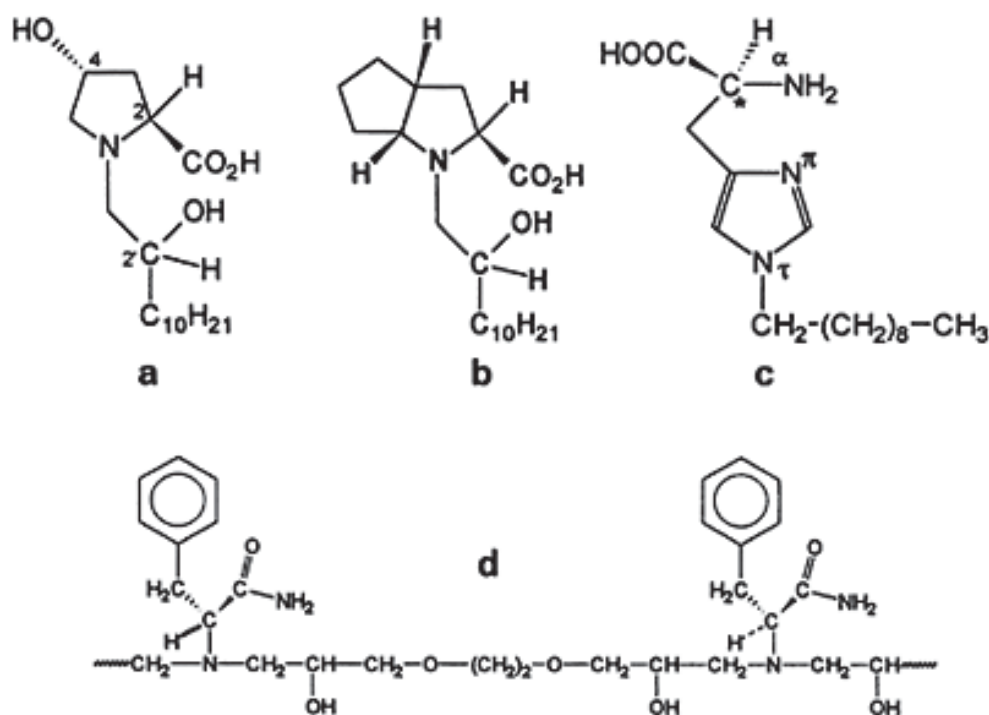


Figure 5 (a) 4-Hydroxyproline selector (206); (b) iminocarboxylic acid selector (214); (c) histidine selector (217); (d) poly-L-phenylalanine amide selector (213).

Remelli et al. (217) described a selector based on histidine. With this chiral selector, *L-Nⁿ-n*-decylhistidine (Fig. 5c), the simultaneous enantiomeric separation of D,L-tryptophan and D,L-phenylalanine was successfully performed on hydrophobic layers with MeOH–acetonitrile–THF–water (7.3:5.9:33.9:52.9) as eluent. Sinibaldi et al. (213) resolved D,L-dansyl amino acids on reversed-phase TLC plates pretreated with a Cu²⁺ complex of poly-L-phenylalanine amide (Fig. 5d). The polymeric ligand was synthesized by the reaction of optically active amide with ethylene glycol diglycyl ether. The method makes use of a sophisticated liquid chromatograph for obtaining the desired polymer fraction, which is subsequently used for the LEG, and this might limit the application of the separation procedure. However, a simple method was used by Bhushan et al. (218). Here L-proline was used as a chiral selector on normal-phase silica gel (218), and amino acids were resolved with the eluent systems *n*-butanol–acetonitrile–water (6:2:3), chloroform–methanol–propionic acid (15:6:4), and acetonitrile–methanol–water (2:2:1).

Until now these selectors showed no eminent advantage compared with the 4-hydroxyproline selector. Therefore, layers using the 4-hydroxyproline selector are the only commercially available ready-to-use plates (Chiralplate[®], CHIR[®]).

C. Applications

1. Examples of TLC Enantiomeric Separations According to Weinstein (207)

a. Chromatographic Conditions

Method: Ascending, one-dimensional development in a TLC chamber with chamber saturation.

Plates: RP-18 TLC precoated plate (Cat. No. 15389, Merck), 20×20 cm, layer thickness 0.25 mm, with fluorescent indicator.

Preparation of plates: Reversed-phase TLC plates were developed (prior to application of the dansyl amino acids) in 0.3 M sodium acetate in 40% acetonitrile and 60% water, adjusted to pH 7 with acetic acid (buffer A). After fan drying, the plates were immersed in a solution of 8 mM *N,N*-di-*n*-propyl-L-alanine and 4 mM cupric acetate in 97.5% acetonitrile and 2.5% water for 1 h and left to dry in the air. The plates are stable and can be stored for further use.

Eluent: Buffer A.

Sample volume: 0.5 μL of a 0.6% methanolic solution (1:1).

Length of run: 16 cm.

Time of run: 1.5 h.

Detection: UV (366 nm).

b. Spectroscopy

Apparatus: Chromatogram spectrometer CD 60 (Desaga, Heidelberg, Germany)

Measuring principle: Monochromator-TLC plate (fluorescence)

Light source: Mercury lamp

Wavelength: $\lambda_{\text{exc}}=366$ nm, $\lambda_{\text{em}}=420$ nm (cutoff filter)

Slit: 6×0.2 mm

Scanning: 0.05 mm

c. Results. R_f values of selected dansyl amino acids are as follows.

Dansyl-D,L-aspartic acid, 0.45 (L)/0.48 (D)

Dansyl-D,L-serine, 0.32/0.34

Dansyl-D,L-glutamic acid, 0.51 (L)/0.58 (D)

See Figs. 6 and 7.

2. Examples of Separations with Chiralplate and HPTLC-CHIR

Under license from Degussa (28), Chiralplate, the first chiral TLC ready-to-use plate based on ligand-exchange chromatography, was developed and commercialized in 1985 in cooperation with Macherey-Nagel, Düren (219). In 1988 followed, again under license from Degussa, commercialization of the chiral HPTLC ready-to-use plate CHIR with a concentrating zone produced by Merck, Darmstadt. The following separation examples focus on elaborations with Chiralplate; however, because they are based on the same separation principle, they can be easily transferred to the HPTLC-CHIR plates. A comparison of separation results on both plates will be given for

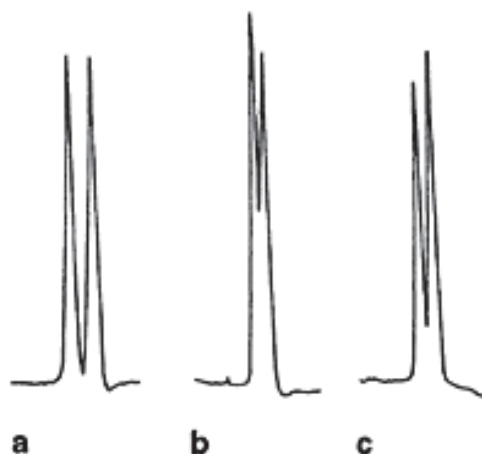


Figure 6 Remission-location curves: (a) Dansyl-D,L-glutamic acid; (b) dansyl-D,L-serine; (c) D,L-aspartic acid.

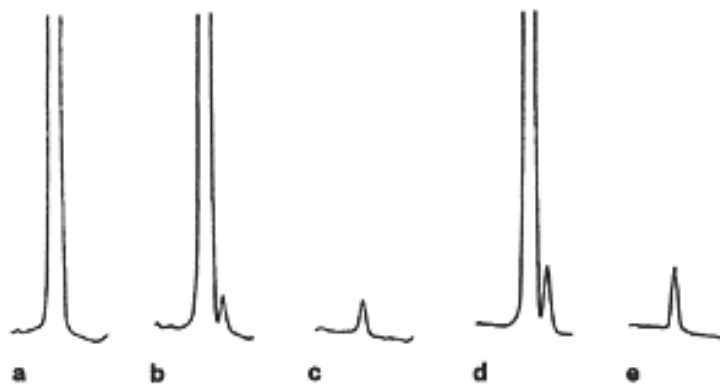


Figure 7 Remission-location curves: (a) Dansyl-L-glutamic acid; (b) 1% dansyl-D-glutamic acid in the L-enantiomer; (c) 1% dansyl-D-glutamic acid; (d) 2% dansyl-D-glutamic acid in the L-enantiomer; (e) 2% dansyl-D-glutamic acid (applied at a total volume of 1 μ L of a 0.1 % methanolic solution as a 10 mm band).

the TLC separation of α -hydroxycarboxylic acids (220). Other applications from external groups (221–241) are summarized in Table 8. This chapter does not discuss the successful application of Chiralplate in forced-flow planar chromatographic techniques such as overpressured layer chromatography (OPLC) and analytical rotation planar chromatography (RPC); we refer to the literature (250, 251).

a. Chromatographic Conditions with Chiralplate

Method: Ascending one-dimensional development in a TLC chamber with chamber saturation.

Plates: TLC precoated plates, Chiralplate (Cat. No. 811 055/056, Macherey-Nagel); size 10 $\times$ 20 cm, layer thickness (0.25 mm).

Eluent: To achieve short analysis times, ternary mixtures of water-miscible alcohol, water, and acetonitrile proved useful. Most racemate separations could be accomplished using one of two eluent systems:

A: Methanol–water–acetonitrile, 50:50:200

B: Methanol–water–acetonitrile, 50:50:30

For some substances, however, different eluent systems were more suitable:

C: Methanol–water, 10:80

D: Acetone–methanol–water 10:2:2

E: Dichloromethane–methanol, 45:5

Sample volume: With eluents A, B, and C, 2 μ L of a 1% solution of the racemate (methanol or methanol–water) was applied. With eluent D, 2 μ L of a 0.5% solution of the racemate of (0.1 M hydrochloric acid–methanol, 1:1) was applied. With eluent E, 2 μ L of a 0.5% solution of the racemate (methanol or methanol–dichloromethane, 1:1) was applied.

Length of run: 13 cm

Time of run: 0.5 h (eluent A), 1 h (eluent B), 1.5 h (eluent C), 0.8 h (eluent D), 0.3 h (eluent E)

Detection: Different detection methods were used, depending on the type of compound. For proteinogenic and nonproteinogenic amino acids, the dried plates were dipped for 3 s in a 0.3% ninhydrin solution in acetone (Tauchfix, Baron) and then dried in a drying cabinet for about 5 min at 110°C. Red derivatives formed on a white background. For α -hydroxycarboxylic acids, 1.82 g of vanadium pentoxide (Merck, Art. 284) was weighed into a 100 mL measuring flask, and 30 mL of 1 M sodium carbonate was added and completely dissolved by treatment in an ultrasonic bath. After cooling, 46 mL of 2.5 M sulfuric acid and acetonitrile to 100 mL were added. The dried plates were briefly

Table 8 Enantiomeric Separations on Chiralplate and HPTLC-CHIR (Selected Applications)

Compounds separated	R_f value	Eluent	Remarks	Ref.	Year
D,L-Norleucine	0.50 (D)/0.61 (L)	Methanol–water– acetonitrile,	Chiralplate (4×6 cm)	221	1985
D,L-Valine and related compounds	0.49 (D)/0.58 (L)	50:50:200	Development time: 5 min Visualization: ninhydrin		
Dipeptides (L,L/D,D) and D,L/L,D pairs	See Ref. 98.	Methanol–water– acetonitrile, 5:5:20 or 5:5:3	Chiralplate Development time: 15 min Visualization: ninhydrin	98	1986
Trp-Trp Ala-Ala and related compounds					
D,L-4-Hydroxy-3- methoxymandelic acid	0.55/0.49	Methylene chloride–methanol, 45:5	Chiralplate Development time: 25 min	222	1986
D,L-3,4-Dihydroxymandelic acid	0.38/0.32		Visualization: 2,6- dichloro-quinone- 4-chloroimide		
D,L- α -Methylmethionine	0.64/0.58	Acetonitrile–	Chiralplate	223	1987

D,L- α -Methylphenylmethyl ester and related compounds	0.72 (D)/0.59 (L)	methanol–water, 4:1:1, and further 8 eluents	Visualization: ninhydrin		
D,L- α -Ethylalanine	0.61/0.55	Acetonitrile–methanol–water, 4:1:1, and further 3 eluents	Chiralplate	224	1987
D,L- α -Propylalanine	0.63/0.55		Visualization: ninhydrin		
D,L- α -Butylalanine and related compounds	0.63/0.51				
D,L-N-Carbamyl-tryptophan	0.55 (D)/0.44 (L)	Solution of 1 mM copper(II) acetate, 5% methanol (pH 5.8)	Chiralplate (10×20 cm) Development time: 4 h (16°C) Visualization: Ehrlich's reagent	225	1987
D,L-2-Phenyl-5,5-dimethylthiazolidine carboxylic acid	0.59 (D)/0.67 (L)	Methanol–water–acetonitrile, 50:50:200	Chiralplate Visualization: ninhydrin	226	1987
D,L-2-(2-Nitrophenyl)-5,5-dimethylthiazolidine carboxylic acid and related compounds	0.65 (D)/0.77 (L)				
α,β -Adenine nucleosides (anomeric forms)	See Ref. 227.	Methanol–water–acetonitrile, 50:50:30–400	Chiralplate (10×20 cm) Development time: 35–105 min Visualization: UV	227	1987
R,S-Thiorphan	See Ref. 228.	No details	Chiralplate (10×20 cm)	228	1988
D,L-4-Mandelic acid	0.50 (D)/0.57 (L)	Methanol–acetonitrile–water, 50:50:20+0.05 mol/L KH_2PO_4	HPTLC-CHIR (10×10 cm and 20×20 cm)	229	1988
D,L-4-Bromomandelic acid	0.33/0.40				
D,L-4-Chloromandelic acid	0.35/0.42		Visualization: for amino acids and peptides, ninhydrin; for hydroxycarboxylic acids, $\text{MnCl}_2/\text{H}_2\text{SO}_4$ (120°C)		
D,L-3-Hydroxymandelic acid	0.47/0.59				
D,L-4-Hydroxymandelic acid	0.45/0.57	Dichloromethane–ethanol, 85:15 +0.1 mol/L LiCl			
D,L-3,4-Dihydroxymandelic	0.33/0.44		Visualization: UV 520, 410, 470 nm		

acid

D,L-4-Hydroxy-3-methoxymandelic acid 0.21/0.26 0.38/0.44 Chloroform–methanol, 90:10 (80% water-saturated)
Dichloromethane–methanol, 90:10

D,L- α -Methylserine No R_f values Methanol–water–acetonitrile, 50:50:30

D,L-Phenylalanine

D,L-Tryptophan

D,L-Tyrosine

D,L-Valine

D-Leu-L-Leu No R_f values Methanol–1-propanol–water, 50:10:40

L-Leu-D-Leu

D,L- α -Bromophenylalanine No R_f values Methanol–water–acetonitrile, 50:50:20

D,L-2-Chloro-6-benzoyl-aminocaproic acid No R_f values

D,L-3,4-Dihydroxycarboxylic acid No R_f values Dichloromethane–ethanol, 85:15 +0.1 mol/L LiCl

R,S-Noradrenaline (after derivatization with salicylic aldehyde) No R_f values Chloroform–methanol, 90:10 (80% saturated with water) HPTLC-CHIR Visualization: in situ evaluation with TLC scanner (410 nm) 230 1988

($\pm$)-2-Azabicyclo[3,3,0]octane-3-carboxylic acid No R_f values Ethanol–1-propanol–water, 60:10:30

D,L-Mandelic acid 0.59 (D)/0.57 (L) Methanol–acetonitrile–water, 50:50:20+0.05 mol/L KH_2PO_4 HPTLC-CHIR with concentrating zone, Merck, Germany 229 1988

DL-4-Bromomandelic acid 0.33/0.40

Compounds separated	R_f value	Eluent	Remarks	Ref.	Year
D,L-4-Chloromandelic acid	0.35/0.42		Visualization		

D,L-3-Hydroxymandelic acid	0.47/0.59 0.45/0.57	Dichloromethane– ethanol, 85:15 +0.1 mol/L LiCl	ninhydrin (for amino acids); MnCl ₂ /H ₂ SO ₄ (for hydroxycarboxylic acids)		
D,L-3,4-Dihydroxymandelic acid					
D,L-4-Hydroxy-3- methoxymandelic acid	0.21/0.26	Trichloromethane– methanol, 90:10, 80% water- saturated			
α -Allylphenylalanine	See Ref. 232.	See Ref. 232.	Chiralplate, Macherey-Nagel, Germany	232	1990
Phenylalanine	0.58 (L)/0.42 (D)	Acetonitrile– methanol–water, 4:1:1	Chiralplate, Macherey-Nagel, Germany	233	1990
2'-Methylphenylalanine	0.54 (L)/0.42 (D)				
2',6'-Dimethylphenylalanine	0.52 (L)/0.38 (D)		Visualization: ninhydrin		
β -Methylphenylalanine	0.56 (S,S)/0.36 (R,R) 0.55 (R,S)/0.47 (S,R)				
2'-Methyl- β - methylphenylalanine	0.57 (S,S)/0.33 (R,R) 0.55 (R,S)/0.48 (S,R)				
β -Methyl- <i>p</i> - nitrophenylalanine	0.62 (S,S)/0.43 (R,R) 0.60 (R,S)/0.52 (S,R)				
β -Methyltyrosine	0.67 (S,S)/0.52 (R,R) 0.67 (R,S)/0.55 (S,R)	Acetonitrile– methanol–water, 4:1:1			

β -Hydroxyphenylalanine	0.63 (<i>S,S</i>)/0.49 (<i>R,R</i>)			
Tyrosine	0.63 (L)/0.51 (D)			
2'-Methyltyrosine	0.62 (L)/0.54 (D)			
2',5'-Dimethyltyrosine	0.67 (L)/0.56 (D)			
2',5'-Dimethyl-4-methoxyphenylalanine	0.57 (L)/0.45 (D)			
Tetrahydroisoquinoline carboxylic acid	0.54 (L)/0.50 (D)			
2'-Methyltetrahydroisoquinoline	0.51 (L)/0.49 (D)			
β -Methyltetrahydroisoquinoline carboxylic acid	0.51 (<i>S,S</i>)/0.45 (<i>R,R</i>)			
2-Aminotetralincarboxylic acid	0.55 (L)/0.47 (D)			
2-Amino-6-hydroxytetralincarboxylic acid	0.64 (L)/0.59 (D)			
<i>N</i> -Methyl-D,L-($\pm$)-aspartic acid	0.42 (L)/0.34 (D)	Acetonitrile– MeOH–H ₂ O, 5:1:1	Chiralplate, 10×20 cm, Macherey-Nagel, Germany	234 1990
	0.45 (L)/0.39 (D)	Acetonitrile– MeOH–H ₂ O, 4:1:1		
	0.49 (L)/0.45 (D)	Acetonitrile– MeOH–H ₂ O, 3:1:1	Visualization: ninhydrin	
	0.52 (L)/0.52 (D)	Acetonitrile– MeOH–H ₂ O, 2:1:1		
	0.60	Acetonitrile–		

	(L)/0.66 (D)	MeOH-H ₂ O, 1:1:1			
	0.58 (L)/0.67 (D)	Acetonitrile- MeOH-H ₂ O, 0.6:1:1			
	0.60 (L)/0.68 (D)	Acetonitrile- MeOH-H ₂ O, 0:1:1			
Leucine	See Ref. 235.	Methanol-water- acetonitrile, 50:50:30	HPTLC-CHIR with concentrating zone, Merck, Germany	235	1990
Proline					
			Development distance: 7 cm		
			Visualization: ninhydrin		
D,L-Asp-D,L-Phe- OCH ₃ (Aspartame)	0.62 (D,D and D,L) 0.50 (L,L and L,D)	Methanol-water- acetonitrile, 50:50:200	Chiralplate, Macherey-Nagel, Germany	236	1991
			Visualization: ninhydrin		
D,L-Asp-acc-OPr (Dipeptide 56410 RP)	See Ref. 237.	Methanol-water- acetonitrile, 50:50:200	HPTLC-CHIR with concentrating zone, 10×10 cm, Merck, Germany	237	1991
			Development distance: 7 cm		
			Visualization: ninhydrin		
Tryptophan	0.52 (D)/0.64 (L)	Acetonitrile- H ₂ O-MeOH, 4:2:1	HPTLC-CHIR with concentrating zone, 10×10 cm, Merck, Germany	238	1992
Leucine					
Isoleucine	0.36 (D)/0.42 (L)	Acetonitrile-H ₂ O- <i>n</i> -PrOH, 3:4:2			
	0.35 (D)/0.52 (L)		Development distance: 7 cm		
Phenylalanine	0.59 (D)/0.71 (L)	Acetonitrile-H ₂ O- <i>n</i> -PrOH, 3:1:1			
D,L-Lactic acid	0.76 (L)/0.70 (D)	Acetonitrile- water, 3:2	HPTLC-CHIR, 10×10 cm, Merck, Germany	239	1993

D,L-Mandelic acid	0.65 (L)/0.57 (D)	
D,L-Hydroxyvaleric acid	0.68 (L)/0.60 (D)	Visualization: ninhydrin (for amino acids); thymolphthalein–NaOH (for hydroxy acids)
D,L-Hydroxycaproic acid	0.69 (L)/0.62 (D)	
D,L-Methionine	0.61 (L)/0.55 (D)	Methanol–water–acetonitrile, 1:1:4
D,L-Valine	0.63 (L)/0.56 (D)	
D,L-Leucine	0.65 (L)/0.52 (D)	
D,L-Serine	0.78 (L)/0.73 (D)	
D,L-Isoleucine	0.63 (L)/0.51 (D)	

Compounds separated	R_f value	Eluent	Remarks	Ref.	Year
D,L-Tyroxine	0.40 (D)/0.48 (L)	Acetonitrile–MeOH–H ₂ O, 60:15:15	Chiralplate, Macherey-Nagel, Germany	243	1994
Phenylalanine, methionine, valine, tryptophan	See Ref. 241.	RP eluents with different organic modifiers (methanol, acetonitrile, dioxane)	Visualization: UV (254 nm) Chiralplate, Macherey-Nagel, FRG	241	1994
Mandelic acid	0.46 (R)/0.53 (S)	Dichloromethane–methanol, 9:1	Chiralplate, Macherey-Nagel, FRG	242	1995

<i>meta</i> -Tyrosine	$\alpha=0.80$	Acetonitrile–methanol– water, 4:1:1	Chiralplate, Macherey- Nagel, FRG	245	1998
3,5-Diiodotyrosine	$\alpha=0.86$				
2',6'-Dimethyltyrosine	$\alpha=1.00$				
4'-Methylphenyltyrosine	$\alpha=0.77$				
1-Amino-2- phenylcyclopropane-1- carboxylic acid	$\alpha=0.77$				
2-Aminotetralin-2- carboxylic acid	$\alpha=0.90$				
6-Hydroxy-2-aminotetralin- 2-carboxylic acid	$\alpha=0.88$				
6-Hydroxy-5,7-diiodo-2- aminotetralin-2-carboxylic acid	$\alpha=1.00$				
1,2,3,4- Tetrahydroisoquinoline-1- carboxylic acid	$\alpha=0.88$				
5'-Methyl-1,2,3,4- tetrahydroisoquinoline-3- carboxylic acid	$\alpha=1.00$				
3-Methyl-1,2,3,4- tetrahydroisoquinoline-3- carboxylic acid	$\alpha=1.00$				
6-Hydroxy-1,2,3,4- tetrahydroisoquinoline-3- carboxylic acid	$\alpha=1.00$				
7-Hydroxy-1,2,3,4- tetrahydroisoquinoline-3- carboxylic acid	$\alpha=1.00$				
7-Hydroxy-6,8-diiodo-1,2,3,4- tetrahydroisoquinoline-3- carboxylic acid	$\alpha=0.82$				
4,5,6,7-Tetrahydro-1 <i>H</i> - imidazo[4,5- <i>c</i>]pyridine-6- carboxylic acid	$\alpha=1.00$				
1,2,3,4-Tetrahydronorharmane-1- carboxylic acid	$\alpha=0.69$				

Alanine	0.47 (D)/0.53 (L)	Acetonitrile– methanol–H ₂ O, 80:20:20	HPTLC chiralplates, Merck, FRG	246 1999
Norvaline	0.50 (D)/0.56 (L)			
Norleucine	0.51 (D)/0.60 (L)			
Glutamic acid	0.52 (D)/0.58 (L)			
Phenylalanine	0.45 (D)/0.54 (L)			
Tyrosine	0.55 (D)/0.65 (L)			
Tryptophan	0.50 (D)/0.58 (L)			
Proline	0.38 (D)/0.46 (L)			
Alanine	0.38 (D)/0.42 (L)	Acetonitrile– methanol–H ₂ O, 80:20:20	Chiralplate, Macherey-Nagel, FRG	248 2001
Phenylalanine	0.47 (D)/0.60 (L)			
Tryptophan	0.49 (D)/0.60 (L)			

Table 9 TLC Separation of Proteinogenic and Nonproteinogenic Amino Acids^a

Racemate	<i>R_f</i> value (configuration)	Eluent ^b
Alanine	0.69 (D)/0.73 (L)	D
Aspartic acid	0.50 (D)/0.55 (L)	A
Glutamic acid	0.54 (D)/0.59 (L)	A
Glutamine	0.41 (L)/0.55 (D)	A

Isoleucine	0.47 (D)/0.58 (L)	A
Leucine	0.53 (D)/0.63 (L)	C
Methionine	0.54 (D)/0.59 (L)	A
Valine	0.54 (D)/0.62 (L)	A
Phenylalanine	0.49 (D)/0.59 (L)	A
Serine	0.73 (D)/0.76 (L)	D
Tyrosine	0.58 (D)/0.66 (L)	A
Tryptophan	0.51 (D)/0.61 (L)	A
Proline	0.41 (D)/0.47 (L)	A
Cysteine (as thiazolidine-4-carboxylic acid)	0.59 (D)/0.69 (L)	A
<i>tert</i> -Leucine	0.40 (D)/0.51 (L)	A
Norleucine	0.53 (D)/0.62 (L)	A
<i>allo</i> -Isoleucine	0.51 (D)/0.61 (L)	A
Norvaline	0.49 (D)/0.56 (L)	A
<i>allo</i> -4-Hydroxyproline	0.41 (L)/0.59 (D)	A
2-Phenylglycine	0.57 (D)/0.67 (L)	A
2-Cyclopentylglycine	0.43/0.50	A
Ethionine	0.52 (D)/0.59 (L)	A
(1-Naphthyl)alanine	0.49 (D)/0.56 (L)	A
(2-Naphthyl)alanine	0.44 (D)/0.59 (L)	A
<i>O</i> -Benzylserine	0.54 (D)/0.65 (L)	A
<i>O</i> -Benzyltyrosine	0.48 (D)/0.64 (L)	A
4-Methyltryptophan	0.50/0.58	A
4-Methoxyphenylalanine	0.52/0.64	A
5-Methoxytryptophan	0.55/0.66	A
Methioninesulfone	0.62 (D)/0.66 (L)	A
Ethioninesulfone	0.55/0.59	A
Selenomethionine	0.53 (D)/0.61 (L)	A
Dopa	0.47 (L)/0.58 (D)	B
<i>S</i> -Methylthiocysteine	0.47 (D)/0.55 (L)	A
<i>S</i> -Methylthiohomocysteine	0.44/0.52	A
2-(2-Thienyl)-glycine	0.55/0.66	A

3,3-Dimethyl-2-aminovaleric acid	0.40 (D)/0.56 (L)	A

^aMigration distance 13 cm; chamber saturation.

^bA, methanol–water–acetonitrile, 50:50:200; B, methanol–water–acetonitrile, 50:50:30; C, methanol–water, 10:80; D, acetone–methanol–water, 10:2:2.

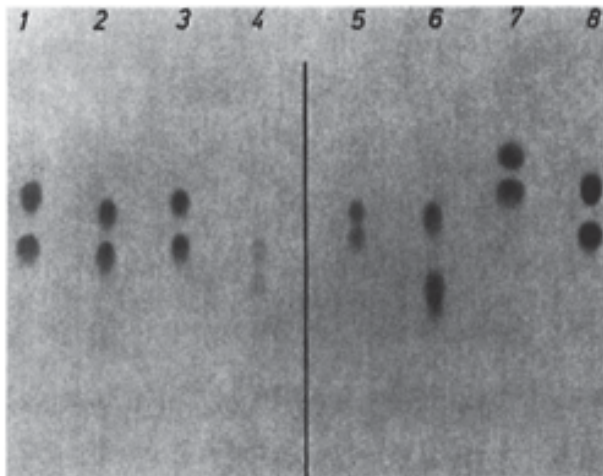


Figure 8 Thin-layer chromatogram of proteinogenic amino acid on Chiralplate. Spots: 1, Phenylalanine; 2, valine; 3, isoleucine; 4, proline; 5, methionine; 6, glutamine; 7, tyrosine; 8, tryptophan.

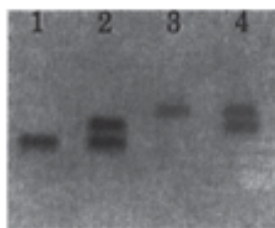


Figure 9 Thin-layer chromatogram of proteinogenic amino acid on Chiralplate. Spots: 1, D-Alanine; 2, D,L-alanine; 3, L-serine; 4, D,L-serine. Application: 10 mm streak (Linomat IV, made by Camag).

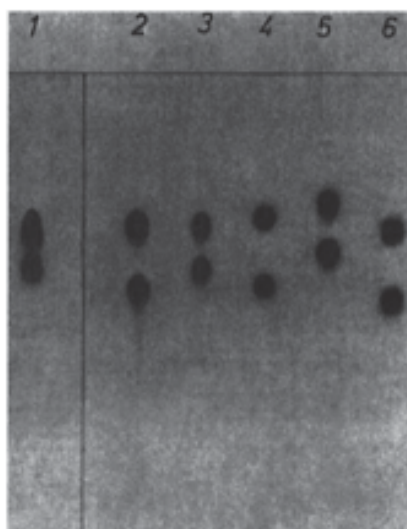


Figure 10 Thin-layer chromatogram of nonproteinogenic amino acid on Chiralplate. Spots: 1, Dopa; 2, *tert*-leucine; 3, *allo*-isoleucine; 4, *O*-benzyltyrosine; 5, 5-methoxytryptophan; 6, (2-naphthyl)alanine.

Table 10 TLC Enantiomeric Separation of Dipeptides^a

Dipeptide	R_f value (configuration)	Eluent ^b
Gly-D,L-Phe	0.57 (L)/0.63 (D)	B
Gly-D,L-Leu	0.53 (L)/0.60 (D)	B
Gly-D,L-Ileu	0.54 (L)/0.61 (D)	B
Gly-D,L-Val	0.58 (L)/0.62 (D)	B
Gly-D-Trp	0.48 (L)/0.55 (D)	B
D-Leu-L-Leu	0.48	B
L-Leu-D-Leu	0.57	B
D-Leu-L-Leu	0.19	A
L-Leu-D-Leu	0.26	A
D-Ala-L-Phe	0.59	B
L-Ala-D-Phe	0.65	B
D-Ala-L-Phe	0.21	A

L-Ala-L-Phe	0.26	A
D-Met-D-Met	0.64	B
L-Met-D-Met	0.71	B
D-Met-L-Met	0.29	A
L-Met-D-Met	0.33	A
D,L-Asp-D,L-Phe-OCH (Aspartame)	0.62 (DD and DL)	A
D,L-Asp-Acc-OPr (dipeptide 56410 RP)	0.50 (LL and LD)	

^aMigration distance 13 cm; chamber saturation.

^bA, methanol–water–acetonitrile, 50:50:200; B, methanol– water–acetonitrile, 50:50:30.

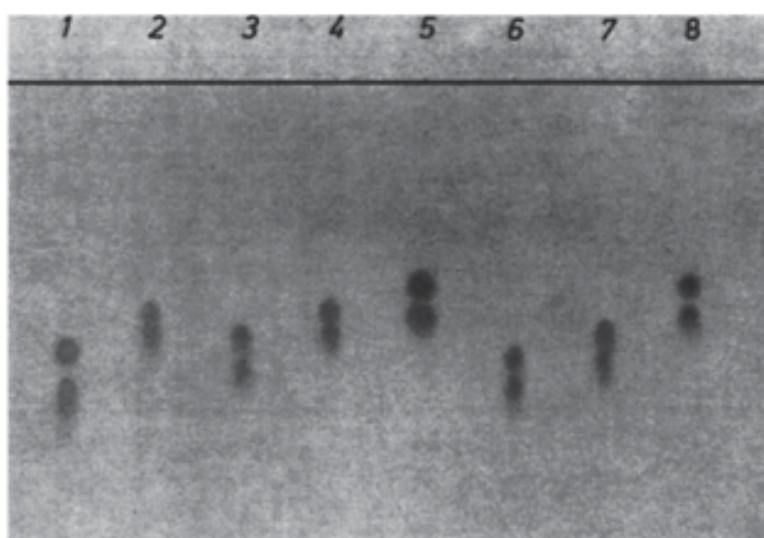


Figure 11 Thin-layer chromatogram of dipeptides on Chiralplate. Spots: 1, D-Leu-L-Leu and L-Leu-D-Leu; 2, Gly-D,L-Val; 3, Gly-D,L-Leu; 4, Gly-D,L-Phe; 5, D-Ala-L-Phe and L-Ala-D-Phe; 6, Gly-D,L-Trp; 7, Gly-D,L-Leu; 8, D-Met-L-Met and L-Met-D-Met.

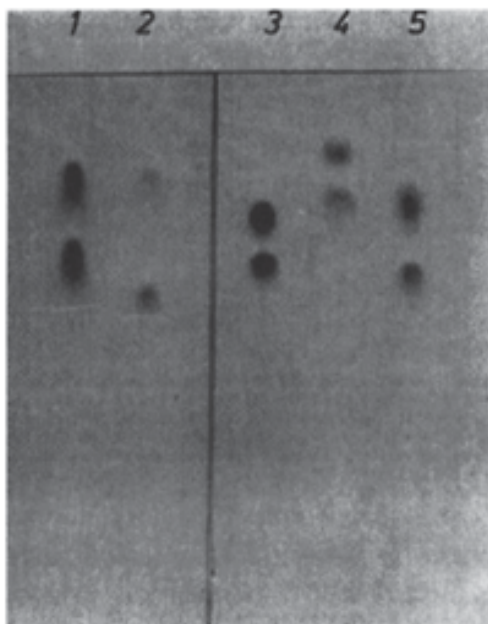


Figure 12 Thin-layer chromatogram of α -methyl amino acid on Chiralplate.

Spots: 1, α -Methylserine; 2, α -methyl dopa; 3, α -methylnmethionine; 4, α -methyltyrosine; 5, α -methylphenylalanine.

(set 2 s on the Tauchfix) dipped into this solution and then left to stand at room temperature for approximately 45 min. Blue derivatives formed on a yellow background.

Spectroscopy

Apparatus: Double-beam TLC scanner CS 930 (Shimadzu, Japan) or chromatogram spectrometer CD 60 (Desaga, Heidelberg, FRG)

Measuring principle: Monochromator-TLC plate (remission)

Light source: Tungsten lamp

Wavelength: See examples that follow.

Slit: 1.2×3 mm (CS 930), 6×0.4 mm (CD 60)

Scanning: 0.05 mm (CS 930), 0.1 mm (CD 60)

Table 11 TLC Separation of Enantiomeric α -Methylamino Acids^a

Racemate	R_f value (configuration)	Eluent ^b
α -Methylmethionine	0.56 (D)/0.64 (L)	A
α -Methylserine	0.56 (L)/0.67 (D)	B
α -Methyltyrosine	0.63 (D)/0.70 (L)	A
α -Methylphenylalanine	0.53 (L)/0.66 (D)	A
α -Methyldopa	0.46 (L)/0.66 (D)	B

^aMigration distance 13 cm; chamber saturation.^bA, methanol–water–acetonitrile, 50:50:200; B, methanol–water–acetonitrile, 50:50:30.**Table 12** TLC Separation of Enantiomeric *N*-Alkyl and *N*-Formyl Amino Acids^a

Racemate	R_f value (configuration)	Eluent ^b
<i>N</i> -Methylleucine	0.49 (L)/0.57 (D)	A
<i>N</i> -Methylvaline	0.65 (L)/0.70 (D)	B
<i>N</i> -Methylphenylalanine	0.59 (D)/0.61 (L)	A
<i>N</i> -Methyl-m-tyrosine	0.36/0.52	B
<i>N,N</i> -Dimethylphenylalanine	0.55 (D)/0.61 (L)	B
<i>N</i> -Formyl-tert-leucine	0.48 (+)/0.61 (–)	A
<i>N</i> -Methylaminobutyric acid	0.65/0.73	D
<i>N</i> -Ethylaminobutyric acid	0.69/0.72	D
<i>N</i> -Methylnorvaline HCl	0.67/0.76	D
<i>N</i> -Ethylnorvaline	0.70/0.74	D
<i>N</i> -Methylnorleucine	0.68/0.77	D
<i>N</i> -Methylalanine	0.64/0.70	D

^aMigration distance 13 cm; chamber saturation; eluent, methanol–water–acetonitrile, 50:50:200.^bA, methanol–water–acetonitrile, 50:50:200; B, methanol–water–acetonitrile, 50:50:30; D, acetone–methanol–water, 10:2:2.

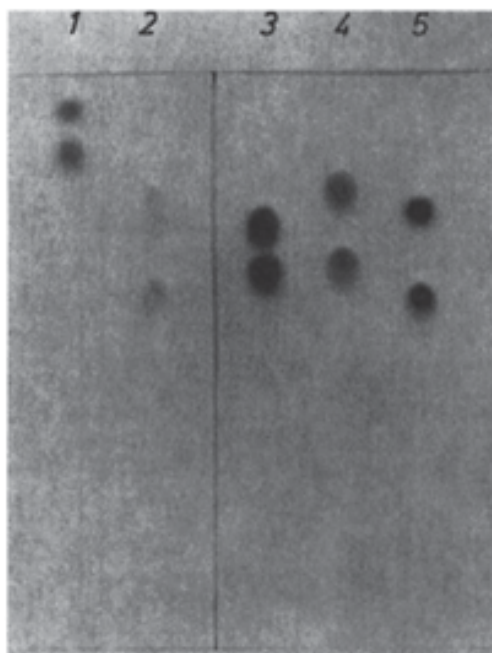


Figure 13 Thin-layer chromatogram of *N*-methyl and *N*-formyl amino acid on Chiralplate. Spots: 1, *N*-Methylvaline; 2, *N*-methyl-*m*-tyrosine; 3, *N*-methylleucine; 4, *N*-methylphenylalanine; 5, *N*-formyl-*tert*-leucine.

Table 13 TLC Separation of Halogenated Amino Acids^a

Racemate	R_f value (configuration)
3-Chloroalanine	0.57/0.64
4-Bromophenylalanine	0.44/0.58
4-Chlorophenylalanine	0.46/0.59
2-Fluorophenylalanine	0.55/0.61
4-Iodophenylalanine	0.45 (D)/0.61 (L)
3-Fluorotyrosine	0.64/0.71
5-Bromotryptophan	0.46/0.58
Thyroxine	0.38 (D)/0.49 (L)

^aMigration distance 13 cm; chamber saturation; eluent, methanol–water–acetonitrile, 50:50:200.

b. Chromatographic Conditions with HPTLC-CHIR

Method: Ascending, one-dimensional development in a TLC chamber with chamber saturation

Plates: HPTLC precoated CHIR plates with concentrating zone (Cat. No. 14285); size 10×10 cm; concentrating zone 2.5×10 cm

Eluent E: dichloromethane-methanol, 45:5

Sample volume: 1 μL of a 0.5% solution (methanol or methanol–dichloromethane, 1:1) applied as a 10 mm streak

Length of run: 5.5 cm

Time of run: 0.1h

Detection: Chiralplate

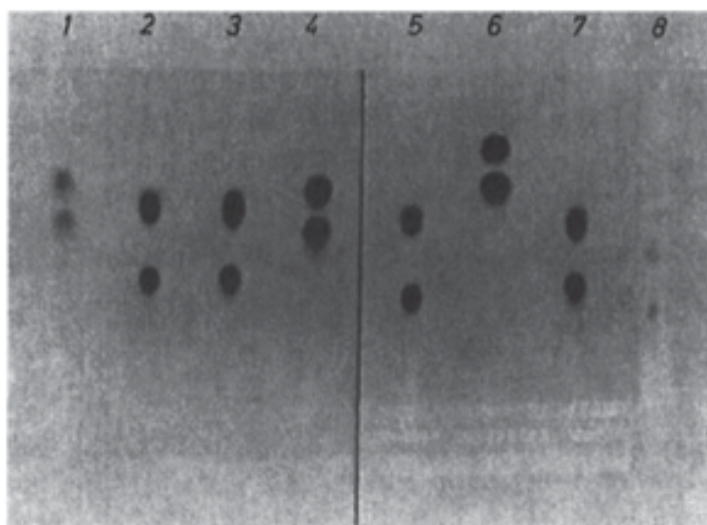


Figure 14 Thin-layer chromatogram of halogenated amino acid on Chiralplate.

Spots: 1, 3-Chloroalanine; 2, 4-bromophenylalanine; 3, 4-chlorophenylalanine; 4, 2-fluorophenylalanine; 5, 4-iodophenylalanine; 6, 3-fluorotyrosine; 7, 5-bromotryptophan; 8, thyroxine.

Table 14 TLC Enantiomeric Separation of Other Classes of Compounds^a

Racemate	R_f value (configuration)	Eluent ^b
Thiazolidine-4-carboxylic acid	0.59 (D)/0.69 (L)	A
5,5-Dimethylthiazolidine-4-carboxylic acid hydrochloride	0.48 (D)/0.62 (L)	A
3-Amino-3,5,5-trimethylbutyrolactone hydrochloride	0.50/0.59	A
Pipecolic acid	0.51/0.58	B

3-Carboxymorpholine	0.45/0.49	A

^aMigration distance 13 cm; chamber saturation.

^bA, methanol–water–acetonitrile, 50:50:200; B, acetone–methanol–water, 10:2:2.

Spectroscopy

Apparatus: Chromatogram spectrometer CD 60 (Desaga, Heidelberg, FRG)

Measuring principle: Monochromator-TLC plate (remission)

Light source: Tungsten lamp

Wavelength: 595 nm

Slit: 6×0.2 mm

Scanning: 0.05 mm

c. Selected Examples of Separation. With the technique described, more than 100 racemate separations have been accomplished by Günther, most of which have been published (206, 219, 220, 251–257). We do not describe all separations accomplished so far but instead demonstrate the versatile applicability of this method for some selected classes of compounds from the field of amino acid and peptide analyses. Additionally, the enantiomeric separation of α -hydroxycarboxylic acids is described.

Amino acids. Thus far, 12 proteinogenic amino acids have been separated without derivatization (Table 9, Figs. 8 and 9). Cysteine can be determined as thiazolidine-4-carboxylic acid, which is formed from cysteine by a simple reaction with formaldehyde. The separation of non-proteinogenic amino acids is shown in Fig. 10.

Dipeptides. For the enantiomeric separation of dipeptides (see Table 10) it is remarkable that the enantiomer with the C-terminal L-configuration always has a lower R_f value than the one with the C-terminal D-configuration (see Fig. 11). This method can also resolve diastereomeric

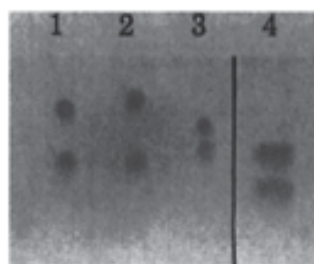


Figure 15 Thin-layer chromatogram of some heterocyclics on Chiralplate. Spots: 1, Thiazolidine-4-carboxylic acid; 2, 5,5-dimethylthiazolidine-4-carboxylic acid; 3, 3-amino-3,5,5-trimethylbutyrolactone hydrochloride; 4, pipecolic acid.

Table 15 TLC Enantiomeric Separation of α -Hydroxycarboxylic Acids

Racemate	R_f value			
	HPTLC-CHIR ^a	Chiralplate ^b		
Mandelic acid	0.36	0.48	0.41	0.48 (L)
3-Hydroxymandelic acid	0.24	0.31	0.28	0.34
4-Hydroxymandelic acid	0.22	0.29	0.25	0.31
3,4-Dihydroxymandelic acid			0.16	0.19
4-Hydroxy-3-methoxymandelic acid	0.24	0.33	0.37	0.41
2-Hydroxy-3-methylpentanoic acid	0.34	0.49	0.46	0.54 (L)
2-Hydroxy-4-methylpentanoic acid (sodium salt)	0.35	0.47	0.45	0.51 (L)
2-Hydroxy-4-methylthiobutanoic acid (sodium salt)	0.33	0.45	0.43	0.50 (L)
2-Hydroxy-3-phenylpropionic acid	0.39	0.51	0.46	0.53 (L)
2-Hydroxy-3-methylbutanoic acid	0.33	0.46	0.42	0.50 (L)
2-Hydroxybutanoic acid (sodium salt)	0.27	0.37	0.35	0.43
2-Hydroxyoctanoic acid	0.36	0.50	0.45	0.51
2-Hydroxytetradecanoic acid	0.34	0.49	0.49	0.55
2-Hydroxy-2-phenylpropionic acid	0.38	0.47	0.50	0.54
Lactic acid	0.19	0.24	0.30	0.34 (L)
2-Hydroxyhexadecanoic acid	0.39	0.56	0.51	0.55
2-Hydroxypentanoic acid	0.25	0.39	0.39	0.47
2-Hydroxydocosanoic acid	0.39	0.56	0.48	0.57

^aMigration distance 6.0 cm (measured from concentrating zone); eluent, dichloromethane–methanol, 45:5.

^bMigration distance 13 cm; eluent, dichloromethane–methanol, 45:5.

dipeptides (253). Wang et al. (98) compared the migration and separation characteristics of dipeptides on Chiralplate with those on cellulose. Marseigne (237) separated D,L-Asp-acc-OPr (dipeptide 56410 RP), a dipeptide with sweetening properties, whereas another group (236) investigated the separation of D,L-Asp-D,L-Phe-OCH₃ (aspartame).

α -Methylamino acids. α -Methylamino acids are very important as specific enzyme inhibitors. Furthermore, they can be directly inserted into numerous biologically active peptides to modify their range of activity. Separations in this field with different eluent systems have been

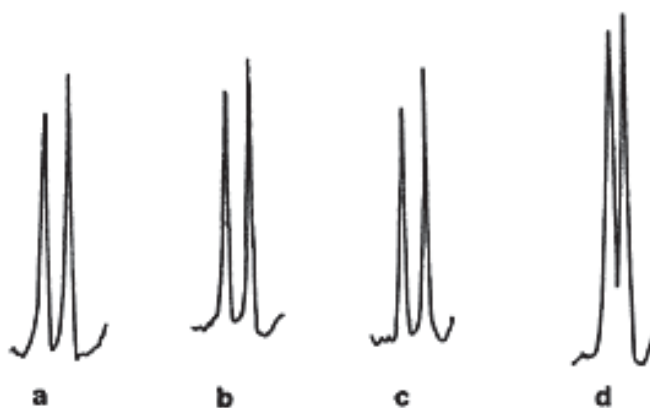


Figure 16 Remission-location curves:
 (a) D,L-2-Hydroxytetradecanoic acid;
 (b) D,L-2-hydroxyhexadecanoic acid;
 (c) D,L-2-hydroxydocosanoic acid; (d)
 D,L-lactic acid.

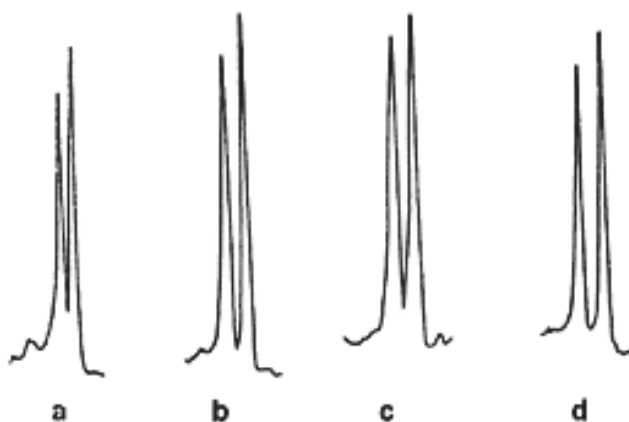


Figure 17 Remission-location curves:
 (a) D,L-2-Hydroxy-2-phenylpropionic
 acid; (b) D,L-2-hydroxy3-
 phenylpropionic acid; (c) D,L-2-
 hydroxybutanoic acid; (d) D,L-2-
 hydroxyoctanoic acid.

published independently (Fig. 12) (223, 224, 256). As can be seen from Table 11, D,L-methyldopa can also be separated without problems (255).

N-Alkylamino acids. Table 12 and Fig. 13 show the separation of enantiomeric *N*-alkylamino acids and *N*-formyl-*tert*-leucine. Further examples have been published recently (116, 219, 251). In contrast to the examples described above, the detection of *N,N*-dimethylphenylalanine was achieved with iodine. The enantiomeric separation of *N*-carbamoyltryptophan has also been described (225).

Halogenated amino acids. Another class of compounds that shows good enantiomeric resolution is the halogenated amino acids (Table 13 and Fig. 14). However, differentiation between 4-chloro-, 4-bromo-, and 4-iodophenylalanines is not possible (219, 251).

Heterocyclic compounds. Thiazolidine-4-carboxylic acid and 5,6-dimethylthiazolidine-4-carboxylic acid are formed by formaldehyde condensation from cysteine and penicillamine, respectively. The derivatization of penicillamine has been published (251). Table 14 and Fig. 15 presents a summary of these results. The chromatographic characteristics of the thiazolidine carboxylic acids formed by the reaction of D,L-penicillamine with various substituted benzaldehydes and heterocyclic aldehydes have also been studied (226). 3-Carboxymorpholine was separated by Günther and Merget (116).

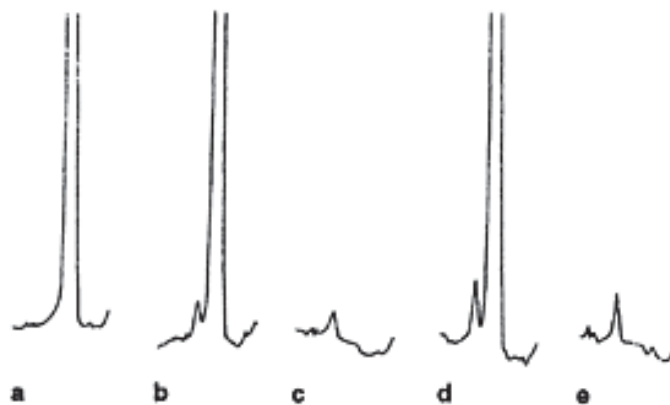


Figure 18 Remission-location curves: (a) D,L-2-Hydroxy-3-phenylpropionic acid; (b) L-2-hydroxy-3-phenylpropionic acid spiked with 1% D-enantiomer; (c) 1% D-2-hydroxy-3-phenylpropionic acid; (d) L-2-hydroxy-3-phenylpropionic acid spiked with 2% D-enantiomer; (e) 2% D-2-hydroxy-3-phenylpropionic acid.

α -Hydroxycarboxylic acids. During investigation of the enantioselective degradation of the biogenic *R*-structured catecholamines norepinephrine (noradrenaline) and epinephrine (adrenaline), Jork and Kany (222) for the first time succeeded in the enantiomeric separation of the resulting 3,4-dihydroxymandelic acid and vanillylmandelic acid, respectively, using the lipophilic eluent mixture dichloromethane-methanol (45:5) and postchromatographic detection with 2,6-dichloroquinone-4-chloroimide (Merck, Cat. No. 3037).

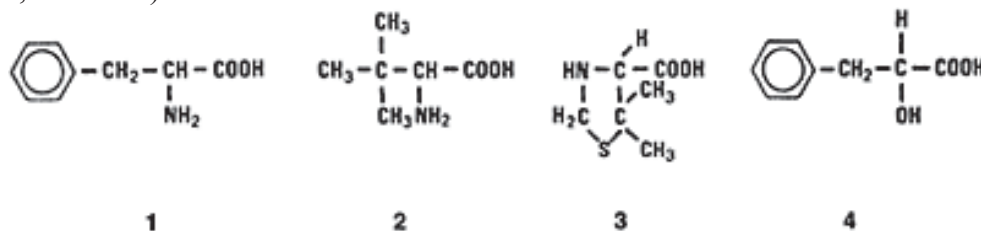
Table 15 shows comparative results for the separation of some α -hydroxycarboxylic acids on Chiralplate and HPTLC-CHIR. The remission-location curves in Figs. 16–18 were recorded with the HPTLC-CHIR plates. Vanadium pentoxide was especially useful

for postchromatographic derivatization (259) of the aromatic and aliphatic α -hydroxycarboxylic acids. For aromatic α -hydroxycarboxylic acids, manganese chloride-sulfuric acid (30 min, 120°C) was also suitable (229).

D. Quantitative Evaluation of TLC-Separated Enantiomers

1. General

Phenylalanine (1), *tert*-leucine (2), 5,5-dimethylthiazolidine-4-carboxylic acid (3), and α -hydroxyphenylalanine (4) have been chosen as models for the direct quantitative evaluation of thin-layer chromatograms. Emphasis has been placed on the evaluation of detection limits for the TLC-separated enantiomers, because exact determination of trace levels of a D- or L-enantiomer in an excess of the other is increasingly important (220, 258, 260–262).



To enhance specificity and sensitivity, postchromatographic derivatization with ninhydrin or vanadium pentoxide was used. Dipping the plates into the reagent solution proved most useful because it could be automated (263). Quantification of the minor enantiomer was achieved by in situ remission measurement with the CS-930 double-beam scanner (Shimadzu) or the densitometer CD 60 (Desaga) and comparison with external standard solutions. Additionally, possible proportional systemic deviations were excluded by the standard addition method (264).

For every substance investigated, the absorption maximum was determined independently prior to the quantification experiments.

2. Preparation of Test Solutions and Standard Solutions

Successful separation of amino acids on the TLC plate depends inherently on the concentration and often on the hydrochloric acid content of the applied solution. Addition of hydrochloric acid generally improves the solubility of the amino acids and often considerably enhances the enantiomeric resolution.

Phenylalanine test solution (U_{Ph}). Weigh 200 mg of D-phenylalanine into a 10 mL measuring flask and fill to the mark with 50% methanolic hydrochloric acid solution (10 g of acid per liter of solution).

Phenylalanine standard solution (V_{Ph}). Weigh 100 mg of L-phenylalanine into a 100 mL measuring flask and fill to the mark with methanol-0.1 M hydrochloric acid (1:1). From this stock solution the standard solutions are prepared for the working range required. Dilute 200 μ L, 400 μ L, 600 μ L, etc., of the stock solution to 10 mL with hydrochloric acid (10 g of acid per liter of solution)-methanol (1:1). Thus 0.1–0.3% solutions of the L-enantiomer relative to the 200 mg of D-phenylalanine are obtained.

Front	V_1	V_1	U	U	V_2	V_2	U	...
	Application:		as spots (U_H/V_H as streaks)					
	Volumes:		U_{Ph} and V_{Ph}		2 μ l each			
			U_L and V_L		1 μ l each			
			U_D and V_D		2 μ l each			
			U_H		1 μ l			
			V_H		1–6 μ l			
	Application unit:		Nanommat II		(Camag)			
			Microcaps		(Hirschmann)			
			Linomat IV		(Camag)			
Start	X	X	X	X	X	X	X	...

Figure 19 Application pattern for direct quantification of the enantiomers of phenylalanine (Ph), *tert*-leucine (L), 5,5-dimethylthiazolidine-4-carboxylic acid (D), and hydroxyphenylalanine (H).

tert-Leucine test solution (U_L). Dissolve 200 mg of L-*tert*-leucine in 10.0 mL of 50% methanol.

tert-Leucine standard solution (V_L). Dissolve 100 mg of *tert*-leucine in 100 mL of 50% methanol. Dilute 200 μ L, 400 μ L, 600 μ L, etc., of this stock solution to 10 mL with 50% methanol to obtain 0.1–1.3% D-enantiomer relative to 200 mg of L-*tert*-leucine.

5,5-Dimethylthiazolidine-4-carboxylic acid test solution (U_D). Add 500 μ L of concentrated hydrochloric acid to 500 mg of D-5,5-dimethylthiazolidine-4-carboxylic acid and make up to 10 mL with isopropanol.

5,5-Dimethylthiazolidine-4-carboxylic acid standard solution (V_D). Add 500 μ L of concentrated hydrochloric acid to 100 mg of L-5,5-dimethylthiazolidine-4-carboxylic acid and make up to 100 mL with isopropanol. Add 500 μ L of concentrated hydrochloric acid to 500 μ L, 1000 μ L, 1500 μ L, etc., of this stock solution, and make up to 10 mL with isopropanol. These solutions correspond to 0.1–0.3% of the L-enantiomer relative to 500 mg of D-5,5-dimethylthiazolidine-4-carboxylic acid.

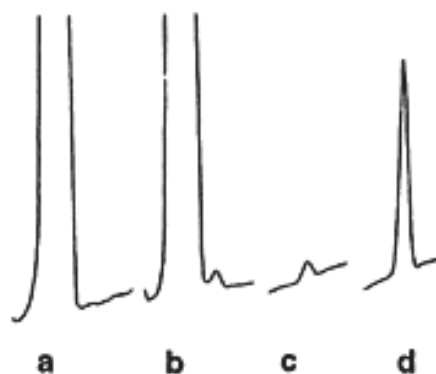


Figure 20 Remission-location curves: (a) D-phenylalanine (Phe); (b) D-Phe spiked with 0.1% L-Phe; (c) 0.1% L-Phe; (d) 1% L-Phe. Conditions: eluent A; $\lambda=540$ nm.

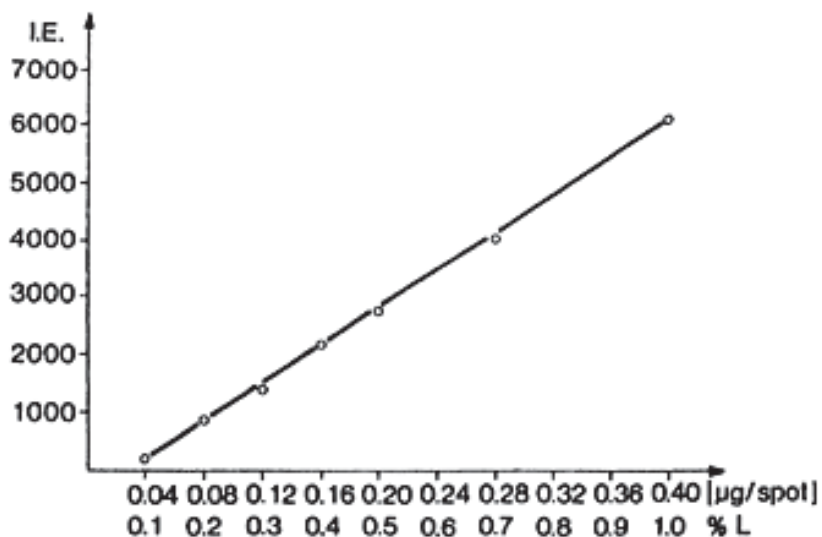


Figure 21 Calibration line for L-phenylalanine (V). I.E.=integration units; $y=-463+16.349x$; $r=0.9992$; $S_{xo}=0.0038 \mu\text{g/spot}$ (207); $\lambda=540$ nm.

Hydroxyphenylalanine test solution (U_H). Weigh 300 mg of L-hydroxyphenylalanine into a 10 mL measuring flask and fill to the mark with methanol.

Hydroxyphenylalanine standard solution (V_H). Dissolve 30 mg of D-hydroxyphenylalanine in 100 mL of methanol; 1 μL , 2 μL , 3 μL , etc., of this stock solution correspond to 1–3% of the D-enantiomer relative to 300 mg of L-hydroxyphenylalanine.

3. Application Pattern and Sample Volumes

For direct quantification of the enantiomers of phenylalanine, *tert*-leucine, 5,5-dimethylthiazolidine-4-carboxylic acid, and hydroxyphenylalanine, the pattern of application shown in Fig. 19 has been used.

4. Chromatographic Conditions

In general, the separation conditions for quantitative evaluation were similar to those for qualitative enantiomer separations by TLC. Any differences are explained below. The plates were TLC precoated Chiralplates (Cat. No. 811058, Macherey-Nagel), 20×20 cm; layer thickness, 0.25 mm. They were activated for 15 min at 110°C in a drying cabinet prior to use. The details of the eluents and detection procedures were as given above for the qualitative separation.

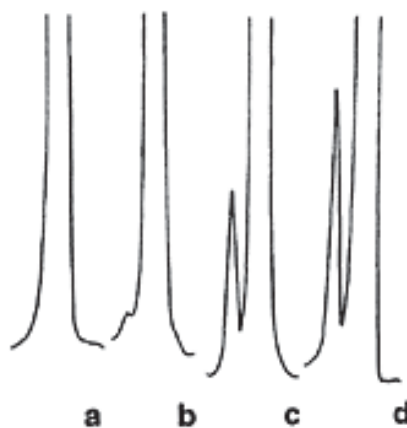


Figure 22 Remission-location curves: (a) L-*tert*-Leucine (Degussa); (b) L-*tert*-Leu+0.1% D-*tert*-Leu; (c) L-*tert*-Leu+1% D-*tert*-Leu; (d) external reference sample. Conditions: eluent C; $\lambda=540$ nm.

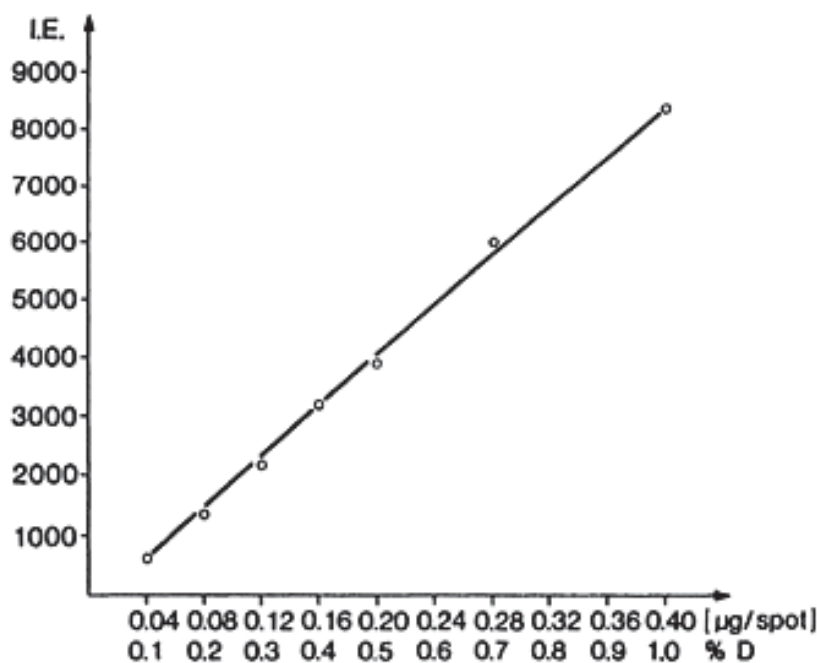


Figure 23 Calibration line for D-tert-leucine. I.E.=integration units;
 $y = -375 + 21.984x$; $r = 0.9980$,
 $S_{xo} = 0.0060 \mu\text{g/spot}$; $\lambda = 520 \text{ nm}$.

5. Spectrophotometric Conditions

Instrument, double-beam TLC scanner CS 930 (Shimadzu, Japan); measuring setup, monochromator-sample (remission); light source, Tungsten lamp; wavelength, see under remission-location curves in figures; measuring area, $1.2 \times 3 \text{ mm}$; feed 0.05 mm .

Instrument, densitometer CD 60 (Desage, Heidelberg, Germany); measuring setup, monochromator-sample (remission); light source, Tungsten lamp; wavelength, see under remission-location curves in figures; measuring area, $6.0 \times 0.4 \text{ mm}$; feed, 0.1 mm .

For the evaluation, the absorption curve was measured in the chromatographic direction. The measured peak areas or peak heights, plotted against the amount of sample per spot, gave the calibration lines.

6. Results

a. Phenylalanine. Figure 20 shows, among others, the remission-location curve for two standard solutions with widely different L-phenylalanine concentrations. The calibration line in

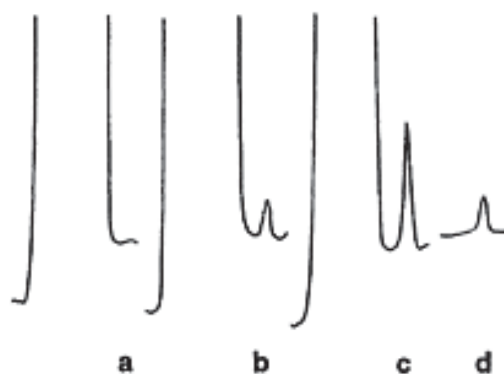


Figure 24 Remission-location curves: (a) D-5,5-Dimethylthiazolidine-4-carboxylic acid; (b) and (c) D-5,5-dimethylthiazolidine-4-carboxylic acid+0.1% and 0.5%, respectively, of the L-enantiomer; (d) 0.1% L-5,5-dimethylthiazolidine-4-carboxylic acid. Conditions: eluent A; $\lambda=370$ nm.

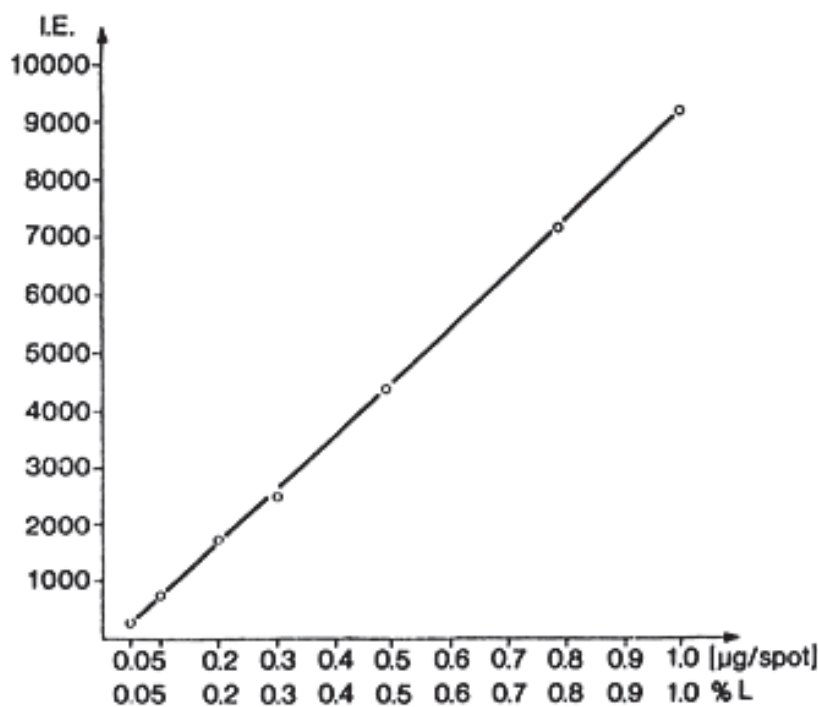


Figure 25 Calibration line for L-5,5-dimethylthiazolidine-4-carboxylic acid. I.E.=integration units;

$$y = -192 + 9224x; r = 0.9985; S_{xo} = 0.015$$

$\mu\text{g/spot}; \lambda = 370 \text{ nm}.$

Fig. 21 shows that quantitative determinations of L-phenylalanine in D-phenylalanine are possible in a working range of 0.04–0.4 $\mu\text{g/spot}$ without any problem.

b. tert-Leucine. The remission-location curve (Fig. 22) and the calibration line (Fig. 23) of tert-leucine show clearly that the D-enantiomer can also be determined with high sensitivity in the L-amino acid.

c. 5,5-Dimethylthiazolidine-4-Carboxylic Acid. The remission-location curve (Fig. 24) and the calibration line (Fig. 25) show that even 0.1–1% of the L-enantiomer can easily be quantified.

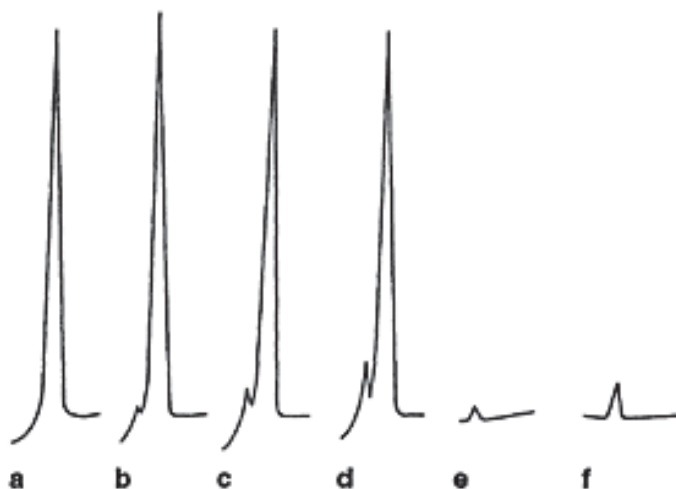


Figure 26 Remission-location curves: (a) L-Hydroxyphenylalanine; (b) 1% D-hydroxyphenylalanine in the L-enantiomer; (c) 3% D-hydroxyphenylalanine in the L-enantiomer; (d) 5% D-hydroxyphenylalanine in the L-enantiomer; (e) 1% D-hydroxyphenylalanine; (f) 1% D-hydroxyphenylalanine.

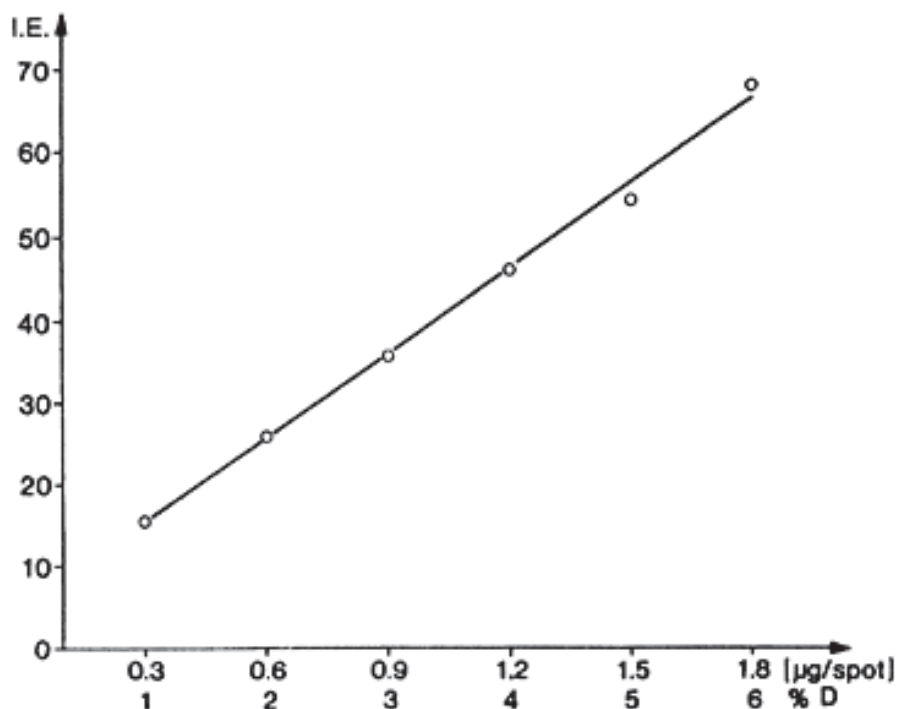


Figure 27 Calibration line for D-hydroxyphenylalanine (V).

I.E.=integration units; $y=4.91+34.2x$;
 $r=0.9981$; $S_{xo}=0.038 \mu\text{g/spot}$; $\lambda=590$
 nm.

d. α -Hydroxyphenylalanine. The remission-location curve (Fig. 26) and the calibration curve (Fig. 27) show good evaluation of the amount of D-enantiomer in the working range 1–6%.

XIII. CONCLUSION

This review does not claim completeness. We intended to demonstrate for a few selected examples the present possibilities of thin-layer chromatographic enantiomeric separations. Emphasis was placed on racemate separations with commercial plates based on cellulose, cellulose triacetate, Chiralplate, and HPTLC-CHIR, with detailed descriptions of the respective separation procedures and applications.

Because precise determinations of minute D- or L-concentrations in an excess of the other enantiomer become more and more important, the quantification of TLC-separated antipodes was treated explicitly. Further optimization of separation parameters and detection by fluorescence should enable improvement of the present detection limit of $\geq 0.1\%$ D- or L-component. Here it is worth mentioning that only the layers based on LEG with the 4-hydroxyproline selector are generally accepted, and these are the only ready-to-use plates commercially available on the market.

Compared to the classical methods of GC and HPLC, the TLC enantiomeric separation technique implies parallel (simultaneous) separations and is therefore especially well suited for economical routine analyses.

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18

Herbal Drugs, Herbal Drug Preparations, and Herbal Medicinal Products

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I. INTRODUCTION

Identification of medicinal plants is one of the oldest fields for the application of thin-layer chromatography (TLC). In the early 1950s, Kirchner et al. (1) used TLC for the analysis of herbal drugs and published many papers. Stahl standardized the methods of TLC by publishing his famous laboratory handbook in 1962 (2). This led to official recognition of TLC and its acceptance as an analytical tool. In the case of identification of herbal drugs by TLC, acceptance was very progressive and led to the publication of numerous methods that are still included in pharmacopoeias worldwide. However, with few exceptions, none of the official methods represents TLC as it is practiced today.

The past few years have seen tremendous growth in the use of herbal medicines worldwide. New products enter the market almost daily, and the demand for analytical methods to ensure safety and quality is rapidly increasing. A new methodology is necessary!

This chapter presents what modern instrumental high-performance thin-layer chromatography (HPTLC) has to offer for the analysis of herbals. Based on our experience with training courses, seminars, and workshops for analysts in the field as well as many discussions with specialists from industry, academia, and regulatory bodies, we propose herein solutions for today's problems and provide guidance for efficient use of HPTLC wherever it is applicable as part of the analytical toolbox. We appreciate the fact that there is more than one way to reach an analytical goal, particularly if a method offers the flexibility of planar chromatography. However, standardization can offer reliability and transferability, features that are important in laboratories that have to comply with good manufacturing and laboratory practice (GMP/GLP). Therefore, the chapter focuses on standardized methodology. The chapter is written primarily for novices who want to use HPTLC for herbal analysis, but we also hope that experienced professionals will find some points of interest to consider for their future work.

A. Scope

Interest in research concerning the constituents and biological activities of medicinal plants has significantly increased in recent years. Clinical studies have provided evidence that there is a great potential for herbal medicinal products. St. John's wort (3), ginkgo (4), and saw palmetto (5) can serve as good examples. The United States and European Pharmacopoeias are continuously revising their monographs on medicinal plants and have begun to include monographs for herbal extracts. Ten monographs including TLC identification are part of the National Formulary 19 (6) (Table 1). The 2001 edition of the European Pharmacopoeia (7) contains 173 monographs on drugs, including essential oils, gums, and resins, and six monographs on extracts. In 2001, Pharmeuropa (8) published 14 monographs on drugs and six on extracts for review and discussion. All but one

Table 1 Monographs on Botanical Raw Materials of the NF 19 (with TLC Identification)

Chamomile (<i>Matricaria recutita</i>)	Oriental ginseng (<i>Panax ginseng</i>)
Feverfew (<i>Tanacetum parthenium</i>)	Milk thistle (<i>Silybum marianum</i>)
Garlic (<i>Allium sativum</i>)	St. John's wort (<i>Hypericum perforatum</i>)
Ginger (<i>Zingiberis officinale</i>)	Saw palmetto (<i>Serenoa repens</i>)
Ginkgo (<i>Ginkgo biloba</i>)	Valerian (<i>Valeriana officinalis</i>)

Source: Ref. 6.

of the monographs includes a section on identification by TLC even though the published methods are still not optimized, as can be seen in Fig. 1.* The proposed method for the identification of lavender oil (*Lavendula officinalis*) requires a TLC plate, which has to be developed twice. If HPTLC silica gel is used as the stationary phase, better separation can be achieved in a single development.

Recently Pharmeuropa adopted the description of the chromatographic result as a table, which, in our opinion, is often inadequate. A well-documented image of an HPTLC plate contains more information (Fig. 2). The TLC atlas of herbal medicines of China (9) (110 illustrated methods in Chinese) can be regarded as a step in this direction. In 1997 the American Herbal Pharmacopoeia (AHP) (10) started to publish comprehensive monographs (13 up to February 2002). For the first time these monographs included HPTLC methods with the corresponding images for fingerprint identification of herbal drugs. They illustrate the potential of modern methodology. All the parameters required for reproducing such fingerprints, carefully selected for high-performance separations, are discussed in Section II.

What began in Europe with the analysis of herbal drugs is nowadays also applicable for extracts and (finished) herbal medicinal products. However, in industry, aside from identification, TLC is often used in quality control during production and in screening programs that eventually lead to new substances with specific medicinal properties. In the United States in particular, another analytical challenge has appeared—the analytical description and quality control of botanical products and dietary supplements. Even though in many cases the analyst has little or no knowledge about the constituents of the

plant and their activity, HPTLC can nevertheless prove the identity and consistency of a material. In Section III, typical tasks are discussed in detail.

B. Definitions

Unfortunately, different organizations often use different terms to describe the same entity. There are also differences between the European and American approaches to the analysis of herbal drugs and botanicals. Even analytical terms are not always defined in the same way. We will use the definitions given by the U.S. Food and Drug Administration (FDA) (11), the International Conference on Harmonisation (ICH) (12, 13), and the European Agency for the Evaluation of Medicinal Products (EMA) (14). The adjective “herbal” is widely used in Europe, whereas in the United States the adjective “botanical” is commonly employed to describe plant material. The definitions given in Appendix A are intended to clarify the terminology used in this chapter.

C. Typical Tasks

1. Identification

Identification can be considered the main application of planar chromatography. Identity is established by comparing a sample to a reference on the same plate. The prevailing value of HPTLC fingerprints is the visual impression, which can be further expanded by multiple detection. A

* A copy of all figures in full color can be obtained from the authors.

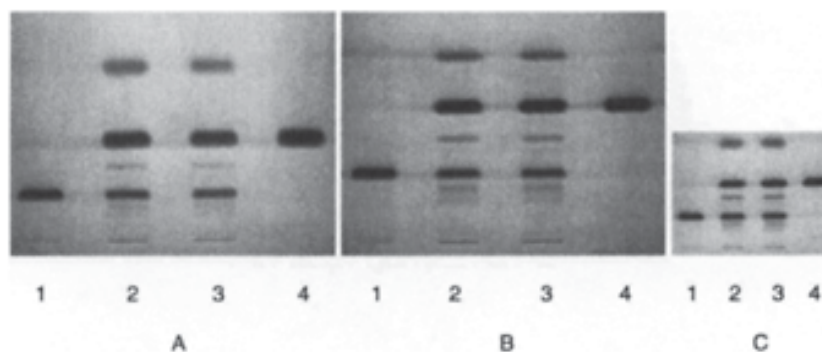


Figure 1 Identification of lavender oil (*Lavendula officinalis*) based on the proposed monograph of Pharmedropa 13.2. Mobile phase toluene–ethyl acetate (95:5); derivatization with anisaldehyde reagent. The upper edge of each image represents the solvent front. (A) TLC plate 20×20 cm Si 60

F₂₅₄. Separation distance (application position to front) 100 mm; developing time 15 min. (B) TLC plate 20 × 20 cm Si 60 F₂₅₄. Separation distance twice 100 mm; 5 min drying in stream of cold air between developments; total developing time 30 min. (C) HPTLC plate 10 × 10 cm Si 60 F₂₅₄. Separation distance 52 mm; developing time 8 min. Track assignment: 1, linalool; 2 and 3, lavender oil; 4, linalyl acetate.

broad spectrum of constituents can thus be detected and described without the need to know the chemical nature of each zone of the chromatogram. It is an added advantage of TLC that the entire sample is seen on the fingerprint and several samples can be compared side by side.

2. Stability Tests

Stability tests on herbal drug preparations and finished products are a new application of HPTLC. During such tests it must be shown that the material being tested does not change over the declared shelf life when stored under defined conditions. On the other hand, any changes that take place should also be detectable.

3. Quantification of Marker Compounds

Quantification of marker compounds is the most demanding application of planar chromatography. Owing to the limited separation power of the technique it is often not possible to obtain baseline separation of all components of complex samples such as plant materials. Therefore, most assays are based on HPLC. However, it is often overlooked that with proper instrumentation and suitable methodology quantitative determinations by HPTLC are not difficult.

D. Standardization of HPTLC Methods

One of the great advantages of planar chromatography is its flexibility. There are many parameters that can affect the chromatographic result (15), some of which are discussed below, and countless combinations that all give different results. Some combinations of parameters are advantageous for solving only one particular task and will not work with others. The extreme flexibility of TLC can turn into a problem unless one understands the influence and interaction of parameters, develops standardized procedures, and adheres to them. Too often the question about which TLC procedure is “better” is answered by choosing an HPLC method instead: Very few parameters must be defined, and those few

are easily kept constant. This is why, in our opinion, standardization should also be considered for HPTLC.

The methodological section that follows includes recommendations for each HPTLC step. These recommendations are applicable to most analytical tasks. Choices of combinations of stationary and mobile phases as well as those for postchromatographic derivatization and multiple detection still provide more flexibility than any other chromatographic technique can offer.

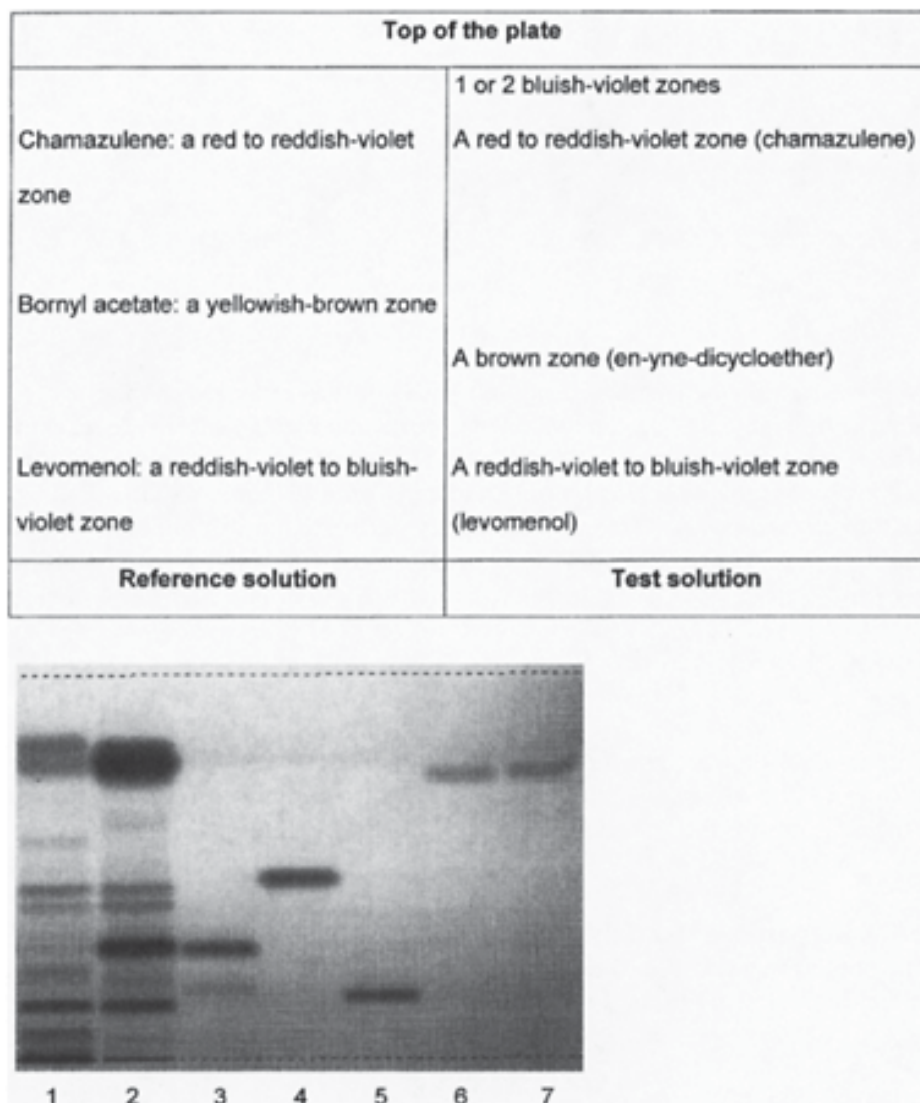


Figure 2 Representation of the chromatographic result of chamomile oil (*Matricaria recutita*, Pharmeuropa 13.2) in a table (top) and as an image (bottom). Mobile phase toluene–ethyl acetate (95:5); separation distance 100 mm. Derivatization: anisaldehyde

reagent. Track assignment: 1, Matricaria (chamomile) flowers (in addition to Parmeuropa method); 2, matricaria oil; 3, levomenol (bisabolol); 4, bornyl acetate; 5, borneol (in addition to Parmeuropa method); 6, guaiazulene (in addition to Parmeuropa method); 7, charnazulene.

E. Resources

Aside from the individual pharmacopoeias, resources on analysis of herbal drugs by TLC are limited. HPTLC methods are even harder to find; only AHP monographs include those. This is in part due to the fact that most analytical methods suitable for quality control in the herbal industry are proprietary. Another factor is the lack of standardization in TLC procedures in general. Efforts such as those by the Association of Official Analytical Chemists (AOAC) to put methods through a thorough validation process require a great deal of time and financial support and have therefore not yet been successful in meeting the growing demand of analysts.

The most useful starting point for solving an analytical problem with herbals drugs is probably *Plant Drug Analysis* by Wagner and Bladt (16), which provides about 200 color images of TLC separations of more than 180 plants arranged according to classes of constituents. A list of useful solvent systems for screening unknown plant material is given in Table 2. Another comprehensive entry is *Hager's Handbuch der Pharmazeutischen Praxis* (17); however, the TLC-related information does not usually include images of the chromatograms. Simplified TLC methods for about 80 herbal drugs that are commonly used in Germany are found in *DC-Atlas, Dunnschichtchromatographic in der Apotheke* by Pachaly (18). For many plants that are not included in the pharmacopoeias, TLC-related information can often be found in the CAMAG Bibliography Service (CBS), a searchable database that can be downloaded free of charge from the Internet (19).

II. GENERAL METHODOLOGY

A. Choices of Stationary Phases—TLC or HPTLC

Modern planar chromatography offers a great selection of stationary phases, which are discussed in detail in Chapter 4 of this Handbook. However, for the analysis of herbal drugs, silica gel is predominantly used. The high selectivity for differences in functionality of the analyte is one of the reasons. For fingerprint analysis particularly, it is desirable to separate and analyze as many compounds of different classes as possible in order to obtain a comprehensive profile of the plant. Adsorption chromatography is well

suited for this task. Another strong point of silica gel is its comparatively simple manufacturing process, which leads to lower costs while maintaining good batch-to-batch consistency. However, there are significant differences between plates of different manufacturers. When used for the predominantly qualitative purpose of identification by fingerprint analysis, this is usually not a question of “good or bad” but can concern the difference in sequence and color of the separated zones of the fingerprint. The binder, for instance, can affect the separation as well as detections based on color reactions. In many Asian countries, self-coated plates using carboxymethylcellulose as binder are widely used. If skillfully prepared, those plates can give quite reproducible results (20), yet compared to precoated plates using polymethacrylate

Table 2 Common Screening Systems for Unknown Herbal Drugs According to Wagner and Bladt

Substance class	Mobile phase (screening)	Detection	Derivatization
Alkaloid drugs	Toluene–ethyl acetate–diethylamine (70:20:10)	UV, 254 and 366 nm	Dragendorff reagent, sulfuric acid reagent, iodoplatinate reagent
Purine drugs	Ethyl acetate–methanol–water (100:13.5:10)	UV, 254 and 366 nm	Iodine–hydrochloric acid reagent
Anthracene derivatives	Ethyl acetate–methanol–water (100:13.5:10) or <i>n</i> -propanol–ethyl acetate–water–acetic acid (40:40:29:1)	UV, 254 and 366 nm	Potassium hydroxide reagent, Natural Products reagent
Essential oils	Toluene–ethyl acetate (93:7) or chloroform or dichloromethane	UV, 254 nm	Vanillin–sulfuric acid reagent, anisaldehyde–sulfuric acid reagent, phosphomolybdic acid reagent
Flavonoid drugs	Ethyl acetate–formic acid–acetic acid–water (100:11:11:26)	UV, 254 and 366 nm	Natural Products reagent and macrogol, Fast Blue salt B reagent
Arbutin, hydroquinone derivatives	Ethyl acetate–methanol–water (100:13.5:10)	UV, 254 nm	Berlin blue reaction, Millons reagent, Gibb’s reagent (dichloroquinonechloroimide)

Source: Ref. 16.

binders they can still be quite different. Unfortunately, none of the above statements can be generalized. A decision must be made for each case.

For stability tests and quantification of marker compounds, two other aspects are of importance: signal-to-noise ratio (a function of surface smoothness and homogeneity) and reproducibility across plates, and from one plate to another over long periods of time (a function of quality control during the manufacturing process). Only plates from a few reputable manufacturers are able to meet this requirement. In any case, it is desirable to develop a method for use with plates of one manufacturer. If so desired, the suitability of other plate material can be shown during the validation of the method. It should be noted

here that silica gel G (for gypsum as binder, nowadays often falsely used, even in many pharmacopoeias, as a synonym for TLC silica gel) is not widely available as a precoated layer today. Gypsum was the preferred binder of “homemade” plates. The layers are soft and not very durable. Modern precoated plates use organic binders, which create harder layers that are not affected during packaging, shipping, and storage.

Another decision to be made is that between TLC and HPTLC plates. There is no general reason why HPTLC plates should not be preferred over the conventional TLC plate. HPTLC plates give better separation and reproducibility and are more sensitive. Although slightly higher in price, HPTLC plates will pay off by significant savings in cost of solvents and analysis time. Typical pharmacopoeial methods require 45 min to 1 h for development on TLC plates. The same or better separation can often be achieved on HPTLC plates in 8–20 min (Fig. 1). Due to smaller particles and more homogeneous packing of the layers, HPTLC plates restrict the flow of the mobile phase more than TLC plates. Therefore the usable separation distance is limited to about 60 mm (see also Sec. II.C).

Upon storage and handling, silica gel will interact with the environment, adsorbing water vapor as well as fumes and dust. This has two consequences: (a) The activity of the plate is dependent on the relative humidity of the surrounding atmosphere and (b) when developed with polar solvents, “dirt zones” can be seen at the position of the solvent front. Whereas for most qualitative analyses plates are typically used “out of the box” without any pretreatment, it is important to consider a standardized cleaning procedure if the analytical method has to be validated (stability test, quantification) and reproducible results are required. The activity of the plate affects the R_f value of the analyte. The higher the activity, the lower the R_f . Heating the plate to 120°C increases its activity. At that temperature adsorbed water is removed from the surface of the silica gel. High activity is not necessarily desirable, because it can cause tailing. Technically it is rather difficult to maintain a specific activity of the silica gel for chromatography. It can be done, though, by conditioning the plate prior to development, over sulfuric acid for an extended period of time (Fig. 3). A TLC system (layer and developing solvent) is more sensitive to changes in relative humidity the less polar the developing system is. Therefore, it is recommended to

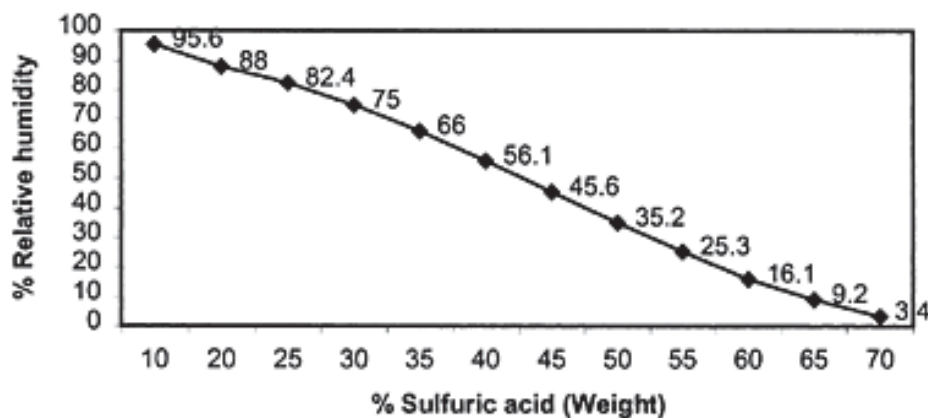


Figure 3 Relative humidity as a function of the concentration of

sulfuric acid at 20°C. (Adapted from Ref. 50.)

develop methods that are not sensitive to small changes in humidity. In any case it is advisable to record the actual relative humidity and the temperature during chromatogram development.

To remove impurities that have accumulated on a plate, prewashing or predevelopment with methanol is recommended (21). The cleaned plate is dried (and activated) in an oven at 120°C for 20 min. In an environment that is free of dust and fumes (e.g., a large empty desiccator), the active plate is cooled to room temperature and equilibrated with the relative humidity of the laboratory atmosphere.

Aside from silica gel as stationary phase, the literature on TLC separation and isolation of natural products such as phenolics and flavonoids often refers to cellulose and polyamide. Both stationary phases have been used for several decades and have their merits with respect to selectivity; however, from an analytical point of view, HPTLC on silica gel will give superior results in most cases. Chemically bonded phases based on silica gel can offer some advantages because of their different selectivity. Figure 4 shows the separation of St. John's wort (*Hypericum perforatum*) on selected stationary phases. The mobile phase was kept constant. One of the most promising phases is diol-modified silica gel. It offers selectivity similar to that of silica gel but is not affected by changes in relative humidity. Bonded phases are, unfortunately, rather expensive, and it seems that batch-to-batch conformity is not always achieved.

Recommended stationary phase for standardized HPTLC. 10×10 cm or 20×10 cm HPTLC plates, silica gel 60 F₂₅₄, predeveloped in methanol, dried at 120°C for 20 min, cooled to room temperature, and equilibrated with the relative humidity of laboratory.

B. Sample Application

It has been discussed in Chapter 5 of this book that sample application determines to a great extent the quality of the chromatographic result. Identifications are predominantly based on comparison of R_f values (migration distances). Therefore the samples must be precisely positioned. Contrary to common practice, it is not advisable to mark the application positions on the layer. This can damage the plate surface, and pencil marks can interfere with the sample transfer onto the layer. Pencil marks will definitely affect evaluation by scanning densitometry.

Samples should not be applied too close to the edges of the plate because there can be irregularities in the layer thickness. For quantitative determinations the left and right margins should not be less than 15 mm for HPTLC plates and 25 mm for TLC plates. When development starts, the application position must be clearly above the level of the developing solvent. When

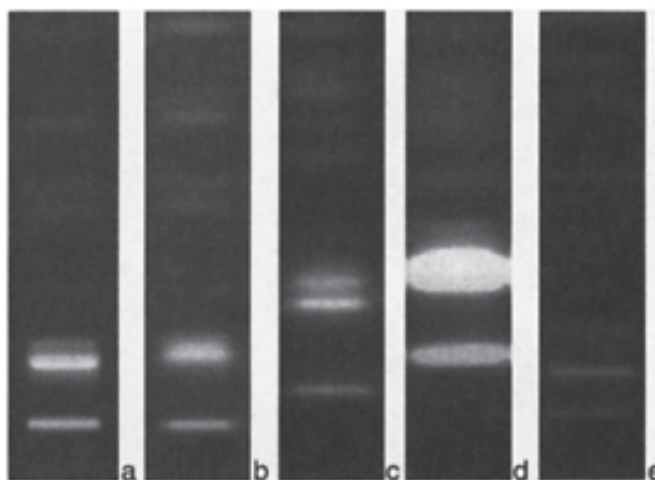


Figure 4 Separation of St. John's wort (*Hypericum perforatum*) on various stationary phases. All HPTLC plates 10×10 cm, separation distance 52 mm, mobile phase ethyl acetate–formic acid–acetic acid–water–dichloromethane (100:10:10:11:25), UV at 366 nm after derivatization with Natural Products reagent/macrojol 400. (a) silica gel 60 F₂₅₄; (b) LiChrospher Si 60 F₂₅₄; (c) silica gel 60 WR F_{254s}; (d) silica gel 60 DIOL F₂₅₄; (e) silica gel 60 NH₂ F_{254s}.

the solvent level is maintained at 5 mm (HPTLC) or 10 mm (TLC), a suitable application position above the lower edge of the plate would be 8 or 15 mm, respectively. The distance between (the edges of) bands should be at least 2 or 4 mm, respectively. The centers of spots are usually spaced 5 mm apart on HPTLC plates and 10 mm on TLC plates.

The volume of the applied sample must also be controlled. This is particularly important for quantitative determinations, but qualitative comparisons also require defined volumes for reproducible results. An applied spot should not be larger than 1.5 mm on an HPTLC plate and not larger than 4 mm on TLC plates. When samples are applied by contact spotting, the volume should not exceed 1 μ L on HPTLC plates or 5 μ L on TLC plates. A very common problem with most pharmacopoeial methods is that the specified sample volumes and the applied amounts of sample are much too large. Even if samples are applied as bands, the plate may be overloaded, in which case separation efficiency decreases. Overloaded samples can be noticed by their excessive tailing.

In general, the separation can be improved if the sample is applied as a band rather than as a spot. However, significant improvement can be achieved only if the spray-on

technique is employed. The applied sample zone must be as small as possible in the direction of chromatography for best results. It is not the “shape” of the applied zone that improves the separation, but the fact that, unlike in contact spotting, properly performed spray-on application avoids any chromatography during application. Very sharp bands of variable length can therefore be obtained (22). Another advantage of the technique is that comparatively large volumes can be concentrated into narrow bands, so the application volume can be chosen according to the concentration of the analyte. In herbal analysis the extraction solvents are often very polar (methanol, ethanol–water, etc.). Application of such samples by contact transfer as spots creates round zones that will migrate without improving their shape (Fig. 5). Even though the fingerprint in the given example is not clearly structured, it can be seen that the spray-on technique offers significant improvement of the separation regardless of the polarity of the sample solvent.

It should be mentioned that neither the application of bands by “streaking” (using a handheld syringe) nor the use of plates with concentration zones to transfer spots into bands can be considered a suitable alternative to the spray-on technique, although the “visual impression” of the developed chromatogram may show an improvement in separation over spot application. Depending on the analytical task, a wide range of more or less sophisticated instrumentation is available for sample application. For details, see Chapter 5, Section II.C.

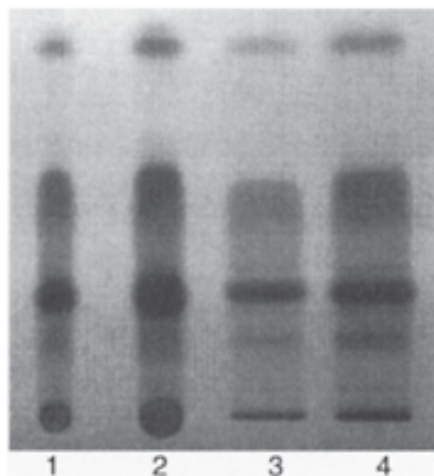


Figure 5 Comparison of HPTLC fingerprint of bearberry leaf (*Arctostaphylos uva ursi*) on silica gel 60 F₂₅₄, following application as spot (left) and band (right). Mobile phase ethyl acetate–formic acid– water (88:6:6), separation distance 52 mm. Derivatization: Gibb’s reagent. Track assignment: 1 and 3, 1 μ L bearberry leaf extract; 2 and 4, 2 μ L bearberry leaf extract.

Recommendation for standardized sample application. Generally band application by spray-on technique, 8 mm bands, 10 mm apart (center to center), 8 mm from lower edge of plate (Y), first application position 20 mm (center) from left edge of the plate (X), not less than 2 μL volume per band. Spot application only for samples with few components or for large numbers of samples. First $X=15$ mm, $Y=8$ mm, distance between spots 5 mm, 0.5 μL sample per spot.

C. Chromatogram Development

It is a unique feature of planar chromatography that in addition to stationary and mobile phases a gas phase (containing vapor of the mobile phase) is present, which can affect the separation significantly (23). The geometry of a chamber and its degree of saturation can therefore greatly influence the chromatographic result. Figure 6 shows three chromatograms of Wu wei zi (*Schisandra chinensis*) visualized under UV light at 254 nm and, after derivatization with anisaldehyde, in visible light. The sample, stationary phase, and mobile phase are the same in both cases, the only differences being the saturation and the type of developing chamber. The choice of chamber is generally based on personal preference and on economic considerations. Because in most cases different chambers will give different results, a decision must be made when establishing a method. Technical details of various developing chambers can be found in Chapter 5, Section III.B. Some practical considerations are added here:

1. Developments in sandwich mode usually give the sharpest zones and reproducible separation. However, solvent mixtures often produce secondary fronts, which may interfere with separation.
2. Unsaturated chambers give sharp zones and allow short analysis time. However, reproducibility can be a problem, and solvent fronts may not be straight ("banana front") if the mobile phase contains volatile components. R_f values are higher than in saturated chambers.
3. Saturated chambers are very stable systems and tend to give reproducible results. However, zones are often slightly more diffuse, and saturation is time-consuming. R_f values are lower than in unsaturated chambers.

Because the mobile phase is moved through the stationary phase by capillary action (except in OPLC), its velocity decreases with increasing migration distance. Consequently, the chromatographic efficiency decreases. The optimum separation distance on HPTLC plates is 50 mm (24). As illustrated in Fig. 7, increasing the separation distance results in an extreme increase in required time and in diffuse chromatogram zones. Even less than 50 mm is sufficient if only a few components are present in the sample. The separation distance on TLC plates should be between 10

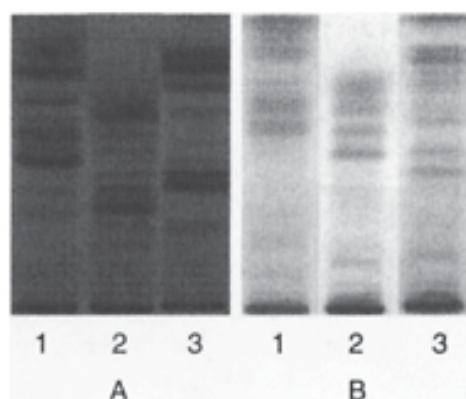


Figure 6 HPTLC of Wu wei zi berries (*Schisandra chinensis*) on HPTLC silica gel 60 F₂₅₄. Mobile phase ethyl acetate–toluene–acetic acid (70:30:3); separation distance 52 mm. (A) UV, 254 nm. (B) Anisaldehyde reagent. Tracks: 1, unsaturated twin-trough chamber; 2, saturated twin-trough chamber; 3, horizontal developing chamber in sandwich configuration.

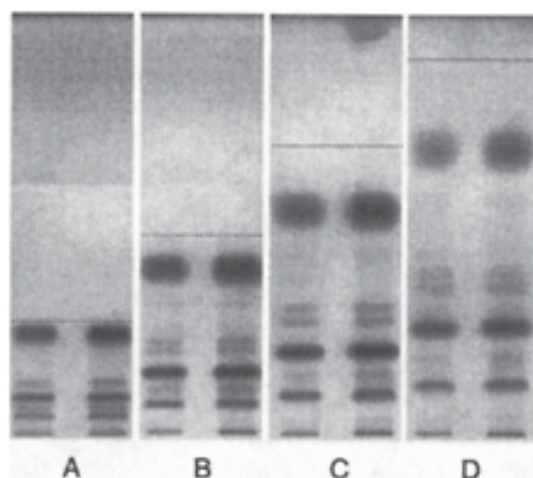


Figure 7 Influence of the developing distance on the separation of chamomile oil (*Matricaria recutita*), 1 and 2 μ L extract each on HPTLC silica gel. Mobile phase toluene–ethyl acetate (95:5). Derivatization with anisaldehyde reagent. Separation

distance (A) 22 mm (required 3 min);
(B) 42 mm (required 7 min); (C) 62
mm (required 13 min); (D) 82 mm
(required 20 min).

and 15 cm. Prior to development the migration distance of the solvent front is marked on one edge of the plate with a short pencil mark. It is not advisable to draw or scratch a line across the plate!

Automated multiple development (AMD) is a gradient technique that uses repeated development of HPTLC plates. The solvent strength of each development is lower than that of the previous step. This is combined with increasing migration distance for each step. After each development the plate is dried by vacuum. AMD has also been used for the separation of herbals (25, 26).

Recommendation for standardized chromatogram development. Saturated twin-trough chamber 5 or 10 mL of developing solvent in the front trough of a 10×10 cm or 20×10 cm chamber, respectively. The rear trough is fitted with a filter paper and contains 5 mL developing solvent for the smaller chamber, 10 mL for the larger one. The chamber is saturated for 20 min prior to plate development. Developing distance is 60 mm from the lower edge of the plate (52 mm from the application position). The plate is positioned in the front trough with the stationary phase facing the inside of the chamber.

D. Derivatization

The possibility of convenient postchromatographic specific or nonspecific chemical derivatization is another strong point of planar chromatography. For the analysis of botanicals, numerous derivatizing agents are available (Table 3). The only decision to be made is about how to transfer the reagent to the plate. For liquid reagents, either spraying or dipping is possible. Although spraying is fast and uses small volumes of reagent, it also generates fumes, which must be removed using a spray cabinet. Achieving a homogeneous derivatization across the plate requires great skill. A homogeneous reagent transfer can be performed by dipping. This requires larger reagent volumes (up to 200 mL for 20×10 cm plates). Although most pharmacopoeial methods specify derivatization by spraying, it is recommended to consider dipping, which requires that the reagent concentration be reduced and the solvent selected to avoid washing off the separated samples during derivatization. Billeter et al. (27) published an illustrative example, i.e., the adaptation of Natural Products reagent for derivatization of flavonoids by immersion.

Often a chemical derivatization is completed with a heating step. It is important that the plate heater maintains a uniform temperature across its surface. For reproducible results the temperature of derivatization should be adjusted so that the plate is heated for at least 5 min.

Table 3 Most Common Nonspecific Derivatization Reagents for Analysis of Plant Materials

Reagent	Substance classes
Anisaldehyde or vanillin with sulfuric or phosphoric acid	Phenols, steroids, glycosides, saponins, carbohydrates (sugars), terpenes (essential oils)
Sulfuric acid	Steroids, saponins, sapogenins, cardiac glycosides, alkaloids, lipids
Fast Blue salt B	Phenols, tannins, coumarins, flavonols, phenol carbonic acid, cannabinoids, amines
Natural Products reagent	Flavonoids, carbohydrates (sugars), anthocyanins
Ammonia vapor	Alkaloids, flavonoids, anthrones

Source: Ref. 51.

Recommendation for standardized derivatization. Derivatization by immersion (rapid immersion and short exposure) in a suitable reagent, for instance 10% sulfuric acid in methanol (general non-specific reagent), followed by heating of the derivatized plate for 5 min at 110°C or until colored chromatogram zones appear.

E. Labeling and Documentation

Chromatographic plates are sensitive to impurities from the environment and must be handled with extreme care. Labeling should be performed only in an area not used for chromatography. Commonly, the area close to the upper edge of the plate is labeled with a soft pencil. When GLP-coded plates are used, the laser-etched number of the plate serves as an ID. The GLP code is also visible under UV light when a narrow strip of white paper is placed under the plate.

To take full advantage of the visual impression that can be obtained from a TLC chromatogram, the plate should be documented. Today video and digital documentation systems are widely used for this purpose. Combined with a suitable lighting unit, multiple detection of the same chromatogram under 254 and 366 nm UV light and visible light (prior to and/or after derivatization) can provide a wealth of data, particularly in fingerprint analysis. For a description of instrumentation, see Chapter 5, Section IV of this Handbook. Modern electronic documentation systems produce instant images, which

- Can be easily edited, archived, and evaluated
- Are reproducible and do not change over time
- Are GMP/GLP-compliant if generated with suitable software
- Can be quantitatively evaluated with video densitometry

Hahn-Deinstrop (28) described several aspects of electronic documentation in detail. When images of HPTLC are used as the basis for stability tests or quantitative determinations, the documentation process must be rigorously standardized.

Recommendation for standardized labeling and documentation. Label the plate with a soft pencil in the upper right corner. The label should contain the project ID, the date, and a consecutive plate number. Documentation is done with a video or digital system under UV light at 254 and 366 nm prior to derivatization and under 366 nm and visible light after derivatization. Image labels should include plate ID and derivatization and illumination mode.

F. Evaluation

Depending on the analytical task, evaluation in HPTLC can be performed in several ways.

1. Visual Inspection

Visual inspection of the chromatogram and comparison to a reference standard is usually done during identification. The result of such an evaluation is usually a yes or no decision, meaning that the specified identity is established or not, based on whether acceptance criteria are met. Typically, the analyte and reference standard are chromatographed side by side on the same plate. However, comparison can also be made to results obtained from other plates or their images (book, electronic library, etc.) or to a verbal description of the expected results, or to both. This is a valuable technique if the identity of the analyte is not known or uncertain and in cases when reference standards are not available. As a prerequisite, the employed HPTLC fingerprint method must be documented in detail and should be validated. If several images of the same plate are generated during multiple detections in order to increase the certainty of the analytical result, it is important that none of the derived decisions about the identity of the analyte contradicts the others. Visual inspection is always subjective, and it is therefore important to properly document the chromatogram in a “durable” form to enable independent peer verification at a later time.

2. Video Densitometry

Video densitometry performed on images of the HPTLC chromatogram allows comparison of analog curves of the individual tracks and, if chromatography was done under identical conditions, comparison from plate to plate. Using this technique for identification purposes, it is predominantly the number, position, and relative area or height of the peaks on the compared tracks that are evaluated. Semiquantitative statements in regard to changes during a stability test can thus be supported.

3. Scanning Densitometry

Scanning densitometry offers the most accurate type of evaluation in HPTLC, particularly for assay and quantification of marker compounds. Absorbance or fluorescence of separated compounds is measured and evaluated against that of reference standards of known quantity. For details see Ref. 29. Scanning densitometry offers a great advantage over visual inspection and video densitometry owing to its spectral

selectivity. Because monochromatic light in the range of 190–800 nm can be used and tuned to the absorption or fluorescence maximum of the individual compounds, the measurement is very sensitive. Typical detection limits are in the low nanogram range (absorbance) or middle picogram range (fluorescence). Densitometry is usually performed prior to derivatization. Substances without chromophoric groups must be chemically altered to render them detectable.

Scanning densitometry can also be used for identification by comparing profiles of the analog curves of individual sample tracks. This includes multiwavelength scanning, i.e., the sequential scanning of each chromatogram track with up to 30 wavelengths, and the evaluation of each track at all wavelengths. UV spectra of all separated substances can also be recorded and used for identification purposes. Meaningful densitometric evaluation requires “good” chromatography; i.e., all previous steps of HPTLC should be done with great care and according to standardized methods.

Recommendation for standardized evaluation. Scanning in absorbance mode with a slit 6×0.3 mm, scanning distance 5–65 mm at 254 nm using a deuterium lamp. When target compound(s) fluoresce, scanning in fluorescence mode with a mercury lamp at 366–>400 nm. Recording spectra from 200 to 400 nm for each detected peak. Performing quantitative evaluation at absorption maximum of target compound(s).

III. PRACTICAL CONSIDERATIONS

A. Identification

1. General Aspects

Regulatory agencies [FDA (11) and EMEA (14)] recommend fingerprint chromatography as the basis for identification of herbal drugs, herbal drug preparations, and herbal medicinal products. In HPTLC the fingerprint of one sample (unknown) is compared to that of another sample (reference substance), which is chromatographed on the same plate. This procedure can be performed for raw materials, extracts, and finished products and also for complex samples such as multidrug preparations (30). The sample must meet certain predefined acceptance criteria such as number, sequence, position, and color of separated zones. The only requirement of the procedure is that it must be specific in the sense that the sample for which the identity is to be established must meet the acceptance criteria and any other material such as adulterants fail the test.

An HPTLC fingerprint or, better, sets of fingerprints based on multiple detection of the same plate can provide a very characteristic visual impression of a sequence of (colored) zones that “describe” the sample. Compared to GC or HPLC chromatograms, such fingerprints usually show less resolution but are still sensitive to minor differences between two samples. Given a reliable methodology, this allows establishing two samples as “equal” within certain limits based on amount (intensity), number, and kind (color) of principal constituents and their relative sequence.

Either a chemically defined compound (standard) or a botanical raw material that was authenticated by a botanist using macroscopic, microscopic, organoleptic, genetic, and other information (voucher specimen) can serve as a reference. Unlike synthetic drugs,

which can easily be characterized, herbal drugs are difficult to deal with, because, technically speaking, no two raw materials are exactly alike. In many cases the detailed chemical composition is not even known. The same can be said for herbal drug preparations and finished products. The constituent profile of the plant material can be affected by several factors (see Table 4). As a consequence there is a certain range within which the identity of a sample must be looked at.

An example that illustrates the complexity of the information that can be obtained during HPTLC fingerprint identification is valerian (*Valeriana officinalis*) according to the AHP monograph (31). Figure 8 shows multiple detections performed on the same plate. On tracks 1–4, four samples of *Valeriana officinalis* are separated, track 5 contains valerenic acid; track 6, *Valeriana sitchensis*; and track 7, *Valeriana wallichii*. Although the three botanical species can be distinguished, it might seem difficult to label the fingerprints of the four samples of *Valeriana officinalis* as equal. However, they are still more similar to each other than to the other species. Furthermore, samples 1 and 2 are more similar, and so are samples 3 and 4. Samples on tracks 1 and 2 were from botanical gardens in the United States, and the samples on tracks 3 and 4 were from Europe. The sample on track 3 was bought at a market in the Netherlands and is the only one not certified. This sample's track contains a zone not present in any of the other samples' chromatograms (32).

Table 4 Factors Influencing the Substance Spectrum of Botanical Raw Material and Botanical Drug Substances

Genetic factors	Chemical races; variability
Environmental factors	Climate (temperature, light, rain); soil (pH, fertilization, heavy metals); insects, pests, microbiological infection
Raw material production	Part of the plant (root, bark, flower, leaf, fruit, etc.); harvest time (before, during, after flowering time); aftertreatment (washing, peeling); drying (method, duration, temperature)
Treatment of raw material to produce drug substances	Pulverization (fine or coarse cut, grinding temperature); extraction (solvent polarity, temperature, duration); distillation (temperature); expression (temperature); fermentation (temperature, duration)
Storage	Light, oxygen (radical building, self-oxidation); humidity (hydrolysis, enzymatic reaction, microbiological infection); temperature (polymerization, decomposition, microbiological infection)

Source: Ref. 52.

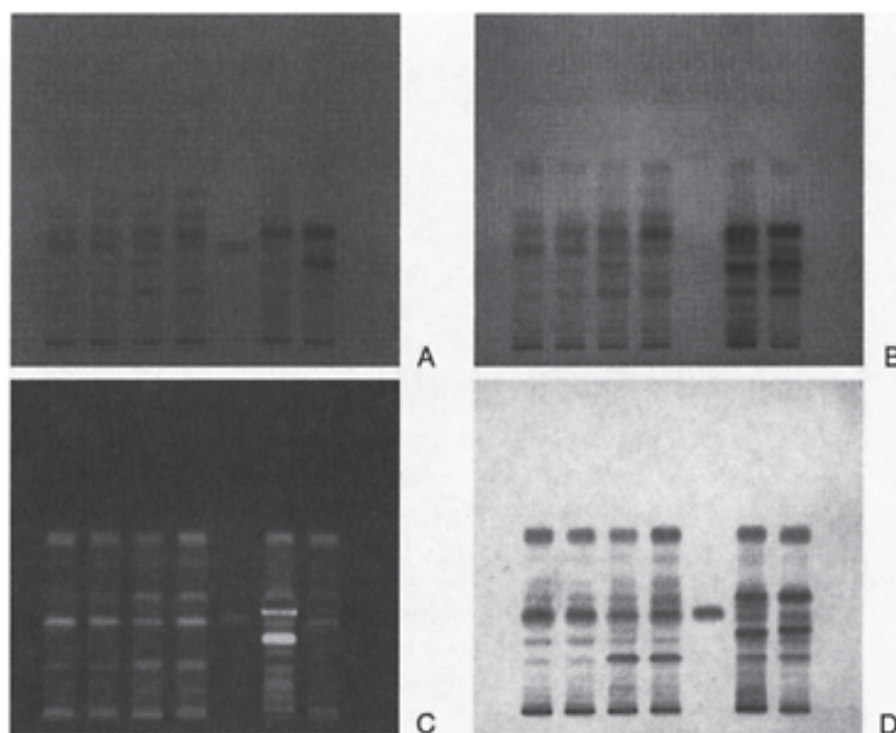


Figure 8 Identity tests on valerian (*Valeriana officinalis*). (A) Fluorescence quenching. All compounds absorbing UV 254 nm are visible. (B) Detection by visible light after derivatization with HCl–acetic acid. Valepotriates appear as colored zones. (C) Fluorescence under 366 nm UV light after additional derivatization with anisaldehyde reagent: general derivatization. (D) Detection by visible light after additional derivatization with anisaldehyde reagent: general derivatization.

Figure 9 illustrates the interesting problem of chemical races. On the basis of their HPTLC fingerprints, three different (chemical) types can be distinguished for ashwaganda (*Withania somnifera*) (33). The phenotypes of the plant samples are identical; therefore, the presence of chemical races can be concluded. If the existence of chemical races had not been known, two of the three samples would not pass as ashwaganda.

Aside from a simple yes-or-no question of whether a sample is of a certain identity and therefore passes or fails quality control, a more difficult problem arises when

adulteration is to be detected. In this case the problem is to find out what adulterant and how much of it is present in a given sample. Black haw (*Viburnum prunifolium*) and cramp bark (*Viburnum opulus*), for example, can easily be confused. As can be seen in Fig. 10, not only can both plants be distinguished by their HPTLC fingerprints, but also the percentage of one species in a mixture with the other can be semiquantitatively assessed based on the intensity of the compounds marked with an arrow. For more precise quantitative measurements, scanning densitometry could be used.

Statements regarding the identity of a raw material can be made as long as its fingerprint is characteristic. This is possible even when there is no information at all about the chemical constituents of a given plant. Figure 11 shows the separation of reishi mushrooms (*Ganoderma lucidum*). The HPTLC fingerprint allows the fruiting body to be distinguished from the mycelium of the same species as well as different species from each other.

An important feature of HPTLC is the large number of samples that can be analyzed in parallel, thus affording rapid results. HPTLC fingerprints are often used to monitor the production of extracts and finished products. During process development, HPTLC can help establish proper extraction parameters, standardize and normalize extracts, and detect any changes or degradation in the material during formulation (34, 35). After the raw material has been identified it must also be demonstrated that during production of the preparation and the final product the “composition”

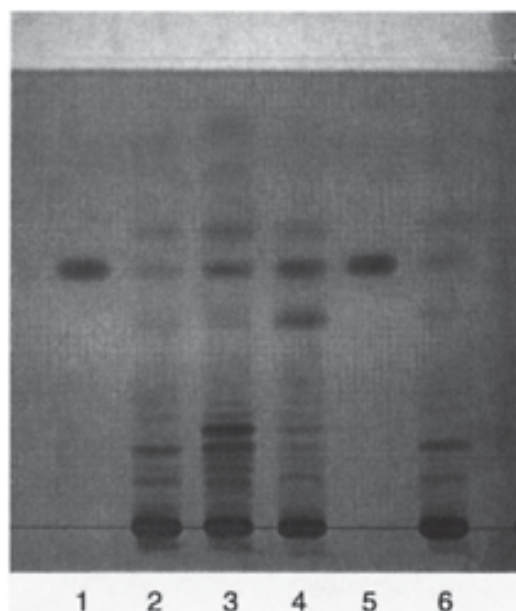


Figure 9 HPTLC fingerprint chromatograms of different chemical races of ashwaganda (*Withania somnifera*). Silica gel 60 F₂₅₄, mobile phase toluene–ethyl acetate–formic acid (50:15:5). Derivatization with sulfuric acid. Separation distance 62

mm. Track assignment: 1, 5: β -Sitosterol; 2, 6, ashwaganda Type 1 (authenticated material); 3, ashwaganda Type 2; 4, ashwaganda Type 3.

of the raw material is preserved in its entirety. It is a requirement for such analyses that none of the excipients affects the fingerprint.

2. Pharmacopoeial Methods

Pharmacopoeial methods are available only for the most commonly used herbal drugs (botanical raw materials) and a few herbal preparations (botanical drug substances). Nevertheless, they pro-

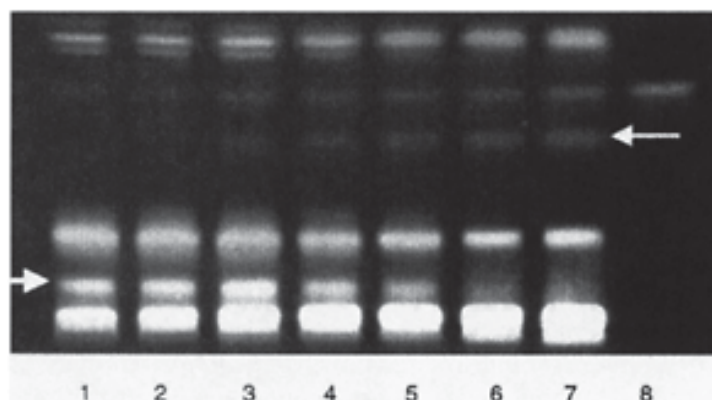


Figure 10 Mixtures of black haw (*Viburni prunifoli cortex*) with cramp bark (*Viburni oplui cortex*). (Chromatogram courtesy of Flachsman, Switzerland.) Mobile phase chloroform–acetone–formic acid (130:53:17). Separation distance 52 mm. Derivatization: Natural Products reagent.

Track	1	2	3	4	5	6	7	8
Cramp bark	100%	90%	65%	50%	35%	10%	0%	Amentoflavon
Black haw	0%	10%	35%	50%	65%	90%	100%	

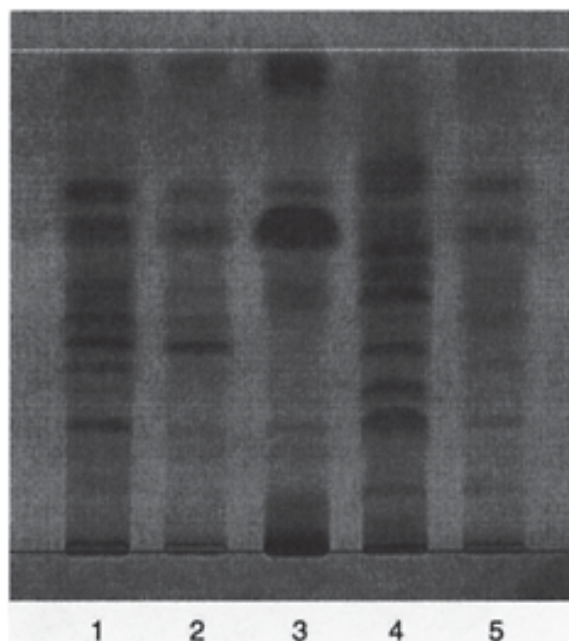


Figure 11 HPTLC fingerprint of reishi mushrooms (*Ganoderma lucidum*). Mobile phase dichloromethane-methanol (9:1), separation distance 62 mm. Derivatization with sulfuric acid. Track assignment: 1, *G. lucidum* fruiting body; 2, *G. lucidum* fruiting body (Duanwood); 3, *G. lucidum* mycelium; 4, *G. appalanatum* fruiting body; 5, *G. lucidum* fruiting body.

vide a starting point when new herbal drugs have to be identified. Table 5 gives a summary of typical solvent systems and detection modes for several substance classes taken from the European Pharmacopoeia.

In our opinion the major problem with most pharmacopoeial methods is that they do not provide images of TLC plates. The description of the chromatogram is often imprecise. Usually there is no range of variation given nor is there a detailed description of the chromatograms of common adulterants. Only the American Herbal Pharmacopoeia addresses these issues. Another shortcoming of many pharmacopoeial methods is that there is no systematic approach to certain classes of constituents and no international accord with respect to the individual herbal drugs. Considering St. John's wort (*Hypericum perforatum*) as an example, most pharmacopoeias specify different solvent systems for TLC fingerprint identification. Additionally scientific literature provides even more choices. All of the nine mobile phases selected in Fig. 12 could be used for identification purposes. However, if more specific analytical questions were asked, or

reproducibility of the result or even quantitative determinations were to become an issue, significant differences would be seen among the various methods (36).

Another example further illustrates this problem (see Fig. 13). Under certain circumstances the question of whether rutin is present in a given sample of hawthorn (*Crataegus* spp.) is of analytical interest. If, for example, the method of the Swiss Pharmacopoeia is used (mobile phase: upper phase of ethyl acetate–formic acid–water 50:10:40), this question cannot be answered. Rutin and vitexin-2"-*O*-rhamnoside are coeluting. Using the method of the European Pharmacopoeia instead (mobile phase: ethyl acetate–methyl ethyl ketone–formic acid–water 50:30:10:10), separation of the two compounds in question is achieved. Further optimization of the method has resulted in a chromatographic system (mobile phase: ethyl acetate–methanol–formic acid–water 50:3:3:6) that allows not only the separation of the target compound rutin from all other components of the chromatogram but also the characterization of various *Crataegus* species (37).

Table 5 Solvent Systems and Detection Methods for Several Substance Classes from European Pharmacopoeia

Substance class	Mobile phase	Detection	Derivatization
Essential oils	Ethyl acetate or methanol and toluene or hexane in various concentrations	UV, 254 nm	Vanillin–sulfuric acid reagent, Anisaldehyde–sulfuric acid reagent
Flavonoids	Formic acid–water–ethyl acetate in various concentrations, with or without ethyl methyl ketone	UV, 254 nm	Natural Products reagent and macrogol
Saponins	Acetic acid 98%–water– 1–butanol (10:40:50) or ammonia–water– ethanol 96%–ethyl acetate (1:9:25:65) or ethyl acetate–water– 1–butanol (25:50:100), upper phase)	UV, 254 or 365 nm	Anisaldehyde–sulfuric acid reagent
Tannins	Formic acid–water–ethyl acetate in various concentrations, with or without acetic acid or ethyl acetate–toluene (2:98) or acetic acid 98%–ether–hexane–ethyl acetate (20:20:20:40)		Natural Products reagent and macrogol, iron(III) chloride reagent, Fast Blue salt B reagent, and sodium hydroxide or ammonia vapor
Alkaloids	Various mobile phases	UV, 254 nm	Dragendorff reagent, hydrochloric acid and iodine reagent
Anthrones	Water–methanol–ethyl acetate (10:17:100) or formic acid–ethyl acetate–petroleum ether (1:25:75) or acetic acid 98%–water– ethyl	UV, 365 nm	Potassium hydroxide reagent

	acetate–1-propanol (1:30:40:40)		
Saccharides	Water–acetonitrile (10:85) or sodium dihydrogen phosphate 1.6%–1-butanol–acetone (10:40:50)		Aminohippuric acid, anisaldehyde–sulfuric acid reagent, diphenylamine–aniline–phosphoric acid reagent
Bitter compounds	Acetic acid–ethyl acetate–cyclohexane (2:38:60) or acetone–methanol–acetic acid–toluene (5:5:10:80)	UV, 254 or 365 nm	Molybdate–tungstate reagent or anisaldehyde–sulfuric acid reagent
Triterpenes	Acetic acid–formic acid–water–ethyl acetate (11:11:27:100)		Anisaldehyde–sulfuric acid reagent
Iridoid glycosides	Water–methanol–ethyl acetate (8:15:77)	UV, 254 nm	Phloroglucine reagent
Hydroquinone derivatives	Formic acid–water–ethyl acetate (6:6:88)		Dichloroquinone chlorimide and sodium carbonate

Source: Ref. 7.

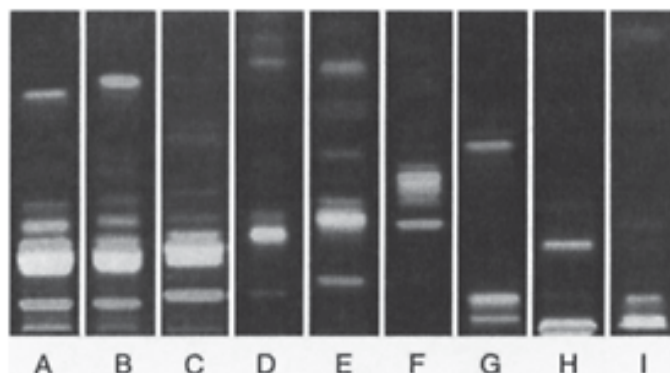


Figure 12 Solvent systems for TLC fingerprint identification of St. John's wort (*Hypericum perforatum*). Separation distance 52 mm. Derivatization: Natural Products reagent. (A) Ethyl acetate–formic acid–water (90:6:9), European Pharmacopoeia; (B) ethyl acetate–formic acid–water (86:6:8); (C) ethyl acetate–formic acid–acetic acid–water–dichloromethane (100:10:10:11:25); (D) ethyl acetate–formic acid–water (20:2:1); (E) ethyl

acetate–formic acid–water (85:10:5); (F) ethyl acetate–formic acid–acetic acid–water (100:11:11:26), National Formulary 19; (G) ethyl acetate–formic acid–toluene (40:10:50); (H) ethyl acetate–formic acid–toluene (40:10:100); (I) chloroform–methanol–water (80:18:2).

B. Stability Tests

1. General Aspects

High-performance thin-layer chromatography is a suitable tool for stability tests on herbal medicinal products. Many samples can be analyzed rapidly, and there is practically no start-up time for instrumentation. It is typical for stability tests that the same analysis has to be repeated at certain intervals (from one month to several months) over long periods of time (up to several years). Each time the test is performed on a fresh HPTLC plate, providing exactly the same chromatographic conditions for each sample. This is an advantage over column chromatographic methods, which face difficulties in keeping the chromatographic system constant and free of contamination over a long time.

Stability tests using HPTLC are based on the visual comparison of fingerprints. Very small variations in the sample may not be detected, but significant changes are easily seen. Acceptance criteria are therefore easily established. Owing to higher separation power, most peaks in HPLC are well separated and precisely characterized by retention times and area or height. Therefore, small variations are easily seen, and over a long period of time “stability” is more difficult to define. It is a great advantage of HPTLC that the sample in its entirety can be visualized on the plate, unlike in column chromatography, where only those components are detected that are separated by a selected gradient. The information obtainable from multiple detection on the same plate, particularly when specific chemical derivatization steps are included, makes it possible to look at a broad spectrum of compounds during the same analysis. The information may be complemented by semiquantitative data from video densitometry.

The EMEA (14) stipulates that variation in content during the shelf life of an herbal drug preparation with constituents of known activity should not exceed $\pm 5\%$ of the initial assay value. When the active compounds are unknown, a variation of $\pm 10\%$ can be accepted. However, the practical aspects of stability tests are still under discussion. As a starting point, the ICH guideline on stability testing of new drug substances and products (38) may be considered for herbal medicinal products. Forced degradation could be used to establish methods that are specific for stability-indicating constituents of the herbal medicinal product. Typically, products are stressed with light, temperature, humidity, and oxidizing, reducing, and hydrolytic environments.

2. Requirements

Unlike typical HPTLC applications, where all samples and standards are on the same plate, in stability tests samples are compared from plate to plate over a long period of time. The first plate

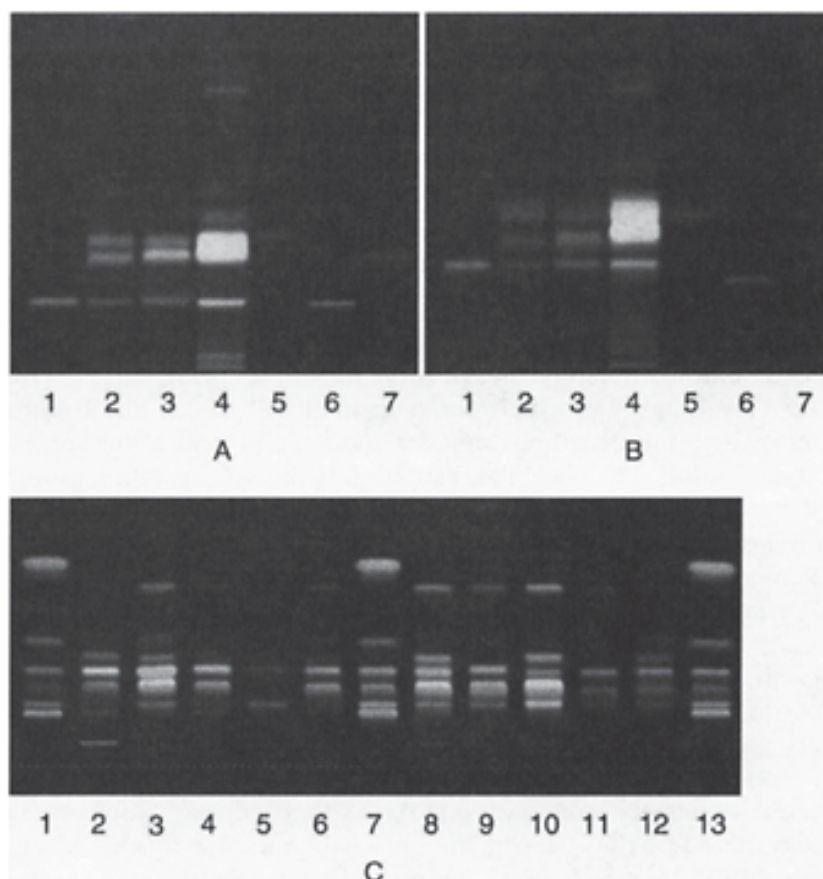


Figure 13 HPTLC fingerprint of hawthorn (*Crataegus*). (A) According to Swiss Pharmacopoeia. TLC plate 20×20 cm Si 60 F₂₅₄. Mobile phase water–formic acid–ethyl acetate (40:10:50, upper phase); separation distance 150 mm. Derivatization: Natural Products reagent. (B) According to European Pharmacopoeia. TLC plate 20×20 cm Si 60 F₂₅₄. Mobile phase water–formic acid–ethyl acetate– ethyl methyl ketone (10:10:50:30); separation distance 150 mm. Derivatization: Natural Products reagent. (C)

According to CAMAG application note. HPTLC plate 20×10 cm Si 60 F₂₅₄. Mobile phase water–formic acid–ethyl acetate–methanol (3:6:50:3); separation distance 52 mm. Derivatization: Natural Products reagent. Track assignment: (A, B) 1, Rutin; 2, *Crataegus* (Swiss Pharmacopoeia); 3, *Crataegus* extract (Swiss Pharmacopoeia); 4, *Crataegus* extract (European Pharmacopoeia); 5, hyperoside; 6, vitexin-2"-*O*-rhamnoside; 7, chlorogenic acid. (C) 1,7,13, rutin, vitexin-2"-*O*-rhamnoside, chlorogenic acid, hyperoside, vitexin, caffeic acid; 2, *C. laevigata* (AHP); 3, *C. monogyna* (AHP); 4, *C. monogyna* (trade sample); 5, *C. monogyna* (fermented); 6, *C. monogyna* (Italy); 8, *C. laevigata* (trade sample); 9, *C. laevigata* "Punicea"; 10, *C. ×macrocarpa*; 11, *C. azarolus* spp. *azarolus*; 12, *C. pentagyna*.

serves as the reference to which all subsequent plates are compared. In this regard, reproducibility of the selected method is of utmost importance. Rigorous standardization is therefore essential as well as the need to use adequate instrumentation in all steps of the HPTLC analysis. Complete validation of the entire method is a must. Whereas HPTLC fingerprints for the purpose of identification have to be specific only with regard to adulterations, methods for stability tests must also be specific with regard to stability-indicating substances (39). In other words, it has to be established that the method can detect changes taking place in the sample over time.

To be able to visually compare several HPTLC plates over a period of time, "durable" images of the chromatograms must be generated. Only electronic images obtained with video cameras, digital (still) cameras, or flatbed scanners can provide the necessary durability. However, the imaging process must be completely controllable, predictable, and reproducible to ensure that differences between two images are caused only by differences in the sample.

Because stability tests are mainly performed on herbal medicinal products, all analytical work has to comply with GMP regulations.

C. Quantification of Marker Compounds

1. General Aspects

Simplified sample preparation, the option of specifically optimizing chromatography for the separation of selected compounds, and the possibility of specific postchromatographic derivatization are advantages of quantitative planar chromatography. Because of the single use of the HPTLC plate, contamination of the system is not an issue as it is for sequential techniques like column chromatography. Nevertheless, most fingerprint methods that are used for identification are not suitable for quantification without adaptation. The principal restriction is that of separation power. If the sample contains too many components, a useful fingerprint may still be obtained but baseline separation of all substances as a requirement for quantification may be difficult to achieve. However, aside from resorting to gradient techniques such as AMD, the best solution is the optimization of the method for the separation or quantification of selected marker compounds. The possibility of successful quantitative HPTLC of herbal medicinal products is documented by the fact that in the last two years over 90 papers were published on this subject. The majority of these publications concern quality assurance of traditional Chinese medicines. The particular advantages of quantitative HPTLC as a complementary technique to HPLC and GC are discussed by Xie (40).

Quantification is usually performed by scanning densitometry. Scanning each track of the plate with a light beam of selectable dimension and wavelength, the absorption or fluorescence of the sample components is measured. The raw data in the chromatogram are integrated and compared to those obtained from a set of known standards chromatographed on the same plate.

2. Requirements

Quantification of marker compounds requires suitable instrumentation (see Chapter 5 of this Handbook) to perform each step of the HPTLC analysis accurately and precisely. All steps of the HPTLC method should be optimized and standardized to ensure reproducible data. It is necessary to validate quantitative methods according to ICH guidelines Q2A and Q2B (12, 13). If possible, quantitative evaluation should be performed prior to derivatization unless the target compound must be detected by a chemical reaction.

3. Example

Pharmacopoeial methods described for the fingerprint identification of bearberry (*Arctostaphylos uva ursi*) typically result in a chromatogram similar to that seen in Fig. 14a when viewed under 254 nm UV light. An important quality-related question aside from the identity of a bearberry preparation or finished product is its hydroquinone content. In the chromatogram hydroquinone is seen as a very faint zone close to the solvent front. The more intense zone just below is that of gallic acid. Figure 14b shows the chromatogram of different amounts of gallic acid [migration distance (MD)=45 mm] and hydroquinone (MD=50 mm) after densitometry at 287 nm. There is a secondary solvent front at MD=52 mm that interferes with the quantification of hydroquinone.

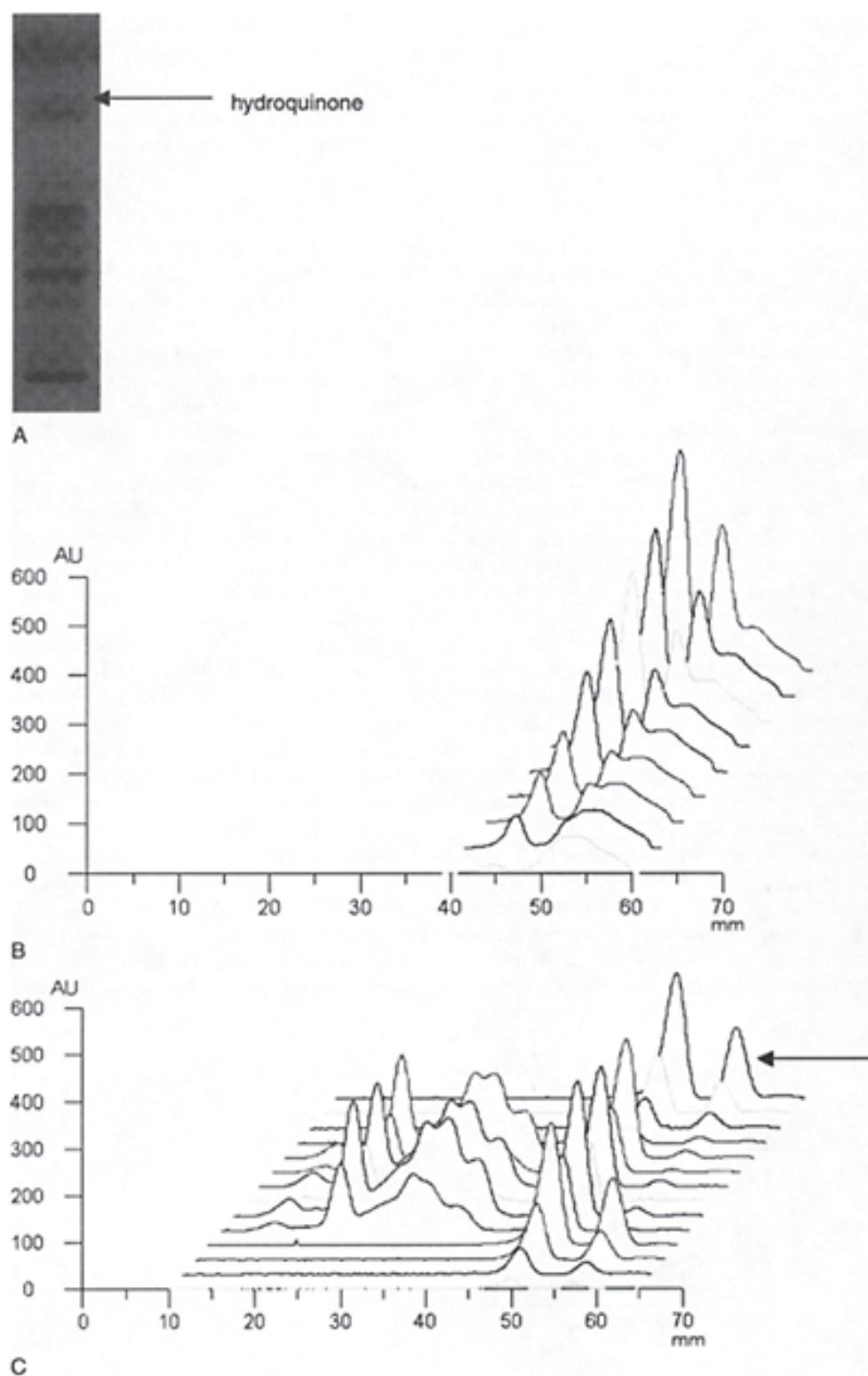


Figure 14 Quantitative determination of hydroquinone in bearberry leaves. HPTLC plate, silica gel 60 F₂₅₄, saturated chamber. Mobile phase ethyl

acetate–formic acid–water (90:10:6)
 (for details see text). (A) Typical
 chromatogram seen under UV, 254
 nm. (B) Scanning densitometry at 287
 nm. (C) Mobile phase ethyl acetate–
 formic acid–water (88:6:6), 10 min
 preconditioning with mobile phase,
 scanning densitometry at 287 nm.

Optimization of the chromatographic conditions (Fig. 14c) results in complete separation of the hydroquinone peak (MD=57 mm) from the solvent front (MD=70 mm) and allows quantification of hydroquinone in bearberry preparations (tracks 5–10).

D. Method Development

There are many new herbal drugs for which no TLC-related information can be found in the literature. In other cases the official methods or other established procedures are not adequate to answer a given analytical question and the usual optimization attempts (HPTLC instead of TLC plates, improved sample application, changing chamber conditions, chemical derivatization, multiple detection, etc.) have failed. At this time it becomes necessary to develop a new HPTLC method. Method development begins with clarification and an understanding of the analytical goal. For example, methods targeting known constituents of herbal drug preparations or herbal medicinal products will be different from those developed for distinguishing suitable raw material from adulterants.

1. General

In most cases and unless the chemical nature of the analyte is not compatible with it, HPTLC silica gel should be used as the stationary phase. At this point a decision has to be made about the developing chamber. A saturated twin-trough chamber is a good choice. All other parameters (application, separation distance, derivatization, documentation) can be selected according to the recommended standardized HPTLC as described in Section II. Method development can now be considered as the selection and optimization of the mobile phase. Many publications deal with theoretical approaches to method development in TLC (see Chap. 3 of this Handbook for details).

As far as semiempirical concepts for planar chromatography are concerned, the “four-solvent approach” (41) and the PRISMA model are particularly well established (42). However, from the perspective of general applicability, the following model should be discussed (43). The great advantage of this strategy is that no calculations are needed and method development proceeds in the form of guided trial and error. Like many other approaches, the CAMAG optimization scheme for the mobile phase is based on two fundamental parameters: the solvent strength, which affects the R_f value of the analyte, and the selectivity, which affects the relative position of the substance zones. Geiss (44) demonstrated that the best separation in HPTLC can be achieved around an R_f of 0.3. Consequently the solvent strength of the mobile phase must be adjusted so that the most

critical substance pair to be separated is in the optimal R_f range (0.2–0.5). For fingerprint chromatograms the situation is slightly different, because there are usually many substances to be separated simultaneously. The solvent strength is appropriate if as many of the substances of interest as possible are spread over the R_f range of 0.1–0.8. The selectivity of the solvent system is adjusted so that the separation of the most critical substance pair is optimized. In the case of a complex fingerprint chromatogram, the zones should be as evenly spaced over the selected R_f range as possible.

Solvents are characterized according to Snyder (45) by the solvent strength parameter P' and the selectivity group (I–VIII). Solvents or solvent mixtures having the same P' value should produce similar R_f values for a given mixture of compounds. Solvents of the same selectivity group should give a similar order of elution of compounds in the mixture.

Level 1. In the first step of the CAMAG optimization scheme (Fig. 15), eight to ten neat solvents representing different selectivity groups are used as mobile phase. Based on the chro-

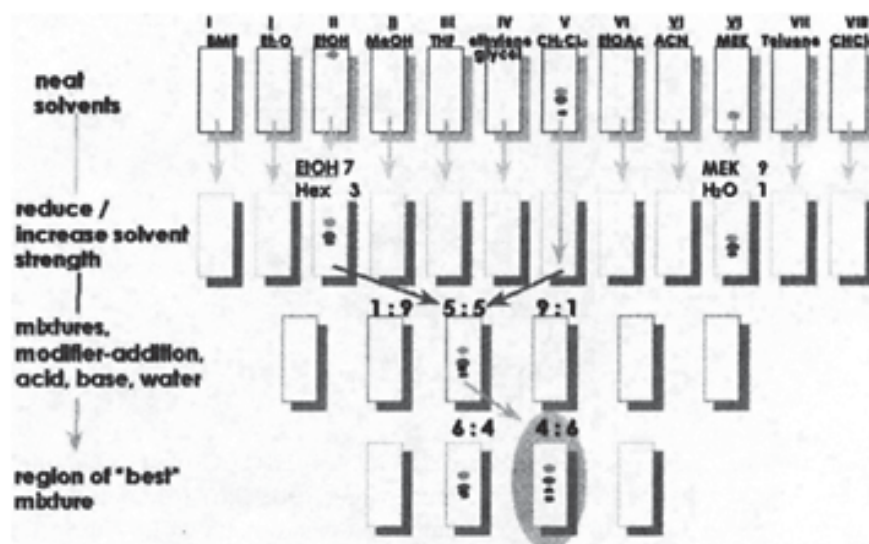


Figure 15 CAMAG scheme for optimization of the mobile phase.

matographic result of these initial tests, three groups of solvents can be distinguished. Group A solvents give suitable R_f values; these solvents can be considered in level 3, provided their selectivity is suitable. The R_f values resulting from group B solvents are too high, and those resulting from group C solvents are too low.

Level 2. If none of the solvents of group A provided suitable selectivity (decision based on number of and resolution between separated zones), optimization continues on level 2. The solvent strength of group B solvents is reduced by adding hexane ($P'=0$). The amount depends on the R_f value achieved at level 1, but typically the hexane content can be increased in steps of 10–20% per trial. The R_f value for group C solvents is increased by adding small amounts (1–5%) of polar modifiers such as water, acetic acid or formic acid, or diethylamine or ammonia. After proper adjustment of the R_f values, solvents are again selected for use at level 3 based on their selectivity.

Level 3. At level 3, optimization proceeds by mixing two solvents of different selectivity groups from level 1 and/or level 2 in order to improve the separation of the target compounds. If the chromatographic result is improved, the proportions of the components may be varied. If the result is not improved, combinations of other solvents must be tried.

Level 4. At level 4 a fine-tuning is performed. This includes minor adjustments to the solvent strength, additions of modifiers to improve the shape of the separated zones, and variations of the chamber configuration.

If the analytical goal is not achieved because of insufficient selectivity, the procedure can be repeated using a different stationary phase. If the separation power of a single development is not sufficient, automated multiple development (AMD) can be tried.

It is important to note that this approach usually offers many different solutions for a given analytical problem. Therefore, it is important to keep the analytical goal in mind when deciding whether a solvent system is suitable or not. Separation must be as good as necessary, not as good as possible. Typically a suitable method for fingerprint analysis can be developed with about 15 test runs. Using the HPTLC VARIO chamber (see Chap. 5), up to six solvents can be investigated in parallel on a 10×10 cm HPTLC plate (46).

2. Fingerprints

An important aspect of methods for HPTLC fingerprinting is the option of using multiple detection. During method development, not only should several nonspecific derivatization reagents be evaluated but also specific reagents that target compound classes of interest should be evaluated. For method development it is advisable to select marker compounds that are specifically found in the given herbal drug. If available, several reference materials for such markers should be included in the method development. In our opinion, it is not good practice to develop methods using a dye or other substance that is not known as constituent of the given herbal drug as a reference point for the fingerprint. If the active compounds of a given herbal drug are known, it is important to develop fingerprints that afford sufficient separation of those compounds from other substances. When developing a method for fingerprint identification of herbal drugs, specificity is the dominant goal. It is advisable to include common adulterants and several different samples of the herbal drug in the method development process in order to create a system that can discriminate adulterants.

3. Stability Tests

Aside from optimized separation within the HPTLC fingerprint and stability of the analyte during chromatography, a method for stability testing must ensure suitable visualization of the chromatogram. During method development these aspects must be addressed with particular regard to reproducibility. Chromatographic parameters can be reproduced if standardized equipment and methodology are used. Optimization of the derivatization (visualization) process requires special attention. Parameters such as type and concentration of the reagent, immersion time, drying time, temperature, and duration of any heating steps as well as the time interval between the end of derivatization and detection must be carefully selected. Also, the imaging process needs to be standardized,

particularly with regard to the camera. Reproducible results can be obtained only with qualified equipment.

4. Quantification

Method development for quantitative determinations usually emphasizes baseline separation of target compounds and elimination of matrix effects. Also, parameters that affect the shape of the separated zones (tailing, peak width) such as proper sample application, layer activity, developing distance, and chamber saturation and preconditioning must be carefully optimized for reliable results. Calibration functions based on absorption measurements over a wide concentration range are generally not linear. Therefore, it is important to select a suitable working range in order to use pseudolinear regression for evaluation of data. To increase sensitivity of detection and lower the detection limits of a method, several parameters of the densitometric evaluation should be considered. Most important in this regard are the dimensions of the scanning slit and the measuring wavelength. In certain cases specific derivatization can improve the measurement.

E. Validation

Validation is the formal proof that a method is suitable for its intended application. Prior to validation several other aspects have to be considered. All steps of the method must be documented in detail, and a validation protocol that specifies acceptance criteria for evaluation of the data must be agreed upon. Validation of HPTLC methods does not usually require an extensive amount of work, because many of the required experiments can be performed during method development.

1. Prevalidation Experiments

An important yet commonly overlooked question to be addressed is that of the stability of the analyte during chromatography. Simple 2-D chromatography can provide the answer (Fig. 16). The sample is applied as a spot in the lower left corner of the HPTLC plate. After development in the first direction according to the selected method, the plate is thoroughly dried in a stream of cold air. Then the plate is turned 90° to the left and a second development is performed under identical conditions (fresh mobile phase). The dried plate is evaluated. The sample (analyte) is stable during chromatography if all components line up on the diagonal that connects the application position with the crossing point of the two mobile-phase fronts. Any spots that appear above or below the diagonal indicate a substance that is generated or altered during chromatography.

Robustness of the method is another aspect that can be addressed during method development. Typically, the effects of small changes in relative humidity, developing distance, chamber saturation, and mobile-phase composition should be investigated.

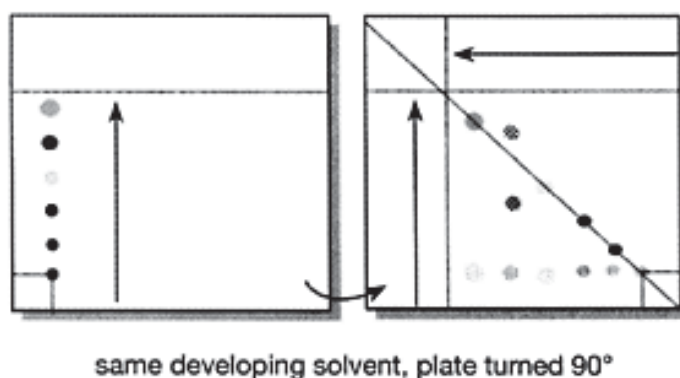


Figure 16 Two-dimensional chromatography (schematically) for evaluation of stability of analyte during chromatography. See text for details.

2. Fingerprints

Specificity of the fingerprint for herbal drugs can be proven if the sample is compared to reference material (authenticated plant material) and commonly known adulterants are chromatographed in parallel on the same HPTLC plate. The method is specific if the sample and the reference give fingerprints that are similar with respect to number, color, relative position, and intensity of the separated zones. The fingerprints of the sample and adulterants must be significantly different. When judging “similarity,” the natural variability of the herbal drug should be assessed. Comparison of several batches of the same material can do this. Specificity of methods for identification of herbal drug preparations and herbal medicinal products can be established by comparison to authenticated plant material and by proving that none of the excipients interfere with the fingerprint of the herbal drug.

3. Stability Tests

For methods intended to be used for stability tests prior to validation, not only must the stability of the analyte during chromatography be demonstrated but also its stability in solution and on the plate. For experimental details of these experiments see Ref. 47. During validation, specificity with respect to degradation products and stability-indicating compounds is established. The repeatability of the method is of great importance. If the stability test is based on the comparison of images, the derivatization and documentation steps have to be carefully investigated.

As an illustrative example the development and validation of a method for stability tests on valerian (*Valeriana officinalis*) extracts should be mentioned (48). Based on the method of the European Pharmacopoeia for identification of valerian, the method was first optimized with respect to the separation of three marker compounds. The derivatization reagent had to be changed from anisaldehyde, which gave variable and

interfering background coloration of the plate, to 15% sulfuric acid in methanol. The documentation procedure using a video documentation system was consequently standardized. Figure 17 presents the results obtained for the same sample on five different plates over a time period of 6 weeks.

4. Quantification

Quantitative HPTLC methods are usually validated according to the ICH guidelines. In addition to the previously mentioned parameters, accuracy, precision, repeatability, intermediate precision,

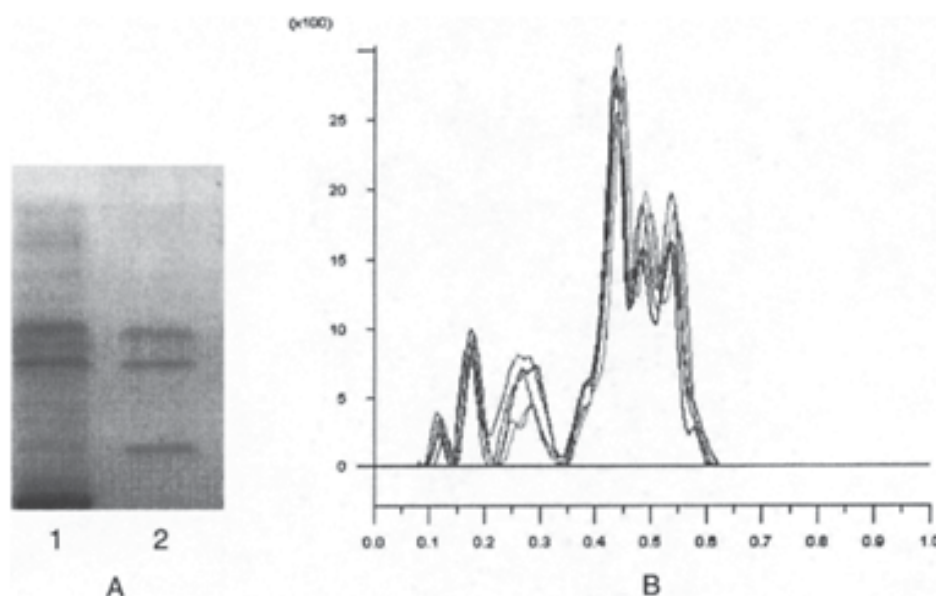


Figure 17 Reproducibility of video densitometry after HPTLC of valerian dry extract (*Valeriana officinalis*). Mobile phase chloroform–ethyl acetate–1-propanol (6:4:0.4); separation distance 52 mm. (A) Derivatized plate (anisaldehyde). 1, Valerian extract; 2, valerenic acid, acetoxyvalerenic acid, hydroxyvalerenic acid. (B) Track 1 of 5 plates developed with the same method in 6 weeks.

detection limit, quantification limit, linearity, and range must be validated. As an example, the development and validation of a quantitative method for the determination of hyperforin in herbal drugs, herbal drug preparations, and herbal medicinal products is

mentioned (49). Hyperforin can be separated from any other component of the samples. The UV spectrum of the analyte is identical to that of the reference material. No matrix effect was found during recovery experiments.

Quantification is performed by scanning densitometry in the absorbance mode at 310 nm. The linear working range for the determination is 40–120 ng absolute. LOD was found to be 3.8

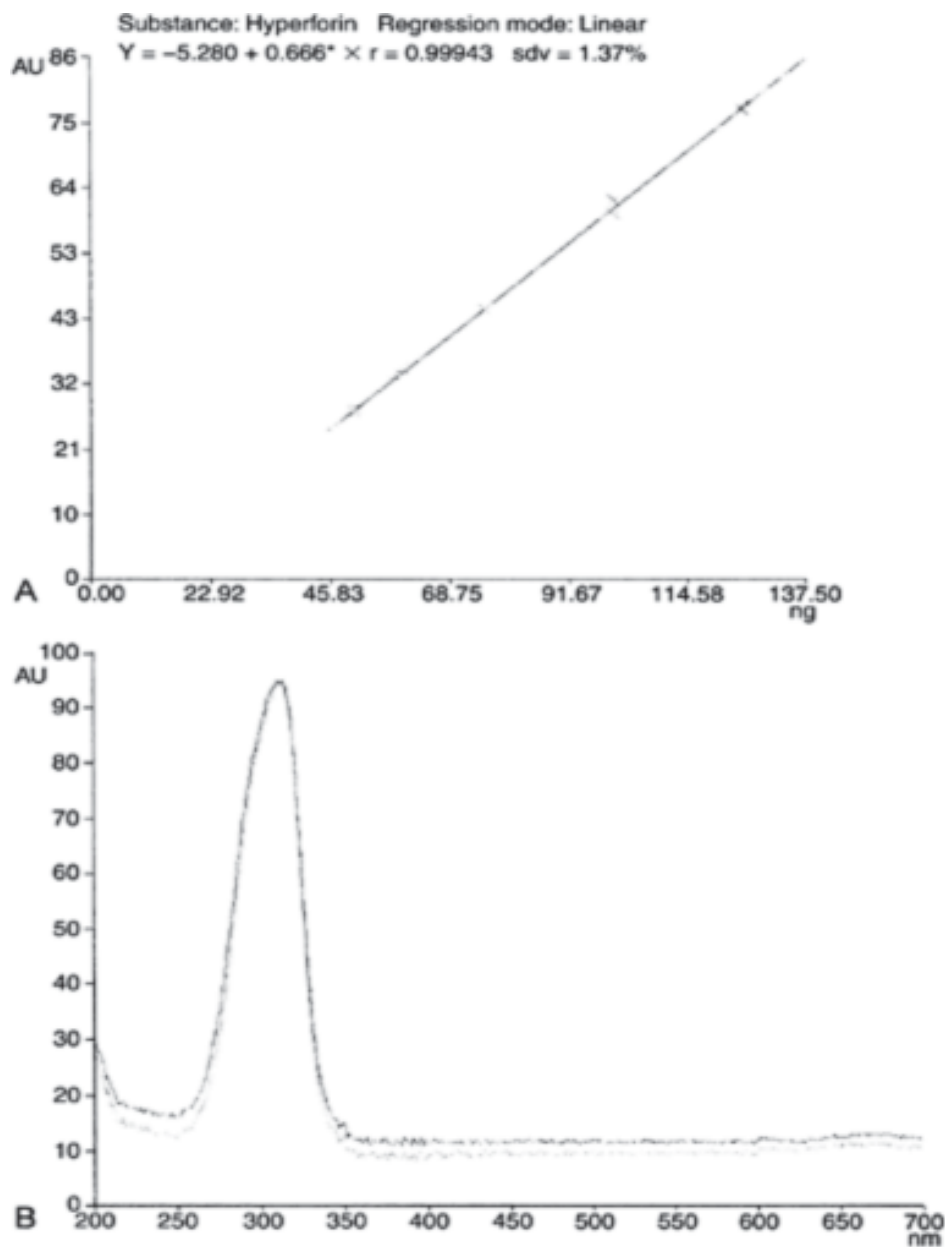


Figure 18 Quantification of hyperforin by HPTLC. (A) Calibration curve; (B) UV spectra of analyte (upper curve) and reference (lower curve).

ng; LOQ, 7.6 ng; and the precision was 1.86% ($n=10$). Quantification of hyperforin gave an intermediate precision (7 days, one determination per plate per day) of 5.2% (Fig. 18).

DEFINITIONS

Herbal drugs (botanical raw materials) are mainly whole, fragmented, or cut plants, parts of plants, algae, fungi, or lichens in an unprocessed state, usually in dried form but sometimes fresh. Certain exudates that have not been subjected to a specific treatment are also considered to be herbal drugs. Herbal drugs are defined by the botanical scientific name according to the binomial system.

Herbal drug preparations (botanical drug substances) are obtained by subjecting herbal drugs (botanical raw materials) to treatments such as pulverization, extraction, distillation, expression, fermentation, or other similar processes. These include comminuted or powdered herbal drugs, tinctures, liquid or dry extracts, oils, expressed juices, gums, syrups, and process exudates. *Native herbal drug preparation* refers to a preparation without excipients.

Botanical products or botanicals, are finished labeled products that contain vegetable matter (plants, algae, fungi). Depending on its intended use, a botanical product may be a food, drug (botanical drug product), medicinal device, or cosmetic.

Herbal medicinal products (botanical drug products or botanical drugs) are botanical products that are intended for use as drugs and prepared from botanical drug substances (herbal drug preparations).

Dietary (nutritional) supplements are products intended to supplement the diet that contain one or more of the following ingredients: a vitamin, a mineral, an herb or other botanical, an amino acid, or an extract, a metabolite, or a constituent of any of these.

Nutraceutical and functional food are not legally defined terms. The manufacturer must decide on the intended use (conventional food or dietary supplement) early in product development.

Reference standards or reference materials are substances prepared for use as the standard in an assay, identification, or purity test. In the case of botanical products, the reference standards may be authenticated botanical samples of the herbal drugs (or preparations), or

chemically defined substances (active constituent, marker, impurity).

Markers are chemically defined constituents of herbal drugs that are of interest for control purposes independent of whether or not they have any therapeutic activity.

Active constituents are chemically defined substances that are responsible for the intended pharmacological activity or therapeutic effect.

Standardization is the adjustment of the herbal drug preparation to a defined content of a constituent or group of substances with known therapeutic activity (standardization to a target value). The expression “standardization” is also used in certain countries to describe all measures (manufacturing process and quality control) leading to reproducible quality (standardization of a process).

Analytical procedure refers to the way of performing the analysis. An analytical procedure should describe in detail the steps necessary to perform each analytical test. This may include the sample, the reference standard, and the reagent preparations; use of the apparatus; generation of the calibration curve; and the use of formulas for calculations.

Specificity is the ability to unequivocally assess the analyte in the presence of components that may be expected to be present. Typically these include impurities, degradants, and the matrix. Lack of specificity of an individual analytical procedure may be compensated for by other supporting analytical procedure(s). This definition has the following implications:

Identification: to ensure the identity of an analyte.

Purity tests: to ensure that all the analytical procedures performed allow an accurate statement of the impurity content of an analyte, i.e., related substances tested, heavy metals and residual solvent content.

Assay (of content or potency): to provide an exact result that allows an accurate statement of the content or potency of the analyte in a sample.

Accuracy of an analytical procedure expresses the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference

value and the value found. This is sometimes termed trueness.

Precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision, and reproducibility. Precision should be investigated using homogeneous authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution. The precision of an analytical procedure is usually expressed as the variance, standard deviation, or coefficient of variation of a series of measurements.

Repeatability expresses the precision under identical operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Intermediate precision expresses within-laboratory variations: different days, different analysts, and different equipment.

Reproducibility expresses the precision between laboratories.

Detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact value. The quantification limit of an individual analytical procedure is the smallest amount of analyte in a sample that can be quantifiably determined with suitable precision and accuracy. The quantification limit is a parameter of quantitative assays for low levels of compounds in sample matrices and is used particularly for the determination of impurities and/or degradation products.

Linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample.

Range of an analytical procedure is the interval between and including the upper and lower concentrations (amounts) of analyte in the sample for which it has been demonstrated that the analytical procedure has suitable levels of precision, accuracy, and linearity.

Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal use.

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19

Hydrocarbons

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I. HYDROCARBON SAMPLES

Hydrocarbons occur together with other related compounds as complex mixtures in nature (e.g., petroleum) and human activities (e.g., products derived from the conversion of petroleum, coal, shale, and oil). Likewise, hydrocarbons are ubiquitous pollutants in soils, water, and air, and some of them have carcinogenic and/or mutagenic activities.

The composition of these mixtures, as well as the occurrence of a particular hydrocarbon family, depends on the type of sample and its source or process history. In the case of environmental samples, it also depends on the method and extent of sample cleanup. As a consequence of this, the composition of hydrocarbon-containing samples varies in molecular structure and size, polarity, and functionality or chemical group.

Hydrocarbon types found in real samples can be summarized as saturates (*n*-alkanes, isoalkanes, and cycloalkanes), olefins (alkenes and cycloalkenes), monoaromatics, polycyclic aromatic hydrocarbons (PAHs, hydrocarbons containing two or more rings of the benzenoid structure), and condensed hydroaromatic structures (1). Furthermore, heterocyclic (N, S, O) aromatic structures—compounds with PAH structure in which carbon atoms have been substituted with oxygen, sulfur, or nitrogen atoms—occur together with PAHs in many cases. The presence of these compounds is so common in hydrocarbon-containing samples that it is customary to refer to all aromatic compounds as polycyclic aromatic compounds (PACs) instead of PAHs. In addition to the cited compounds, other types of polar molecules can be found together with hydrocarbons depending on the origin of the sample. These include nitroaromatics; condensed aromatics with phenolic, ketonic, or carboxylic groups; and others in environmental samples or even as porphyrins in heavy petroleum residues or fullerenes from fossil fuel combustion samples.

This picture becomes more complicated as the sample boiling range becomes higher due to the increase in the number of isomers for each family, the increase in concentration of polar compounds, and the increasing number of possible combinations of compound types (e.g., alkylaromatics).

Apart from the samples derived from fossil sources, hydrocarbons can also be found in samples derived from organic reactions or synthesis (e.g., fullerenes) or other natural products (e.g., terpene hydrocarbons).

II. TYPES OF ANALYSES REQUIRED

Given that complete molecular separation of the components of hydrocarbon-containing samples is not usually possible owing to their complex nature and the limitations of current separation techniques, the types of analysis required for these samples include either the determination of targeted, individual hydrocarbons or the separation and quantification of hydrocarbon types (2). The latter is commonly required in industry at either the analytical or semipreparative scale. In effect, the thermal or catalytic behavior of a hydrocarbon-containing product is usually better defined in terms of principal groups or hydrocarbon types rather than performing an extensive separation of all the components. Such analyses can be performed using thin-layer chromatography and are useful in petrochemistry, carbochemistry, geochemistry, and environmental sciences for process monitoring, compliance with environmental regulations, and the evaluation of product quality, catalyst performance, and feed processability as well as for determining hazardous compounds and understanding and solving basic research problems.

III. INTRODUCTION TO THIN-LAYER CHROMATOGRAPHY OF HYDROCARBONS

Thin-layer chromatography (TLC) is a particularly well adapted technique for separating liquid or soluble hydrocarbon-containing samples and, in general, complex or dirty samples, all of which include fractions or impurities that can contaminate the stationary phase. The 1960s were a period of intense research activity into the TLC of hydrocarbons (3). In this period, techniques such as argentation and charge-transfer TLC of unsaturated and aromatic hydrocarbons were developed (4, 5). Some of the methods developed in this period with the technology then available are still useful or have the potential to be adapted to modern equipment. The following decades of the 1970s and 1980s saw a decline in this research area, mainly due to the development of both gas chromatography (GC) and high-performance liquid chromatography (HPLC) and the stagnation in TLC technology development. Improvements in instrumentation (plate performance and manufacture, automatic sample application systems, scanners, software) during the 1990s made it possible for TLC to become a useful and reliable technique (6). There has been a resurgence in this technique for hydrocarbon analysis.

One of the most important advantages of TLC over HPLC for hydrocarbon analysis is the possibility of scanning the whole sample without prior sample preparation steps (e.g., deasphalting of petroleum products). All of the compounds (even those that do not migrate) contribute to the resulting chromatogram. In column techniques, some heavy and/or polar hydrocarbons can be irreversibly adsorbed on the stationary phase, producing column deterioration, unelution, and hence quantitative errors.

These advantages have been highlighted in a number of reviews dedicated to TLC and concerned mainly with particular aspects that are partially related to TLC of hydrocarbons. These cover industrial application of TLC itself; comparison with other chromatographic techniques in coal and oil fields (7), in heavy organics (8), and in the petroleum industry (2); application to the analysis of a PAH (4, 9); and application of a specific combination of TLC and flame ionization detection (TLC-FID) to coal and oil (10).

The purpose of this chapter is to give a general overview of all thin-layer chromatographic systems applied to hydrocarbons, including saturates and other related compounds that are usually found in real samples. We emphasize the two most popular techniques in hydrocarbon analysis: TLC-densitometry and TLC-FID. In the case of TLC-densitometry, we distinguish between TLC of pure hydrocarbon mixtures, which is used mainly to validate the developed separation methods, and the application of these methods to real samples in various fields of interest.

IV. TLC-DENSITOMETRY

A. Pure Hydrocarbons and Their Mixtures

As previously mentioned, pure hydrocarbons and their mixtures are used for developing new analytical methods and validating them before they are applied to real samples. Because of the complexity of samples submitted to analysis, many of the procedures are carried out in a first step on model compounds. Their application is considered in Section IV.B.

1. Saturated Hydrocarbons

Saturated hydrocarbons are found in high concentrations in petroleum-derived products. TLC-densitometry has seldom been applied to hydrocarbon analysis because of difficulties in the detection of saturated hydrocarbons. In effect, these molecules yield neither UV nor fluorescence spectra under the usual analytical working conditions. Moreover, they have traditionally been considered inert molecules. However, in 1947 it was found (verified in 1981) that alkanes show a visible green fluorescence when eluted in a solution of berberine from a silica gel column (11, 12). This phenomenon was applied to TLC by Marsh and Hiekane (13) in 1991 to detect saturated hydrocarbons in bitumen using berberine-impregnated silica gel plates, elution with *n*-hexane, and fluorescence scanning densitometry ($\lambda_{\text{exc}}=264$ nm (see Sec. IV.B.1.c). However, this phenomenon was neither systematically studied nor applied to other products until 1999, when it was investigated by Cebolla and coworkers (14–16) (Fig. 1). They used 365 nm as excitation wavelength. They showed that the fluorescence intensity increases with the mass of alkane and the alkane chain length and that the fluorescent emission is due to a ion-induced dipole interaction between the berberine cation and the corresponding saturated hydrocarbon. This model allows the experimental results to be explained.

Although TLC is not efficient enough to molecularly separate all the saturated hydrocarbons in real samples, several methods have been proposed to separate and

determine saturated hydrocarbons as a group (14). Likewise, it has also been possible to separate alkanes and isoalkanes from cycloalkanes, and determine both families, in middle distillates (17). Details of these methods are given in Section IV.B.1.c.

2. Alkenes and Terpene Hydrocarbons

Olefins are present in products derived from petroleum cracking, whereas terpene hydrocarbons are present in samples derived from natural products and organic reactions. Argentation chromatography in TLC is a well-known method for the separation of all these unsaturated compounds, as shown in Table 1. A review has been published on this useful technique (5). Argentation is carried out by spraying the plates with a saturated aqueous solution of silver nitrate; dissolving the silver salt in a solvent or a mixture of solvents in the desired concentration and then incorporating it in the silica gel slurry; inserting the edge of the plate into a solution of 10% aqueous silver nitrate, about 1 cm high, and allowing the solution to travel the length of the plate; and using developing solvents containing silver salt (18).

Silver nitrate-impregnated TLC plates were used to investigate cyclopentene and cyclohexene (19), and separation of allylic derivatives of benzene or cyclohexene from their propenyl isomers was also carried out (20). In this case the compounds studied were pulegone, isopulegone, estragole, anethole, eugenol, isoeugenol, safrole, and isosafrole. Only the allylic isomers formed complexes with silver nitrate, because the propenyl derivatives showed about the same R_f values on both silica and impregnated silica.

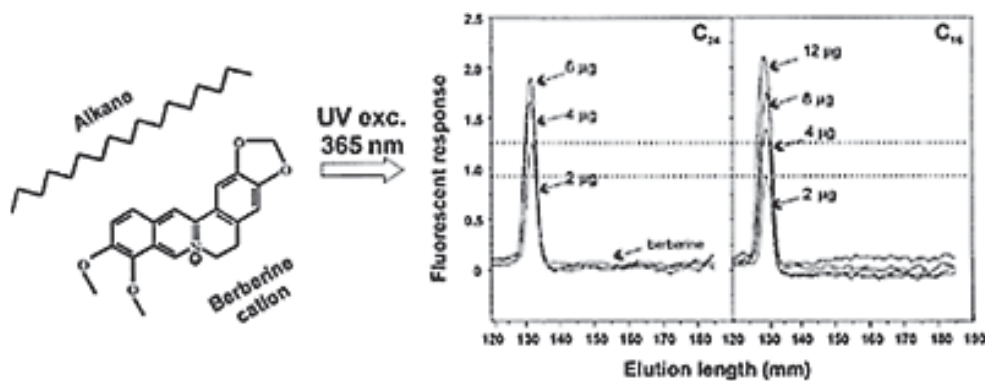


Figure 1 TLC chromatograms of n -C₂₄ and n -C₁₆ determined by enhancement of fluorescence response using berberine-impregnated silica gel plates (development: 9 min with n -hexane). (From Ref. 16. Copyright 2000 American Chemical Society.)

Table 1 TLC-Argentation of Unsaturated Hydrocarbons and Other Related Compounds with a Solution of Silver Nitrate

Solvent	Salt/solvent concentration (%)	Salt/support concentration (%)	Compounds separated	Ref.
H ₂ O-methanol ^a	5		Tetracyclic triterpenes	22
H ₂ O		2.5	Olefins	18
H ₂ O	12.5	26	Allylic-propenylic isomers	18
Acetone-10% H ₂ O	29	13	Sesquiterpenes, diterpenes	18,23
Acetone-10% H ₂ O	29	13	Terpenoids	5,18
H ₂ O-60% ethanol	54	13	Sesquiterpenes	24

^aBy spraying. All others, by using impregnated plates.

Source: Adapted from Ref. 18.

The influence in electron density around the double bond on the R_f values was also studied by Fuggerth (21) in the case of substituted stilbenes separated into their cis and trans isomers. They were successfully separated on silica gel+2% aqueous solution of silver nitrate (5% w/w). Spots with higher R_f values were assigned to Z structures.

In the case of terpenes, the original procedures involving the use of silica gel layers containing silver nitrate and gypsum are still used, although some authors claim that silver perchlorate in the absence of gypsum is the optimum combination for terpene separation (5, 18). This was concluded after an R_f study of several terpenes (longicyclene, isolongifolene, longifolene, α -gurgujene, α -bergamotene, β -bisabolene, α - and β -himachalene, and cembrene).

Terpene detection is usually performed by spraying with a solution of chlorosulfonic acid in acetic acid, phosphomolybdic acid in ethanol, or antimony perchlorate in chloroform.

3. Poly cyclic Aromatic Hydrocarbons

Much more work has been performed on conventional TLC and HPTLC of polycyclic aromatic compounds (PACs) compared to other hydrocarbons, due to their importance in environmental problems. In general, the polycyclic aromatic hydrocarbons (PAHs) usually encountered in environmental or fuel-related samples cannot be completely separated using TLC and HPTLC. It is not even possible to separate the 18 PAHs included in the EPA list. However, in a typical analysis it is only necessary to identify or quantify a few PAHs and this is compatible with the capabilities of TLC and HPTLC.

There is no universal or single TLC method superior to all others for PAH analysis. Apart from the development of adequate elution sequences, it is also necessary to use selective fluorescence detection to determine some PAHs.

a. Normal-Phase TLC. In general, separation of PAHs on silica gel and other normal-phase adsorbents (e.g., alumina) is not successful because of poor solvent selectivity. However, these stationary phases are used for determining PAHs as a group in fuels due to the compatibility of normal-phase eluants with fuels (see Sec. IV.B.1.c).

A mixture (1+1) of silica gel and kieselguhr and elution using *n*-hexane–toluene (45:5, v/v) was able to sufficiently separate some PAHs: 1,2-benzanthracene, dibenz[2,4]anthracene, pyrene, benzo[*a*]pyrene, and benzo[*g,h,i*]perylene (25). The same conditions can be used to detect benzo[*a*]pyrene among benzofluoranthenes. In the same research, aluminum oxide plates were also used, and eluants were either *n*-hexane–toluene–chloroform (45:5:10) or *n*-hexane–toluene–carbon tetrachloride (45:5:5). This allowed 1,2-benzanthracene, dibenz[*a,h*]anthracene, pyrene, benzo[*a*]pyrene, and chrysene to be separated. In all cases the plates were dried and observed under UV illumination ($\lambda=254$ nm).

Poly amide TLC plates were also used for separating PAHs. Elution was carried out using dichloromethane–methanol (60:40), and detection was by fluorescence densitometry (26). In addition, laser mass spectrometry was used to identify the separated PAHs. This technique can determine whether broad TLC spots are due simply to tailing or to overlapping of two compounds. Polyamide does not interfere with mass spectra and does not alter compound identification. Polyamide TLC plates have also been used for separating isomeric ortho-, meta-, and para-disubstituted benzenes as well as PAHs using aqueous solutions of urea-solubilized β -cyclodextrin as mobile phase (27). Ascending elution using 4 M urea–0.10 M β -cyclodextrin–10% (v/v) *t*-butyl alcohol allowed anthracene, fluorene, fluoranthene, and phenanthrene to be separated. These compounds were located under UV light by quenching of fluorescence at 254 or 366 nm.

b. Charge-Transfer TLC. One of the most studied types of normal-phase TLC applied to PAH separation has been charge-transfer TLC. This technique was established in the 1960s (28–34). The migration of PAHs over silica gel or alumina adsorbents impregnated with electron donors is selectively retarded to a degree that depends on the strength of the charge-transfer complex formed. Thus, the strength of the complexation is a direct function of the number of aromatic rings. The use of this technique up to 1992 was reviewed by Cagniant (4). Many electron acceptors have been used for PAH separation (Table 2). Impregnation of TLC plates was done by using a precoating method during plate preparation, by dipping or spraying commercial TLC and HPTLC silica gel plates, or by adding the acceptor (or donor) compound to the solvent of development.

As far as quantitative determination is concerned, UV detection at wavelengths longer than 300 nm has been used to avoid interactions with caffeine in the cases where this compound has been used as acceptor. Recently, fluorescence densitometry has been used because caffeine does not present a fluorescence response at the wavelengths used for detection.

According to Cagniant (4), trinitrofluorenone (TNF) is the most valuable acceptor for forming strong complexes with PAHs that have at least three rings. Caffeine, pyromellitic dianhydride, and tetramethylureic acid gave good results as impregnating agents. In contrast, picric acid, urea,

Table 2 Charge Transfer TLC of Aromatic Hydrocarbons

Layer	Acceptor	Ref.
Silica gel	Caffeine	28, 29, 33, 35, 36
	Pyromellitic dianhydride	33, 34
	Tetracyanoethylene	33
	1,3,5-Trinitrobenzene	32
	2,3,7-Trinitrofluorenone	28, 32
	2,3,7,9-Tetranitrofluorenone	37
	Picric acid	34, 37
	Styphnic acid	34
	<i>p</i> -Benzoquinone	42
	Chloranil	33, 38
	2,3-Dichloro-5,6-dicyanobenzoquinone	38
	Nucleic acid bases	39
	Urea	28
	Dimethylformamide	28
	Silver nitrate	28
	<i>N</i> -Methylated cyclic ureids	39
	Bile acids	39
	Bromanil	42
	Amino acids	43
Aluminum oxide	Styphnic acid	34
	2,4,7-Trinitrofluorenone	34, 38

Source: Adapted from Refs. 4 and 18.

dimethylformamide, sodium deoxycholate, and tetracyanoethylene (TCNE) had no effect, were not satisfactory under the TLC conditions used, or provided no reproducible results. However, some results obtained in the 1960s could be questioned because of either the strong dependence of R_f on experimental conditions or the poor separations obtained.

HPTLC plates impregnated with caffeine have provided for efficient separation of PAHs. They were commercialized in the 1990s due to the work of Funk et al. (35, 36). These plates provided peak-to-peak separation of several heavy PAHs in drinking water that are included in the EPA list: benzo[*ghi*]perylene, indeno[1,2,3-*cd*]pyrene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, and fluoranthene. Sample application was carried out in bands using a Linomat system; elution was carried out with

dichloromethane at -20°C (8 cm in 20 min), and detection was done by fluorescence densitometry at 365 nm (excitation), with emission collected at 436 nm. These plates were used by Matt et al. (37) to carry out an in-depth study of PAH and S-PAH elution order, using a horizontal developing chamber, as a previous step to developing a method for PAH separation according to the number of aromatic rings in petroleum middle distillates.

c. Reversed-Phase TLC. Reversed phase (e.g., octadecyl-bonded silica gel) shows higher selectivity for the separation of PAHs and gives higher fluorescence intensity in the corresponding hydrocarbon response (40). Therefore, reversed phase is mainly used for separating PAHs in water-compatible samples. Development solvents usually contain methanol, acetonitrile, or tetrahydrofuran in water (binary solvents) and methanol-water containing acetonitrile or dichloromethane (ternary solvents). Multiple development, which takes advantage of a spot reconcentration mechanism, improves resolution of PAHs with regard to conventional elution techniques (40).

Although the most successful phase for PAH separation has been octadecyl-bonded silica gel, other reversed-phase systems have also been studied (9, 25, 41, 42). For example, acetylated cellulose showed complementary separation characteristics with regard to octadecyl-bonded silica gel (41). Coelution characteristics with regard to PAHs using these phases are shown in Table 3.

The best choice for PAH detection is fluorescence densitometry. Some PAHs that are coeluted can be selectively detected using fluorescence. Table 4 shows selected conditions for determining PAHs by fluorescence after separation by HPTLC (40). The paper by Butler et al. (40) includes an interesting explanation of interferences in PAH determination. A criterion to confirm peak homogeneity after separation is that of using normalized emission response ratios. This criterion, together with that of coincidence in retention of standards and sample components, has been used to assess peak identity.

4. Heterocyclic Polyaromatic Compounds with S, N, and O

The problem of separating sulfur-containing polyaromatic compounds (S-PACs) from PAHs has long received a lot of attention (43). It is still unsolved in petroleum products.

Silica gel or acidic alumina plates loaded with mercuric acetate (44) were useful for the separation of mercaptans, sulfides, and disulfides from S-PAC+PAH in standard mixtures by a ligand-exchange mechanism. In this case, *n*-hexane was used as developing solvent in a sandwich chamber, and detection was carried out by UV inspection (at 254 nm) and by spraying with a

Table 3 Some Examples of PAH Coelution Characteristics Using Reversed-Phase TLC

Stationary phase	Coeluted PAHs
Octadecyl-bonded silica gel	Benzo[<i>a</i>]anthracene, chrysene, benzo[<i>a</i>]pyrene, benzo[<i>e</i>]pyrene, pyrene, benzo[<i>fluoranthene</i> isomers Benzo[<i>g,h,i</i>]perylene, indeno[1,2,3- <i>cd</i>]pyrene, anthracene, phenanthrene
Acetylated cellulose	Fluorene, benzo[<i>e</i>]naphthalene, benzo[<i>ghi</i>]naphthalene, coronene

benzo[*a*]anthracene, dibenzo[*a,h*]anthracene

Source: Adapted from Ref. 46.

palladous chloride solution. Spots colored by spraying were scanned at 380 nm, compensating background at 600 nm, for sulfur compound analyses. An orange spot indicated the presence of dibenzothiophene, and a yellow one indicated the presence of sulfides.

Alkyl-phenyl sulfides, which are compounds typically present in fossil fuels, were separated on either cadmium acetate or silver nitrate-silica gel impregnated TLC plates with a salt/support concentration of 25% (45).

Other polar functional groups in PACs have been detected by spraying the plates with specific reagents. Tyrpien et al. (46) described the characteristics of these compounds before and after spraying with Fast Blue salt B.

5. Fullerenes

The retention and separation selectivity of C₆₀ and C₇₀ fullerenes have been evaluated on silica gel neutral and basic alumina, amino, C-18, diol plates, and silica gel impregnated with aqueous solutions (0.5–2.5%) of polymers such as polyvinylalcohol (PVA), polyvinylpyrrolidone, and poly(ethylene oxide) (47–49). The ability of fullerenes to form complexes with various polymers is useful for separating, purifying, and transforming them into water-soluble moieties.

Among the systems studied, the use of diol HPTLC plates and development with isooctane and of HPTLC silica gel plates and development with hexane–pyridine (95:5) gave successful separations. Although all the studied polymers increased retention of fullerenes with regard to silica gel, the use of PVA-impregnated plates and hexane elution improved separation selectivity. In all cases, separated peaks were inspected under UV light at 254 and 366 nm.

B. Applications

1. Petro-, Carbo-, and Geochemistry

Almost all coal, petrochemical, and geochemical applications of TLC involve the use of normalphase adsorbents, usually silica gel (50). Usually, the separation of compounds is carried out by developing TLC plates with solvents of increasing or decreasing eluotropic strengths.

Applications of TLC involve (a) preparative fractionation of products, (b) qualitative identification of functional groups, (c) semiquantitative or quantitative hydrocarbon type analysis, and (d) planar size-exclusion chromatography.

a. Preparative Fractionation of Products. Sample fractions are collected either for further analysis by other analytical techniques (51–53) or for their use as external standards for quantitative determination of hydrocarbon types (17). Preparative fractionation is sometimes used as a rapid way to obtain quick comparative class

separation of fossil fuel-derived products. Table 5 shows examples of preparative TLC of typical hydrocarbon-containing samples.

The thickness of the preparative silica gel layers used depends on the amount and nature of the sample. For fractionation of sedimentary organic matter this is about 0.25 mm for 10–20 mg and 2.5 mm for 20–90 mg (52). Shale oil (150–200 mg) was fractionated using a 0.75 mm layer (4). A gas oil (500 mg) was applied as a 180 mm band using the Linomat IV applicator and fractionated using a 2 mm thick silica gel layer (17).

Preparative TLC was useful for obtaining separation of bitumens with quantitative recovery of sample (52). This procedure is an alternative to traditional methods based on extraction. In this case, cyclohexane was used as developing solvent. Development time was 1–5 h depending on the thickness of the layer used. Spraying half of the layer with a solution of berberine sulfate in methanol and subsequent inspection under UV light gave the location of the following separated fractions: (a) saturated and unsaturated compounds; (b) aromatics, naphthenoaromatics, and thiophenic compounds/ and (c) N, S, and O compounds including resins and asphaltenes. As usual, fractions were scratched, extracted with solvents, and further analyzed by GC/MS to identify the particular compounds of each fraction. It is possible to carry out a further separation between saturates and olefins on a second silica gel plate (0.5 mm thickness) that has been impregnated with an aqueous solution of silver nitrate (5 g/120 mL).

A similar scheme was followed to fractionate a shale oil (53). A rapid and reproducible separation into 14 fractions was obtained for this sample without requiring prior extraction of

Table 4 Selection of Fluorescence Conditions for Determining PAHs After Separation by HPTLC

PAH ^a	Conditions for maximum sensitivity (nm)				Comments on selectivity
	First choice		Second choice		
	Excitation	Emission	Excitation	Emission	
Ant	254	UV-D2	254	400	254:UV-D2 major interference from Phen; 254 and 313:400 minor interference from Phen; 365:400 no interference from Phen, but signal is weak.
BaA	254	400	365	400	365:400 no interference from Chr; 254 and 266:UV-D2 and 400 large interference from Chr; may be determined at 500 nm at all excitation wavelengths without interference, but signal is weak.
BaP	365	450	365	400	365:450 major interference from Per and BxFlt; lower interference from Per and BxFlt at 365:400.
BbFlt	365	400	365	500	At 450 emission interference at all excitation wavelengths from BaP ReP

					Per, and BxFlt. At 500 interference from BeP diminished.
BjFlt	313	550	313	500	Minor interference from BkFlt and BbFlt at 313:600
BkFlt	365	400	365	450	Interference from BaP, BeP, and BxFlt.
BeP	313	400	254	400	No interference at 266:UV-D2 and 254:UV-D2. Major interference from BxFlt and BaP at 400 emission excited at all wavelengths.
BPer	365	450	365	400	365:400 and 450 no interferences. 266 and 313:450 interference from DBahAnt. At 500 emission interference from IncdPyr observed at all excitation wavelengths.
Chr	254	UV-D2	254	400	Interference from BaA at all wavelengths. No response at 365 excitation. 254 and 266: UV-D2 gives a stronger response for Chr than BaA. At 254, 266, and 313:400 response of BaA is greater than that of Chr.
Cor	313	450	313	500	Interference at 313:450 and 500 from DBaiPyr.
DBahAnt	311	400	313	450	No response at $\lambda_{\text{ex}}=365$; weak response at $\lambda_{\text{ex}}=254$. At 313:400 and 450 interference from BPer (lower at 400).
DBaiPyr	365	450	365	500	No interference at 365:450. Cor interferes at 313:450, 500, and 550 (lowest at 550). Weak response at 254 excitation.
Flt	365	450	365	500	Also responds at 550 and 600 emission with a reduction in signal (2–10-fold compared to 500) at all excitation wavelengths. Possible interference from Pyr at 450 emission and all excitation wavelengths.
Flu	254	UV-D2			
IncdPyr	365	500	365	550	No interferences at 313:600; minor interference from BPer at 313:500 and 550.
Per	365	450	254	450	Interference from BaP and BxFlt at 254, 266, and 365:450 and 365:500. Interference from BaP is low at 254:500. If BxFlt levels are low an approximate value for Per can be obtained at 254:550.
Phen	254	UV-D2	254	400	Major interference from Ant.
Pyr	313	400	254	UV-D2	254 and 266:UV-D2 no interferences. At 400 emission Flt interferes at all excitation wavelengths.

^a Anthracene (Ant) benzo[*a*]anthracene (BaA) benzo[*a*]pyrene (BaP) benzo[*e*]pyrene (BeP)

benzo[*g,h,i*]perylene (Bper), chrysene (Chr), coronene (Cor) dibenzo[*a,h*]anthracene (DBahAnt), fluoranthene (Flt), fluorene (Flu), perylene (Per), phenanthrene (Phen), pyrene (Pyr), benzo[*b*]fluoranthene (BbFlt), benzo[*j*]fluoranthene (BjFlt), benzo[*k*]fluoranthene (BkFlt), indeno[1,2,3-*c,d*]pyrene (IndcPyr), dibenzo[*a,i*]pyrene (DBaiPyr).

Source: Ref. 40, by permission of Preston Publications, a division of Preston Industries, Inc.

Table 5 Examples of Preparative TLC
Fractionation of Hydrocarbon-Containing Samples

Sample	Technique	Development	Hydrocarbon type	Detection	Ref.
Bitumen (up to 20 mg)	Silica gel layer (up to 2.5 mm)	Cyclohexane	1. Unsaturates	Berberine, UV inspection	53
			2. Aromatics	UV inspection	
			3. Polars	Visual	
Bitumen	Chromatotron (silica gel, 2 mm)	1. Petroleum ether	1. Saturates, monoaromatics, resins	Gravimetric	59
		2. Petroleum ether-ether (8:2)	2. Polyaromatics, resin-1	Inspection by UV at 254 nm	
		3. Ethyl acetate	3. Resin-2	Visual	
		4. THF	4. Asphalthenes	Visual	
Shale oil (150–200 mg)	Silica gel layer (0.75 mm)	1. <i>n</i> -Pentane	1. Nonpolars	2,7-Dichlorofluorescein (in MeOH), UV inspection	54
		2. <i>n</i> -Pentane–diethyl ether (5:1) (total: 25 min)	2. Polars		
Gas oil (500 mg)	Silical gel layer (2 mm)	<i>n</i> -Hexane (until the top)	Alkanes, cycloalkanes, aromatics	Fluorescence induced by berberine (densitometry)	17
Pitches	Silica gel layer	1. THF (5 cm)	Different types of aromatics and polars	UV at 254, 366 nm (densitometry)	58
		2. Chloroform-methanol (4:1) ($\Delta=+5$ cm)			
		3. Toluene ($\Delta=+5$ cm)			
		4. <i>n</i> -Pentane ($\Delta=+5$ cm)			

		cm)			
Mesophase pitches	Silica gel layer	I: 1. Pyridine	1. Black (immobile)	UV at 254, 366 nm (densitometry)	57
		2. Acetonitrile	2. Brown (mobile in solvent 1)		
		II: 1. Pyridine 2. <i>N,N</i> - Dimethylformamide	3. Orange (mobile in both solvents)		
		III: 1. THF 2. Toluene			

asphaltenes, acids, or bases. Development was carried out with *n*-pentane to elute the nonpolar compounds and *n*-pentane–diethyl ether to separate the polar components. Bands were visualized by spraying with a solution of 2,7-dichlorofluorescein in methanol (0.2%) and irradiating with UV light. The separate bands, which appeared purple or blue under UV light, were scraped and the fractions recovered by extraction. Fractions were subsequently identified by GC-MS, NMR, and IR.

Recently, densitometry was used to monitor preparative TLC fractionation (17, 54–57). A gas oil was preparatively fractionated to isolate calibration standards (17). Elution with *n*-hexane allowed alkanes (linear+iso), cycloalkanes, and aromatics to be separated. The alkanes and cycloalkanes were detected by fluorescence, using a strip of the plate that was previously impregnated with berberine. The cutting point between alkanes and cycloalkanes was fixed by detection of cycloalkanes by UV at 210 nm. The distance of migration of the aromatic fraction was detected by UV at 254 nm. The purity of the isolated fraction was verified by the corresponding HPTLC analytical runs.

Coal tar and other types of pitches and coal liquefaction liquids have also been fractionated by preparative TLC following a variety of schemes (54–57). Characterization of coal tar pitch is important with regard to the quality of carbon-derived products and other environmental and occupational health problems. Pitch-derived fractions underwent subsequent characterization using external techniques. Different elution systems have been used after solubilization of pitch with pyridine and application as spots or bands: (a) a series of decreasing polarity [successive development in THF, chloroform–methanol (4:1), toluene, and pentane]; (b) pyridine followed by acetonitrile, or THF followed by toluene (in both cases, each successive solvent advanced the solvent front from the origin by approximately 5 cm); and (c) multiple development with one of these solvents. In case (b), fractions were located by UV densitometry or by visual inspection: black (immobile), brown (mobile in THF or pyridine but immobile in toluene or acetonitrile), and orange (mobile in both solvents) (Fig. 2).

Preparative, centrifugally accelerated, radial TLC of asphalt was achieved by using a commercially available apparatus, the Chromatotron (58), which consists of a rotor coated with a thin (2 mm) layer of silica gel. The mobile phase and sample solution are introduced through an inlet placed near the center of the rotor. A radial elution leads to the formation of concentric bands that can be collected. This device has been suitable for

quantitative analysis of asphalts. In addition, the collected fractions can be used for the reconstitution of asphalts with special properties.

b. Qualitative Identification of Functional Groups. As in the works cited in the previous subsection, colorless compounds are detected under UV light if they show absorption in the UV

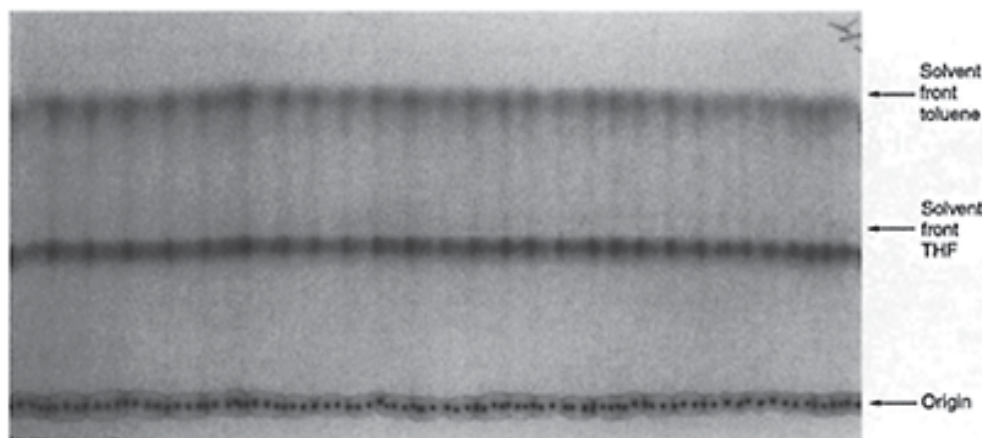


Figure 2 Preparative development of a synthetic naphthalene mesophase pitch applied in pyridine slurry and developed in tetrahydrofuran and toluene. Copyright 2000 Academic Press. (From Ref. 56.)

region or if they can be excited to produce fluorescence by either shortwave (254 nm) or longwave (365 nm) UV excitation. Chromogenic or fluorogenic reagents have also been used to detect functional groups and hydrocarbon types, some of these being selective reagents for particular compounds. For instance, polar compounds present in extracts of lignite, coal tars, and organic matter present in carbonate rocks of various origins were characterized by silica gel TLC using dichloromethane as mobile phase and detection under UV light with a series of chromogenic detection reagents (59).

A procedure has been applied to lube stocks and high-boiling petroleum distillates to distinguish sulfides and dibenzothiophenes. After elution of samples with *n*-hexane on a TLC silica gel or acidic alumina plate impregnated with mercuric acetate, spots were visualized by spraying with a palladous chloride solution and scanning at 380 nm. An orange spot indicated the presence of dibenzothiophene, and a yellow one the presence of sulfides (44).

A quick, inexpensive, qualitative TLC method was developed for residues from petroleum products (e.g., gasoline, kerosene, and diesel fuel) generally encountered as accelerants in fires and arson cases. After a previous extraction of samples with ethyl ether, silica gel TLC is carried out by development with heptane or isooctane mobile phases and UV visualization of zones (60, 61).

c. Semiquantitative or Quantitative Determination of Hydrocarbon Types. In general, quantitative analysis using UV visualization is carried out with TLC scanners in either

absorbance or fluorescence mode. There have been few studies on the quantitative analysis of fossil fuel samples by TLC or HPTLC plates (Table 6). For example, bitumen samples were analyzed by TLC with UV absorbance/fluorescence measurements (13). For this analysis, the sample was spotted on a silica TLC plate to be developed sequentially with *n*-heptane (8.5 cm), dichloromethane (4.5 cm), and tetrahydrofuran (2.5 cm). The plate was then scanned at 254 nm with a scan width of 1.5 cm in the UV absorption mode to determine aromatics and polar compounds. The plate was subsequently dipped for a few seconds in a solution of berberine sulfate (0.004%) in methanol for the determination of saturates in the fluorescence mode using an excitation wavelength of 264 nm and a scan width of 1.5 cm.

The phenomenon of detection of saturates using berberine has recently been studied, and precise, sensitive methods for hydrocarbon type determinations have been developed and adapted to a variety of products with different boiling ranges (e.g., gas oil, heavy oil, and lubricants) using TLC and HPTLC silica gel plates (14–17). In these cases, a methanolic solution of berberine was used to preimpregnate the plates because berberine does not affect separation. The most sensitive wavelength of excitation was 365 nm, and detection sensitivity can be modulated through the concentration of berberine and impregnating time.

A typical analysis for a gas oil on TLC plates includes elution with *n*-hexane (9 min) and then dichloromethane (4.5 min) and detection by berberine-induced fluorescence ($\lambda_{\text{exc}}=365$ nm for saturates) and UV (UV 254 nm for aromatics) (14) (Fig. 3). The use of HPTLC plates (elution: 4 min with *n*-hexane) led to shorter analysis times, an improvement in sensitivity, and improved separation of saturates in alkanes (linear+iso) and cycloalkanes. In addition, determination of total aromatics is possible, using a nonimpregnated silica gel plate after *n*-hexane elution, by merging the broad peaks of aromatics into one narrow Gaussian peak by elution with acetone or ethanol (17). Quantitative analysis is done by external calibration using preparatively isolated fractions.

d. Planar Size-Exclusion Chromatography. A planar size-exclusion chromatographic technique has been developed that allows the molecular mass distribution of bitumens to be determined (62). The chromatography is carried out on a 0.25 mm layer silica gel TLC plate placed in a special sealed chamber that is then tilted to a suitable angle. This enables control of the run time and affects the profile of the chromatogram, which, according to the authors, leads to a separation according to molecular mass rather than chemical classes. Thus, bitumen solutions (5% m/v in THF) are applied onto the plate and developed for a distance of 45 mm. After this, the plate is dried at 80°C for 10 min and then detected with UV at 254 nm. Postimpregnation with a solution of berberine and excitation at 265 nm is then carried out to obtain additional information about

Table 6 Examples of Quantitative Determination of Hydrocarbon Types by TLC-Densitometry

Sample	Technique	Development	Hydrocarbon types	UV detection (nm)	Fluorescence detection (λ_{exc} , nm)	Ref.
Bitumen	TLC	1. <i>n</i> -Heptane (8.5 cm)	Saturates		Postimpregnation, berberine, 264 nm	13
		2. Dichloromethane (4.5 cm)	Aromatics	254		
		3. THF	Polars	254		
Lubricant, gas oil	TLC	1. <i>n</i> -Hexane (9 min)	Saturates		Preimpregnation, berberine, 365 nm	14
		2. Dichloromethane (4.5 cm)	Aromatics	254		
Heavy oil, vis-breaking fuel	TLC	1. <i>n</i> -Hexane (30 min)	Saturates		Preimpregnation, berberine, 365 nm	14
		2. Toluene (8 min)	Aromatics	254		
		3. Dichloromethane (3 min)	Polars, noneluted	254		
Gas oil	HPTLC	1. <i>n</i> -Hexane (4.5 min)	Alkanes, cycloalkanes		Preimpregnation, berberine, 365 nm	17
		2. Acetone to merge all aromatics	Total aromatics	254 ^a		

^a Determination of aromatics exclusively on a nonimpregnated silica gel layer.

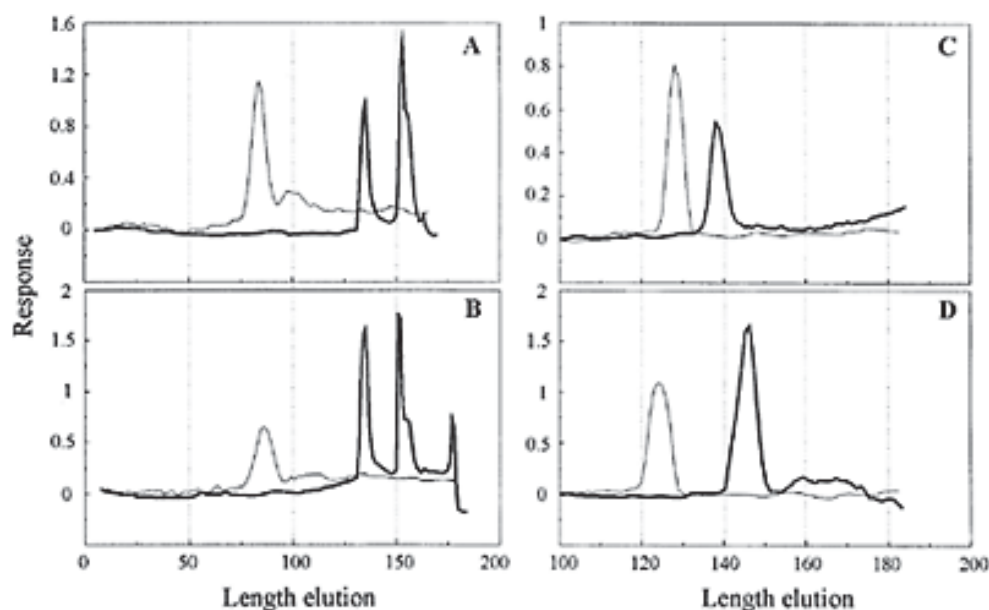


Figure 3 TLC chromatograms corresponding to saturate fractions determined using fluorescence scanning densitometry on berberine-impregnated silica gel plates (lightweight lines) and aromatic fractions determined using UV scanning densitometry on the same plates (heavy lines). (A) Heavy oil; (B) visbreaking fuel, (C) lubricant, (D) gas oil. Copyright 1999 Preston Publications. (From Ref. 14.)

the saturated fraction of the bitumen. According to the authors, qualitative identification of bitumens of different grades and/or from different refineries is possible with this technique.

2. Environmental Sciences, Toxicology, and Health

Hydrocarbons, especially PAHs, are ubiquitous environmental contaminants. In general, PACs enter the environment primarily as by-products of incomplete combustion from a wide variety of sources. Because some PACs are known or suspected to be animal carcinogens, there is considerable interest in the detection of their presence, concentration, and distribution in the environment. Their analysis by TLC was reviewed in 1996 by Bladec (63) in a study concerned with the application of TLC to environmental analysis.

All environmental samples (from water, air, soil and food) require a previous step of sample preparation or cleanup before analysis by TLC. This step depends on sample origin. This aspect is not considered in this chapter.

a. Water and Marine Samples. With regard to environmental applications to marine samples, on-line coupled HPLC-TLC with two-dimensional development has been used for PAH characterization in sediments (64). Reversed phase on C₁₈ bonded silica and acetylated cellulose were used. Developing solvents were methanol–diethyl ether–water. The second dimension of elution resolved the aromatic compounds satisfactorily. PAH peaks were detected by fluorescence densitometry.

Prediction of *n*-octanol–water coefficients and related biological activities was attempted through quantitative structure–activity relationships obtained from *R_f* data of polynuclear parent and heteroatomic hydrocarbons, which were in turn obtained on C₁₈ reversed-phase TLC plates (65). Elution was carried out with methanol–deionized water–phosphate buffer, and detection was done by UV (254 and 350 nm).

A good example of direct application of a method developed with standards is the previously cited work by Funk et al. (35) (see Sec. IV.A.3.b), in which silica gel HPTLC plates were im-pregnated with caffeine to separate and quantify six heavy PAHs according to the German drinking water specifications.

b. Air and Combustion Gases. A method developed by Buttler et al. (40) was adequate for quantitatively determining PAH in the soluble organic fraction of air particulate matter. Determination of 0.4–20 ng benzo[*a*]pyrene per cubic meter of air was achieved by using acetylated cellulose TLC plates (42). Elution was carried out using ethanol–dichloromethane (8:2), then pyridine–methanol–water (3:5:2). Detection was done by fluorescence. Acetylated cellulose was also able to separate some PAHs in diesel exhaust gases (66). Elution was carried out using *n*-hexane–toluene (90:5.5), then MeOH–diethyl ether–water (60:40:10).

Thin-layer chromatographic and HPTLC methods for the separation and identification of some nitrogen derivatives of PAHs in airborne particulate matter have been described (46, 67). Tyrpien (67) used semipreparative TLC to separate chemical families in airborne particulate matter and sewage sludge and analyzed them by GC-MS. Elution was with DCM–*n*-hexane followed by DCM–*n*-hexane–MeOH in a DS sandwich chamber. After scraping and extracting the layer, an aromatic fraction was isolated. Detection of PAHs was carried out by UV illumination at 254 and 366 nm. Other functional groups were visualized using specific reagents.

Separation of methyl-substituted benzo[*c*]acridine from air pollution sources by TLC has been achieved on normal-phase and reversed-phase systems. Detection was done by fluorescence after derivatization with trifluoroacetic acid (68). Benzacridines in both diesel and gasoline vehicle exhaust in air samples taken in a road tunnel, in coal tar, and in river and marine sediments were separated using two-dimensional elution on alumina, kieselguhr, and acetylated cellulose (69).

Nitrogen-containing PAHs were identified in airborne particulate matter using dichloromethane extraction, semipreparative silica gel TLC, and analytical TLC of the separated fractions on C₁₈F layers developed with acetonitrile–water (for nitroarenes) and methanol–water (azaarenes); specific dyeing reagents and viewing under 254 and 365 nm UV light were used for detection (70).

c. Soil. Application of TLC to soil has been done using both portable and laboratory equipment. Portable TLC equipment has been used for analytical field screening of PAHs in soil (71) and included extraction of the soil sample, elution of the extract on a TLC plate, and visualization of contaminants from the developed plate. Visualization was accomplished by using iodine staining, UV absorbance detection, and reaction of the iodine-stained material with α -naphthoflavone-based spray to enhance detection.

Practical approaches have been developed for PAH determination in laboratory TLC. As an alternative to the separation of PAHs in single peaks by reversed-phase TLC, a low-resolution separation into a few "compressed" bands with a characteristic pattern (fingerprint) and a subsequent rapid simple semiquantitative evaluation of their visual fluorescence is possible. This method is reproducible and avoids the use of toxic acetonitrile (72). By using this concept, a simple, rapid, and cheap HPTLC screening method for the estimation of the PAH content in soil samples has been developed (73). This method is based on a highly significant correlation between the visual fluorescence fraction of PAH and the total EPA PAH₁₆ content in mineral soils. It is carried out through a deliberately incomplete reversed-phase HPTLC separation of PAHs (EPA list) into a few fingerprint-like compressed bands within a determined "PAH window." This minimizes the actual costs and time spent per sample when using the reference method (HPLC).

Nitrogen-containing aromatic compounds in soil and sediments have been semipreparatively fractionated by TLC on silica gel for analysis by GC (74). Elution was carried out with DCM–*n*-hexane. Detection was done by UV, in comparison with the UV results of reference compounds.

Thin-layer chromatography has contributed to PAC determination in soils and also to monitoring the ozonation of [¹⁴C]pyrene and [¹⁴C]benzo[*a*]pyrene in both silica and contaminated soil (75). Ozonation of PAHs in soil is a process that can be used for in situ remediation or in combination with bioremediation techniques. A combination of TLC and GC-MS analysis of extraction products from artificially contaminated silica and soil revealed a large number of aromatics: PAH-quinones and 10-ring fission products with formyl and carbonyl groups of both pyrene and benzo[*a*]pyrene. TLC was done using a 50% mixture of ethyl acetate and petroleum ether (60–80°C) for elution, UV at 254 and 366 nm, and ¹⁴C scanning for detection and ¹⁴C mass balances.

Qualitative and quantitative determination of PAHs in petroleum-contaminated soils (76) was described. This method included extraction, TLC separation on caffeine-impregnated kieselguhr plates, and fluorometric detection and quantification.

d. Toxicology and Health. Although benzo[*a*]pyrene (BaP) causes tumors in animals, particularly in the upper gastrointestinal tract, the role of dietary intake of BaP on cancer in humans is not clear. For this reason, a BaP database with data from 200 food items has been created (77, 78). The quantities of BaP were measured using TLC-fluorescence densitometry and HPLC. After saponification of a sample, extraction with isooctane, and cleanup by column chromatography on Florisil, the cleaned sample was developed on silica gel TLC plates with cyclohexane and then with benzene. PAHs were located in the benzene fraction. This fraction was applied as a spot (100 μ L) on a 20% acetylated cellulose plate and further developed using an ethanol–dichloromethane mixture. BaP was detected by fluorescence densitometry (λ_{exc} =387 nm and λ_{em} =428 nm). The limit of detection was 0.005 ppb. Results were highly correlated with those obtained from HPLC.

A method for identification of irradiated fat-containing foods was developed. It involves the determination of 1,2-unsaturated hydrocarbons and 2-alkylcyclobutanones as fat radiolysis products. This was performed by isolation from the food, derivatization, labeling with a fluorophore, and coupled TLC-HPLC (79).

The determination of carcinogenic PAC-DNA adducts is considered as a possible method to monitor human exposure to environmental PAC carcinogens. One of the methods developed to analyze these DNA adducts is the TLC ^{32}P postlabeling assay. After nuclease digestion of DNA samples and further postlabeling with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, the adducted nucleotides are separated by multidirectional, multisolvent anion-exchange polyethyleneimine cellulose TLC. Chromatography directions and development solvents are usually denoted as D1, D3, D4, and D5, with D2 generally omitted. D1 and D2 solvents wash labeled normal nucleotides from the origin of the chromatogram onto areas of the plate that are then cut off and discarded. D3 and D4 represent the crucial steps because they determine adduct resolution and separation. D5 is used in a cleanup step to further remove radioactive contaminants remaining on the layer. Chromatograms are visualized by autoradiography at -80°C for various periods of time.

The composition of D4 solvent has been studied, and several alternatives exist. Thus, 0.2 M ammonium hydroxide was proposed for separation and resolution of a wide array of adducts derived from highly lipophilic PAHs (80). Likewise, it was proposed that boric acid be incorporated into D4 solvent solvent. This facilitates the separation of (+)-*syn*- and (–)-*antibenzo[a]pyrene* dihydrodiol epoxide–DNA adduct (81). Adducts from blood DNA of workers exposed to coke oven PAHs and from DNA modified in vitro with *benzo[a]pyrene*, *benz[a]anthracene*, *benzo[k]fluoranthene*, *dibenz[a]anthracene*, and other PACs have been studied using these methods (82).

V. TLC-FID

In the TLC-FID system, chromatographic separation is carried out on chromatorods that consist of a thin adsorbent layer ($75\text{ }\mu\text{m}$) formed on the surface of a quartz rod ($0.9\text{ mm diameter} \times 15.2\text{ mm length}$) by sintering inorganic binders together with silica gel or alumina (particle size $5\text{ }\mu\text{m}$, pore diameter $60\text{ }\text{\AA}$). In a typical experiment, a set of 10 chromatorods are preassembled in a frame, and after application of the sample, subsequent development with solvents, and drying at a certain temperature (e.g., 70°C), they are sequentially passed at a constant speed through an $\text{H}_2\text{--O}_2$ flame of a flame ionization detector for quantification of the peaks.

This system is more limited than the planar ones from the point of view of compound separation. In addition, FID does not provide structural information on separated peaks and is a destructive method. In spite of these limitations, TLC-FID has been useful for hydrocarbon type analysis of heavy fuels. There have been many publications related to fossil fuel analysis as well as a book (10) and several reviews (83, 84), mostly applied to other fields of chemistry that also include aspects of interest for hydrocarbon analysis.

A. Suitability of TLC-FID for Hydrocarbon Analysis

Although controversy was reported regarding the acceptability of the quantitative results when using TLC-FID (84, 85), an evolution in TLC-FID instrumentation has clarified the situation. Improvements include relatively recent changes in detector design together with the use of automatic sample spotters, technological advances in chromatorod manufacture, and the evolution of electronics in data acquisition and treatment. The use of TLC-FID techniques in various stages of development and their application to excessively volatile samples have been blamed for confusing results in the past.

The Mark 5 Iatroscan model includes the newer detector (FID) configuration in which the ion collector is closer to the chromatorods than it was in the older models (Mark II, III, IV, TH-10). It has been reported to be more sensitive and to give better reproducibility. The performance of this system was tested on alkanes and PAC standards to evaluate its suitability for quantitative hydrocarbon type analysis of coal and petroleum products (86). Using the Mark 5 detector configuration, linear regression provides adequate regression coefficients and intercepts for sample loads higher than 1 μg , with unimportant relative errors and with adequate repeatability.

Absolute response factors obtained for different loads of a high molecular weight PAH (rubrene) did not vary significantly with scan speed, except in the case of the slowest speeds (i.e., 60 s per scan). In this case, smaller, though linear, signals were obtained. Data seem to support the hypothesis that differences in sensitivity for the slower scan speeds could also be caused by more complete combustion of the sample and production of fewer ions in the FID detector.

TLC-FID response factors for compounds of several homologous series were studied to differentiate the effects of volatility from those due exclusively to chemical nature (86). There are clear differences in response factors between different compounds and between different scan speeds, although the absolute response factors are reasonably uniform for each homologous series of alkanes longer than C_{24} and aromatics with four or more rings.

Measurements of chromatorod temperatures were also carried out in order to evaluate whether evaporation of compounds might take place outside the H_2 flame. From this study, it can be concluded that volatilization of rubrene should not take place prior to combustion. However, volatilization of other compounds near the flame should not be discounted.

It can be seen TLC-FID is especially suitable for heavy petroleum or coal products. However, its application to lower boiling range products cannot be dismissed a priori, and studies should be undertaken on each particular sample.

Repeatability of TLC-FID experiments on bitumen composition has often been questioned. Masson et al. (87) recently reported that the effect of chromatorod aging on reproducibility causes a 2–5% variation in the saturates, aromatics, and resin A and B content, and the time between bitumen dissolution and its analysis causes a 50–75% variation in the aromatic and resin A content.

B. Applications

1. Hydrocarbon Type Analysis for Petro-, Carbo-, and Geochemistry

Hydrocarbon-type analysis is almost exclusively the only application of TLC-FID in the hydrocarbon field. This analysis has been used to solve many different problems in petrochemistry, carbochemistry, geochemistry, and environmental sciences. Examples of this are the quantitative characterization of hydrotreated petroleum products (88), lubricant base oils (89), high-boiling residues or fractions derived from crude oils and coal products (88–94), asphaltenic tank oils (95), oil shale bitumen (95, 96) or coal tar pitch (94, 97); characterization of petroleum-contaminated soils (98); compositional analysis of bitumen (87, 99, 100); analysis of heptane insolubles and the paraffin content of bitumen (101); evaluation of the hydrocarbon oil-degrading capability of marine organisms (102, 103); compositional analysis of geochemical sources (104); determination of the relationship between fuel properties and exhaust emissions (105); and determination of PAHs (as a group) in the industrial atmosphere where large amounts of coal or coke are burned (106).

After a few micrograms of the sample have been spotted, the chromatograms are developed sequentially with several solvents or their mixtures in decreasing or increasing eluotropic strengths to separate the hydrocarbon types. Some examples are shown in Table 7. All the solvent schemes for hydrocarbon type analysis are very similar. They are based on aliphatic and aromatic solvents (*n*-hexane or *n*-heptane, toluene) and, for the more polar components, contain either chlorinated hydrocarbons (dichloromethane, chloroform) or alcohols (methanol, ethanol). Sequential developments usually involve two to four eluants (pure or mixtures) of those categories. Although each type of sample requires a particular adaptation, numerous solvent systems exist that can work well for a given sample.

Separation is carried out, in most cases, by using a series of eluants of increasing eluotropic strength. For instance, a deasphalted heavy oil (DAO) and its derived hydrocracking products were separated into saturates, alkylaromatic, aromatic, and polar components plus a noneluted fraction using a sequential elution with *n*-hexane (38 min), toluene (3 min), and dichloromethane–methanol (95:5) (30 s) (88).

The use of solvents of decreasing polarity has sometimes been the preferred choice. It has been stated that this allows separation of component classes with superior baseline, better resolution of the hydrocarbon classes, and polarity-based distribution of aromatic as well as polar constituents (89). For example, petroleum oils have been developed first with a 9:1 chloroform–methanol mixture (3 min), which mobilizes all hydrocarbons and separates the polar components (less retained) into two peaks. Then toluene (5 min) is used for the separation of saturates plus aromatics from the polar components. In the second development with *n*-heptane (30 min), the polar components are not displaced and the saturates are separated from the aromatics. During this step, the aromatics are also distributed broadly according to their polarity. The latter is due to an incremental displacement of aromatic components as their polarity decreases, providing a distribution of aromatics according to the number of aromatic rings (Figs. 4 and 5).

Table 7 Typical Development Systems for Hydrocarbon-Type Determination by TLC-FID

Sample	Development system	Hydrocarbon type	Ref.
Heavy oil and hydrotreated products, vacuum residues, coal tar pitches	1. <i>n</i> -Hexane (38 min)	Saturates	88
	2. Toluene (3 min)	Alkylaromatics, aromatics	
	3. Dichloromethane–methanol (95:5)	Polars, noneluted	
Crude oils	1. <i>n</i> -Hexane (35 min)	Saturates	104
	2. Cyclohexane (14 min)	Mono- and diaromatics	
	3. Toluene (14 min)	Polyaromatics	
	4. Dichloromethane–methanol (93:7) (4 min)	Resins	
Oils	1. Chloroform–methanol (9:1) (3 min)	Polars (two peaks)	89
	2. Toluene (5 min)	Aromatics	
	3. <i>n</i> -Heptane (30 min)	Saturates	
Bitumen	1. <i>n</i> -Heptane (45 min)	Saturates	87
	2. Toluene (20 min)	Aromatics	
	3. THF (5 min)	Resins	
Coal-derived liquids	1. <i>n</i> -Hexane	Saturates	94
	2. Benzene–hexane (80:20)	Aromatics	
	3. Methanol–dichloromethane (40:60)	Polars	

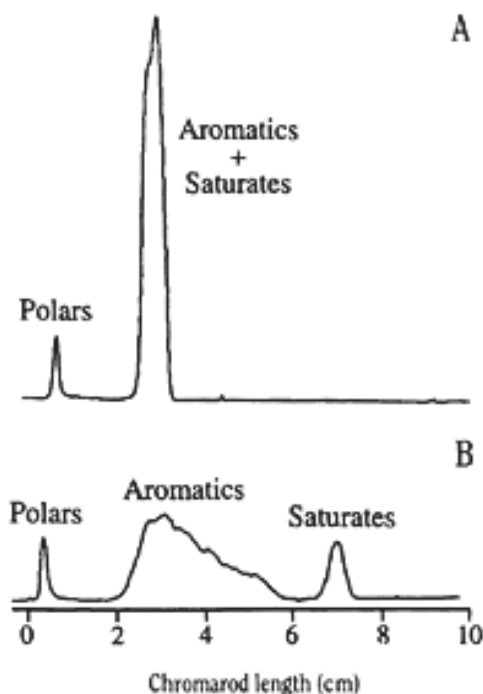


Figure 4 Two-step chromatogram (TLC-FID) development of an aromatic extract. (A) Development with toluene for 5 min; (B) development with *n*-heptane for 30 min following the toluene development. Copyright 1996 Preston Publications. (From Ref. 89.)

There are few applications of TLC-FID other than hydrocarbon-type analysis. One interesting use is the determination of relative asphaltene solubility in naphthas (107). A variety of TLC-FID experiments were done with a series of eluants that ranged from 100% THF to 90% naphtha. While THF moves the asphaltenes with the solvent front in the elution process on chromatorods, the chromatograms become progressively wider and spread with increased proportions of naphtha in the THF eluant. Quantitative evaluation can be achieved using area-slicing of the integrator output and cumulative graphs and calibration with pure precipitants (*n*-hexane, *n*-heptane).

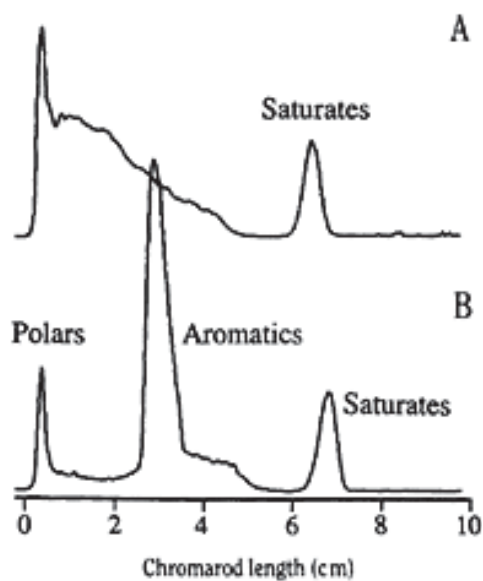


Figure 5 Two-step Chromarod (TLC-FID) development of the same aromatic extract as in Fig. 4. (A) Development with *n*-heptane for 30 min; (B) development with toluene for 5 min following the *n*-heptane development. Copyright 1996 Preston Publications. (From Ref. 89.)

a. Calibration. The main question concerning the need for a calibration step regards the influence of the nature of the sample on the TLC-FID results. Apart from the qualitative similarity of chromatograms for fossil fuel products, there is a widespread tendency for researchers to consider the FID response for fossil fuel samples as homogeneous for all the peaks. This is because TLC-FID has been used in some cases, with good results, for hydrocarbon-type determination without prior calibration (89). However, different response factors for different types of hydrocarbons have also been obtained (86). In principle, the supposed homogeneity in the TLC-FID response should be verified for each sample type, and therefore the need for calibration should not be excluded a priori.

An explanation of this has been proposed (86): When the hydrocarbons in the sample to be analyzed are of a very similar chemical nature or sufficiently high molecular size (i.e., lubricants), they exhibit the same kind of behavior in response to TLC-FID (ionization or combustion). This is the case for samples analyzed by Barman (89), and for similar cases percentages in area can be used directly as mass percentages. However, when the peaks from the sample to be analyzed show an appreciable range of variation in chemical nature or molecular size (i.e., oils) (88, 91, 92), the previous hypothesis is no longer acceptable, and a calibration step becomes necessary.

This range of variation can be used to reduce the number of reference products to be used for calibration. For example, Bharati et al. (91, 92) used for calibration a product

obtained by mixing 31 different oils, in this case, a mixture of all the samples to be studied for preparing calibration standards. Therefore, the calibration averaged the deviations for the samples.

In any case, the above-mentioned procedures (direct integration and preparation of synthetic standards) are acceptable for samples of a particular nature but are not general enough to be applied to an unknown, general, hydrocarbon-containing sample. In general, calibration in the hydrocarbon field is done by using external standards (88). Sample fractions derived from the fossil fuel itself and isolated using a preparative method are the most suitable and most common calibrating standards.

A fast calibration method based on a variation of the internal normalization procedure has been proposed and tested on a variety of petroleum and coal products (88, 108). It is based on the principle that if the response of DID versus the whole sample mass can be linearized for each peak with forced zero intercept, then mass and area percentages can be used indistinctly in this mass range. The linearity (mass) interval for application must be determined by the analyst after inspection of regression data. Results from the external standard calibration method and from this variation of the internal normalization procedure have been in accordance, although the latter is faster and allows a rapid determination of the linear range of the detector.

2. Monitoring of Preparative Fractionations

Preparative column-based techniques (LC) have been used to fractionate fuels. The selection of solvent volume for an eluted fraction is sometimes a delicate matter because of possible fraction overlaps. Subfractions are usually collected, and later off-line characterization is carried out to check the purity of a fraction. Refractive index (RI) values or an ultraviolet (UV) detector are mostly used for this purpose. TLC-FID (88, 89) has been reported to be a useful tool for monitoring the separation and verifying the purity of fractions obtained by LC methods. Thus, TLC-FID allowed the observation to be made that both aromatic and polar fractions were found in a saturate fraction when excess *n*-hexane was used to elute saturates from a heavy oil (88). In another work, cross-contamination in saturates and aromatic and polar fractions from lubricant base stocks obtained by ASTM D2007 were ascertained when these fractions were analyzed by TLC-FID (89). Data also showed incomplete recovery of materials from the adsorbent. This is important for the gravimetric determination of polar components by extraction from the clay adsorbent using a 1:1 acetone-toluene mixture.

TLC-FID was useful in determining a significant amount of non-PACs in the PACs obtained by fractionation using the IP346 standard, and vice versa. According to Barman (109), the bias was significant for samples with both low and high PAC content. It was found that PACs are overestimated at low concentrations (<1.0% w/w) and underestimated at high concentrations (>4.0% w/w).

VI. CONCLUDING REMARKS

In the past, TLC was overshadowed by other chromatographic techniques such as GC, SFC, and HPLC for the analysis of hydrocarbons in various types of fuels and related

samples. However, TLC currently has an important potential in regard to certain separations and quantifications that are otherwise done by more difficult, less effective, or tedious techniques.

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20

Hydrophilic Vitamins

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I. INTRODUCTION

The benefit of using TLC for identification of unknown vitamins and related compounds by comparing R_f values of the unknown compounds with those of authentic vitamins is beyond doubt. The quantification of the separated vitamins can be performed by the use of modern densitometry. TLC as a powerful separation and analytic tool is used particularly for pharmaceutical preparations and food products. Because amounts of most hydrophilic vitamins are low or very low in tissue or body fluid, bioautography or derivatization is used before densitometry. Various high-quality precoated plates with small, uniform particle diameters are available for TLC or high-performance TLC (HPTLC). Stationary phases of silica gel, cellulose, or various reversed phases are available. TLC has great advantages (simplicity, flexibility, speed, and relatively low cost) for the separation and analysis of hydrophilic vitamins.

II. THIAMINE (VITAMIN B₁)

The chemical structure of thiamine is shown in Fig. 1. A pyrimidine moiety (2-methyl-4-amino-5-hydroxymethylpyrimidine) and a thiazole moiety (4-methyl-5-hydroxyethylthiazole) are connected by a methylene group. The double salt form of thiamine with hydrochloric acid is readily soluble in water. Thiamine in water is most stable between pH 2 and 4 and unstable at alkaline pH; it is heat-labile, with its decomposition dependent on pH and length of exposure to heat.

The structures of the phosphate esters of thiamine are also shown in Fig. 1. Thiamine monophosphate (TMP), thiamine pyrophosphate (TPP), and thiamine triphosphate (TTP) are commonly found in organisms. About 80–90% of the total thiamine content in cells is TTP, the coenzyme form of thiamine.

To investigate thiamine metabolism in mammals, thiamine (R_f values 0.16, 0.04, and 0.03), thiamine metabolites excreted in urine [thiochrome (R_f values 0.31, 0.28, and 0.33), thiazole (R_f values 0.85, 0.79, and 0.81), 2-methyl-4-amino-pyrimidinecarboxylic acid (R_f values 0.42, 0.21, and 0.26)], and the related compounds pyrimidinesulfonic acid

(R_f values 0.48, 0.39, and 0.46), α -hydroxyethylthiamine (R_f values 0.23, 0.09, and 0.06), and N' -methylnicotinamide (R_f values 0.31, 0.06, and 0.05) were analyzed and identified by TLC on silica gel with acetonitrile–water (40:10 v/v) adjusted to pH values of 2.54, 4.03, and 7.85, respectively, with formic acid as solvent (1). Although N' -methylnicotinamide and thiochrome could not be separated in single-phase chromatography at pH 2.54, a second phase at right angles to the first in pH 4.03 solvent separated these quite clearly without affecting the resolution of the other compounds (1).

The quantitative analysis of thiamine hydrochloride (vitamin B₁) using HPTLC on silica gel plates with two different mobile phases was elaborated (2). After the TLC separation, vitamin B₁ was derivatized by the use of *tert*-butyl hypochlorite or potassium hexacyanoferrate(III)–sodium hydroxide as reagent. The *tert*-butyl hypochlorite reagent formed yellow fluorescing derivatives

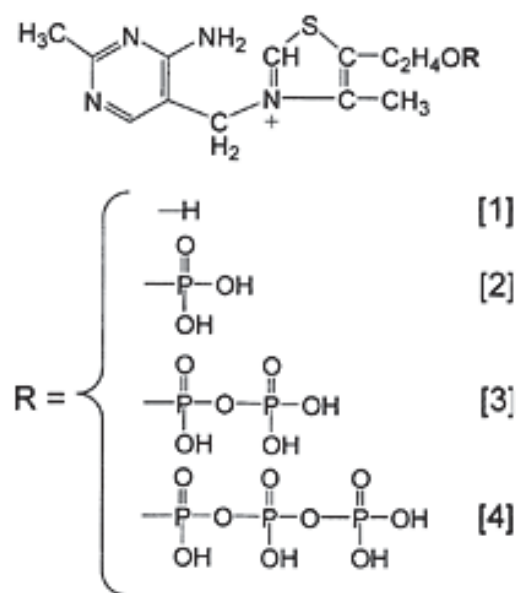


Figure 1 Structural formula of thiamine and partial structures of thiamine compounds. The latter show only those portions of the molecule that differ from thiamine. 1, Thiamine; 2, thiamine mono-phosphate (TMP); 3, thiamine pyrophosphate (TPP); 4, thiamine triphosphate (TTP).

with a limit of detection of less than 3 ng per chromatogram zone. The potassium hexacyanoferrate(III)–sodium hydroxide reagent led to a bluish fluorescing derivative with a limit of detection of 500 ng per chromatogram zone.

Water-soluble vitamins (B₁, B₆, B₁₂, C) in “Kombucha” drink (a curative liquor) were separated by TLC on silica gel plates with water as the developing solvent (3). The plates were visually examined under UV light at wavelengths of 254 and 366 nm. The four

water-soluble vitamins were identified and determined by comparing their R_f values with those of references (vitamin B₁, 0.21; vitamin B₆, 0.73; vitamin B₁₂, 0.34; vitamin C, 0.96) (Table 1).

Separation of vitamin B complex (vitamin B₁, B₂, B₆, B₁₂, and folic acid) by TLC was achieved on plates impregnated with various transition metal ions (4). The metal ions used were Mn²⁺, Fe²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, and Hg²⁺. CuSO₄ at 0.4% impregnation in all the solvent systems employed resulted in the simultaneous resolution of constituents of the vitamin B complex with appreciable differences in R_f values.

III. RIBOFLAVIN (VITAMIN B₂)

Riboflavin (RF), 7,8-dimethyl-10-(1'-D-ribityl)isoalloxazine, a yellow-green light-sensitive compound, is widely distributed in animal and plant cells (Fig. 2). The two coenzyme forms of the

Table 1 Hydrophilic Vitamin Contents (mg/mL) in the Kombucha Drink and Tea Decoctions

	Kombucha drink	Tea	Increasing factor
Vitamin B ₁	0.74±0.003	0.46±0.023	1.61
Vitamin B ₁₂	0.84±0.008	0.36±0.003	2.31
Vitamin B ₆	0.52±0.038	0.29±0.005	1.83
Vitamin C	1.51±0.033	0.71±0.036	2.14

($n=5$).

Source: Ref. 3.

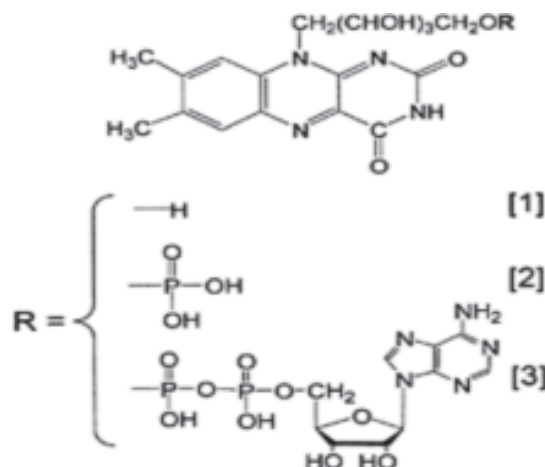


Figure 2 Structural formula of riboflavin and partial structures of riboflavin compounds. The latter show

only those portions of the molecule that differ from riboflavin. 1, Riboflavin (RF); 2, flavin mononucleotide (FMN); 3, flavin adenine dinucleotide (FAD).

vitamin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), are predominant in cells. RF and FMN are very sensitive to UV light. RF is photolyzed to form lumiflavin in basic solution as the main component. Because lumiflavin can be easily extracted from biological samples with chloroform and measured photometrically at 450 nm, the lumiflavin method has been widely used for analytical RF determination in various sources.

Following the administration of oral (20, 40, 60 mg) and intravenous (11.6 mg) doses of RF to healthy humans, 7 α -hydroxyriboflavin (7-hydroxymethylriboflavin) was identified in blood plasma by fluorescence after TLC [benzene–1-butanol–methanol–water (1:2:1:1)], and by its absorbance spectrum (5). Plasma peak concentrations of 40 nmol/L in males and 20 nmol/L in females were achieved within 2 h. No significant influence of different oral RF doses on 7 α -hydroxyriboflavin kinetics was found.

Thin-layer chromatography on silica gel 60 plates was used for both determination and identification of flavin derivatives in baker's yeast (6) and food (7, 8). In yeast samples, in addition to FAD and FMN, small amounts of RF and traces of 10-formylmethylflavin were found (6). The distribution of FAD, FMN, RF, and 10-formylmethylflavin in total flavin content were estimated to be 71.5%, 25.8%, 1.7%, and below 0.05%, respectively. Small amounts (0.8% of total flavins) of a new flavin derivative have been identified as 4',5'-riboflavin cyclic phosphate.

As shown in Tables 2 and 3, 11 TLC solvent systems were used to confirm the presence of flavins in plain yogurt and raw egg white and egg powder (7). The mean contents of individual flavins and total flavin were analyzed in plain yogurts and bioyogurts (8).

Light of wavelengths below 500 nm triggered rapid photoreactions of RF with vinblastine, vincristine, and videsine in aqueous solutions (9). The photoreactions altered the absorption spectra of these alkaloids and yielded degradation products that could be separated by silica gel 60 TLC. The riboflavin-mediated photoreaction results in reliable determination of sensitivity and resistance to *Vinca* alkaloids.

IV. PYRIDOXINE (VITAMIN B₆)

The substance known by the generic term vitamin B₆ exhibits the biological activity of pyridoxine (or pyridoxol) in mammals. Two other forms of vitamin B₆, pyridoxal and pyridoxamine, differ from pyridoxine in the locations of an aldehyde and an amine group at the 4-position of the

Table 2 R_f Values of Flavin Standards and Compounds 2 (7 α -Hydroxyriboflavin) and 6 (Riboflavin- β -Galactoside) in Plain Yogurt

Flavin	R_f							
	I	IIa	III	IV	VIII	IX	X	XI
FAD	0.21	0.23	0	0	0.37	0	0	0
FMN	0.36	0.41	0	0.05	0.37	0.03	0.04	0.05
Riboflavin- β -galactoside	0.56	—	0.14	0.10	—	0.06	0.21	0.21
7 α -Hydroxyriboflavin	0.59	0.57	0.32	0.21	0.53	—	—	—
10-Hydroxyethylflavin	—	0.63	0.71	0.40	0.57	—	—	—
RF	0.68	0.64	0.55	0.32	0.64	0.38	0.50	0.52
10-Formylmethylflavin	0.80	—	0.86	0.76	—	—	—	—
Flavin 2	—	0.57	0.32	0.21	0.53	—	—	—
Flavin 6	0.56	—	0.14	0.10	—	0.06	0.21	0.21

TLC on silica gel: (I) 1-Butanol–glacial acetic acid–water (2:1:1); (IIa) 1-butanol–acetic acid–water (5:2:3); (III) chloroform–methanol–ethyl acetate (5:5:2); (IV) 1-butanol–benzyl alcohol–glacial acetic acid (8:4:3); (VIII) 1-butanol–ethanol–water (10:3:7); (IX) isoamyl alcohol–ethyl methyl ketone–glacial acetic acid–water (40:40:7:13); (X) 1-butanol–isopropanol–water–glacial acetic acid (30:50:10:2); (XI) ethyl methyl ketone–acetic acid–methanol (3:1:1).

Source: Ref. 7.

pyridine ring structure (Fig. 3). The 5'-phosphoric ester of pyridoxal, pyridoxal-5'-phosphate, is the metabolically active form of vitamin B₆. Pyridoxamine-5'-phosphate and pyridoxine-5'-phosphate are also widely distributed in animal and plant tissues.

Table 4 shows R_f values of vitamin B₆ compounds obtained by TLC in different solvents (10). When adsorbents containing fluorescent indicators are used, all forms and derivatives of vitamin B₆ can be detected through fluorescence or through quenching of indicator fluorescence

Table 3 R_f and t_r Values of Flavin Standards and Flavin 4 (4',5'-FMN) Isolated from Raw Egg White and Egg Powder

Flavin	R_f^a								HPLC ^b t_r (min)
	I	IIa	IIb	III	IV	V	VI	VII	
FAD	0.14	—	0.16	0	0		0.62	0	5.27
FMN	0.30	—	0.25	0	0.03	0.13	0.53	0.06	7.05
4',5'-FMN	0.46	0.41	0.32	0.23	0.11	0.45	0.57	0.08	8.22

RF	0.63	0.53	0.44	0.59	0.30	—	0.41	0.32	10.74
Flavin 4									
From raw egg white	0.46	0.41	0.32	0.23	0.11	—	0.57	—	8.18
From egg powder	0.46	—	—	0.23	—	0.45	0.57	0.08	8.20

^aTLC: (I) 1-Butanol–glacial acetic acid–water (2:1:1), silica gel; (IIa, IIb) 1-butanol–acetic acid–water (5:2:3), silica gel and cellulose, respectively; (III) chloroform–methanol–ethyl acetate (5:5:2); (IV) 1-butanol–benzyl alcohol–glacial acetic acid (8:4:3); (V) collidine–water (3:1), cellulose; (VI) 5% NaHPO₄·12H₂O, silica gel; (VII) 1-butanol–formic acid–water–diethyl ether (77:10:13:15), silica gel. ^bHPLC: A symmetrical C₁₈ column, mobile-phase gradient of methanol–0.05 M ammonium acetate, pH 6.0.

Source: Ref. 7.

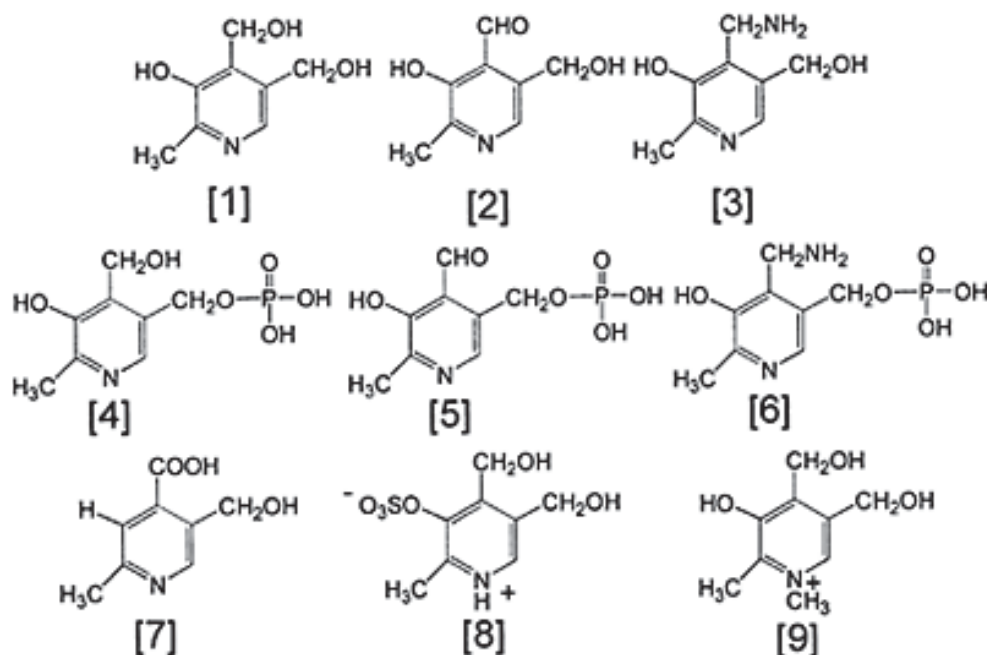


Figure 3 Structural formula of vitamin B₆ and related compounds. 1, Pyridoxine; 2, pyridoxal; 3, pyridoxamine; 4, pyridoxine-5'-phosphate; 5, pyridoxal-5'-phosphate; 6, pyridoxamine-5'-phosphate; 7, 4-pyridoxic acid; 8, pyridoxine 3-sulfate; 9, N-methylpyridoxine.

in UV light (254 nm). The limit of detection with UV light is 1 μg . The limit of detection can be extended to approximately 0.1 μg by using either Gibbs reagent or diazotized *p*-nitroaniline.

To evaluate vitamin B₆ metabolism in adult domestic cats, [¹⁴C]pyridoxine hydrochloride (0.97–490 μmol) was orally supplemented (11). Although about 70% of the radioactive component given was excreted in urine within 24 h, very little pyridoxic acid was found in the urine. Cation-exchange liquid chromatography revealed that two unknown radioactive compounds [compounds X (about 50%) and Y (about 20–25%)] were excreted in the urine. The *R_f* values of compound X (*R_f* values 0.95, 0.83, 0.2, 0.5, and 0.62) and Y (*R_f* values 0.35, 0.20, 0.22, 0.32, and 0.25) were identical to those of pyridoxine-3-sulfate and *N*-methylpyridoxine, respectively, but not to those of pyridoxine (*R_f* values 0.73, 0.83, 0.78, 0.52, and 0.62) in various solvent systems [0.5% ammonium hydroxide, 95% ethanol, chloroform–methanol (3:1), isoamyl alcohol–acetone–triethylamine–water (24:18:8:6), and 2-butanol–1.5 N ammonium hydroxide (3:1), respectively] by TLC on silica gel plates.

The biosynthetic pathway of pyridoxine in *Rhizobium meliloti* was studied (12). Pyridoxine formation from 1-deoxy-D-xylulose and 4-hydroxy-L-threonine as substrates was examined with an intact cell system of *R. meliloti*. Pyridoxine was formed only when both of the substrates were present. The pyridoxine formed in the reaction mixture was analyzed and identified by bioautograms on silica gel 60 TLC plates [CHCl_3 :MeOH (3:1) as solvent] using *Saccharomyces carlsbergensis* ATCC 9080 as an indicator strain (13). Formation of 1-deoxy-D-xylulose (the former substrate) from pyruvate and D-glyceraldehyde as substrates by the enzyme system of *R. meliloti* was analyzed and identified by silica gel 60 TLC [ethyl acetate–pyridine–water (90:5:3) as solvent]. Formation of 4-hydroxy-L-threonine (the latter substrate) from glycine and glycolaldehyde as substrates by the intact cell system of *R. meliloti* was also analyzed and identified by reversed-phase C₈ silica TLC [CHCl_3 :MeOH (3:1) as solvent].

Table 4 *R_f* Values (X100) of Vitamin B₆ Compounds on TLC

Compound	Layer	I	I	I ^a	I	I ^b	II ^b	II ^b	II ^b	III ^b	III ^b
	Solvent	A	B	B	F	F	C	D	G	C	E
Pyridoxol (pyridoxine)		62	47	— ^c	—	—	—	—	—	—	—
Pyridoxal		68	56	36	—	—	—	—	—	—	—
Pyridoxamine		12	05	05	—	—	—	—	—	—	—
Pyridoxal ethyl acetal		54	84	83	—	—	—	—	—	—	—
4-Pyridoxic acid		91	49	—	—	—	—	—	—	—	—
4-Pyridoxic acid lactone		91	18	—	—	—	—	—	—	—	—
Pyridoxol phosphate		95	00	—	30	18	47	68	70	21	69 ^d
Pyridoxal phosphate		95	00	—	54 ^e	33 ^e	64	80	78	37	79 ^d
Pyridoxamine phosphate		86	00	—	41	28	07	36	62	01	57 ^d

Pyridoxal phosphate	—	—	—	76	55	—	—	—	—	—
phenylhydrazine										

Layers: I, Silica gel HF₂₅₄; II, cellulose MN 300 G; III, Chromagram cellulose sheets. Solvents: A, 0.2% NH₄OH in water (1:139 v/v conc. NH₄OH–H₂O); B, chloroform–methanol (75:25 v/v); C, 1-butanol saturated with 1 N HCl, upper layer; D, 1-butanol–conc. HCl–H₂O (25:5:10); E, methanol–1-butanol–benzene–water–triethylamine (20:10:10:10:5); F, methy ethyl ketone–ethanol–conc. NH₄OH–H₂O (15:5:5:5); G, 1-butanol–acetic acid–water (15:10:10).

^aH₃BO₃-treated plate.

^bChamber saturation.

^cIn the strip, compound completely retarded.

^dSpots show tailing.

^ePhenylhydrazine test is negative.

Source: Ref. 10.

V. COBALAMIN (VITAMIN B₁₂)

Vitamin B₁₂ or cyanocobalamin (B₁₂ or CN-B₁₂) belongs to the corrinoids, which are compounds having in common a corrin nucleus. Vitamin B₁₂ (molecular weight 1355.4) is stable in aqueous solutions between pH 4 and 7 and can be heated at 120°C without significant loss. B₁₂ compounds with different upper ligands (L) (Fig. 4), especially MeB₁₂ and AdoB₁₂ as coenzyme forms of the vitamin, occur naturally. Corrinoids carrying a base other than 5,6-dimethylbenzimidazole in the lower ligand (cobalt-coordinated nucleotide) were also found in nature.

The usual dietary sources of B₁₂ are animal food products (meat, milk, egg, and shellfish) but not plant food products (14). Some food plants, edible seaweeds and microalgae, however, contain large amounts of B₁₂, although in what appears to be inactive B₁₂ compounds, so they may not be bioavailable to mammals (15). To evaluate whether some edible shellfish and algal foods contain true B₁₂ or inactive corrinoids, some B₁₂ compounds were purified and characterized using silica gel 60 TLC. Although dried green and purple lavers (nori) (Fig. 5) (16–18), some algal health foods (19, 20), and most shellfish (21) contained considerable amounts of true B₁₂, an inactive B₁₂ compound, pseudovitamin B₁₂, predominated in spirulina tablets (Table 5) (22, 23).

The destruction of vitamin B₁₂ is probably brought about by radicals generated by vitamin C in the presence of copper. Vitamin C alone or the Cu²⁺ metal ion alone did not decompose B₁₂. However, vitamin B₁₂ was destroyed significantly when mixed with both vitamin C and Cu²⁺ (C–Cu²⁺ system) (24). Many B₁₂ degradation compounds (ladderlike red spots) were separated from the B₁₂ treated with the C–Cu²⁺ system by silica gel 60 TLC.

Significant loss of vitamin B₁₂ also occurred in foods during microwave heating due to the conversion of B₁₂ to inactive B₁₂ degradation compounds, which were analyzed by TLC on silica gel sheets (25, 26).

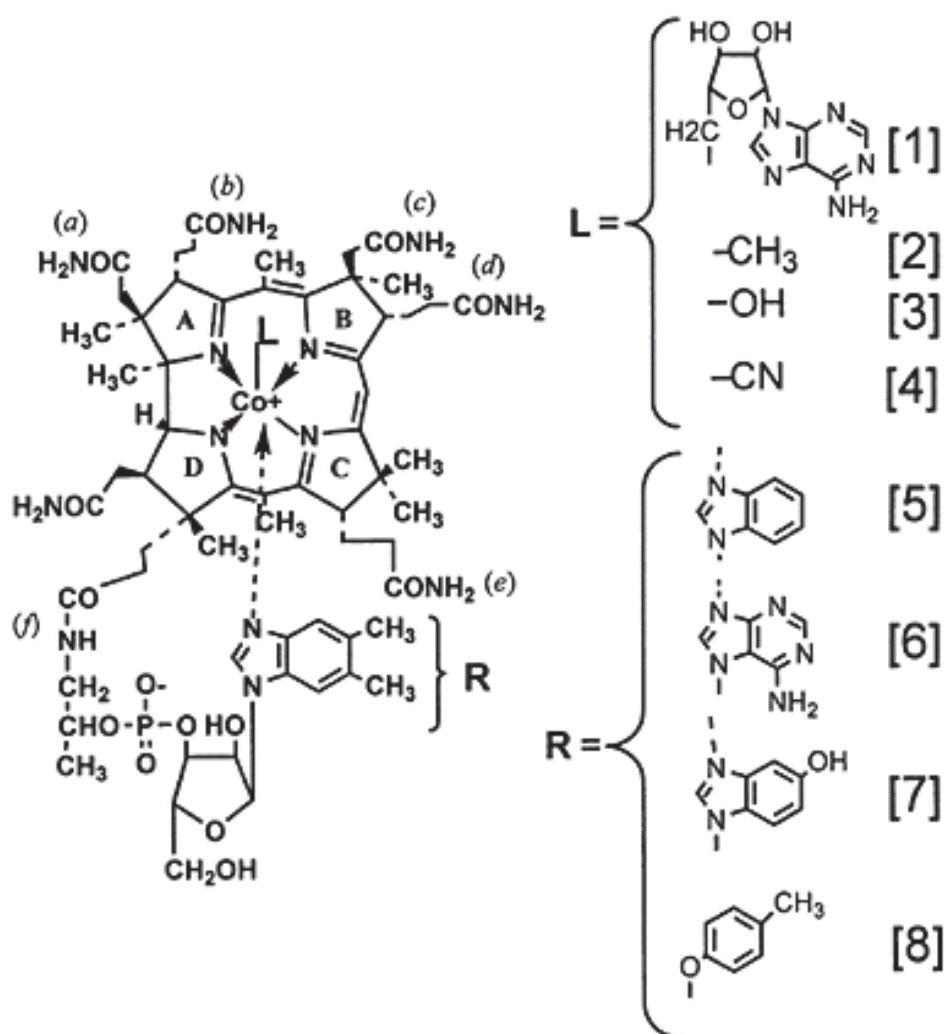


Figure 4 Structural formula of vitamin B₁₂ and partial structures of vitamin B₁₂ compounds. The latter show only those portions of the molecule that differ from vitamin B₁₂. 1, 5'-Deoxyadenosylcobalamin (AdoCbl or AdoB₁₂); 2, methylcobalamin (MeCbl or MeB₁₂); 3, hydroxocobalamin (OH-Cbl or OH-B₁₂); 4, cyanocobalamin (CN-Cbl, CN-B₁₂, or B₁₂); 5, benzimidazolyl cyanocobamide; 6, pseudovitamin B₁₂; 7, 5-hydroxybenzimidazolyl cyanocobamide; 8, *p*-cresolyl cyanocobamide.

Plasma and buffy coat specimens of chronic myelogenous leukemia (CML) patients with untreated disease were analyzed for the B₁₂ patterns using bioautography after TLC separation (27, 28). Plasma concentrations of all forms of vitamin B₁₂ were increased in CML; the proportion of MeB₁₂ was significantly lower than in a reference population. Buffy coat cells and splenic tissue had a higher proportion of AdoB₁₂ and a lower proportion of MeB₁₂ than plasma.

Two types of hydrophobic derivatives of vitamin B₁₂ (long-chain alkylcobalamin and long-chain acyl-B₁₂) (29) and the diaquo forms of several incomplete corrinoids (cobiric acid, co-binamide, and three isomeric cobinic acid pentaamides) (30) were prepared and characterized by TLC behavior. Evaluation of the coupling of vitamin B₁₂ to antisense oligonucleotides by TLC also has been reported (31).

VI. NICOTINIC ACID AND NICOTINAMIDE

Nicotinic acid (pyridine-3-carboxylic acid) and nicotinamide (pyridine-3-carboxamide) have been designated as antipellagra vitamins (Fig. 6). Pellagra affects the skin and the digestive and nervous systems (dermatitis, diarrhea, and dementia). Nicotinamide was found to be an integral part of

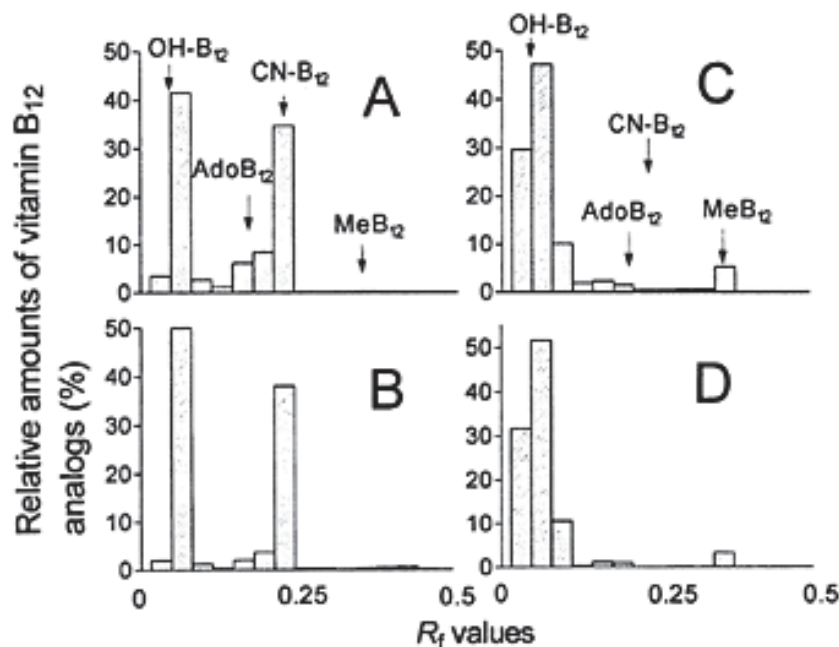


Figure 5 Silica gel 60 TLC analysis of B₁₂ compounds of dried green and purple lavers. The green laver B₁₂ compounds were determined according to the IF-chemiluminescence B₁₂ assay (A) and microbiological B₁₂ assay (B) methods. The purple laver B₁₂

compounds were determined according to the IF-chemiluminescence B₁₂ assay (C) and microbiological B₁₂ assay (D) methods. The R_f values of authentic OH-B₁₂, AdoB₁₂, CN-B₁₂, and MeB₁₂ given by this TLC system were 0.03, 0.20, 0.33, and 0.36, respectively. Data present a typical migration pattern of B₁₂ compounds on the TLC from three experiments. (Adapted from Ref. 16.)

nicotinamide adenine denucleotide (NAD⁺) or its phosphorylated form (NADP⁺), which is a coenzyme of dehydrogenases. Its excretion in urine is essentially in the form of *N*¹-methylnico-tinamide (trigonelline amide), *N*¹-methyl-6-pyridone-3-carboxamide, and *N*¹-methyl-4-pyridone-3-carboxamide.

Table 6 shows R_f values of nicotinic acid and nicotinamide and their derivatives in various solvents (32). The detection is performed by illumination under short-wavelength (257.3 nm) UV light. Urinary metabolites of the vitamin described above were also separated by TLC (33).

Thiamine, riboflavin, and nicotinic acid, all of which occur together in foods, were separated by TLC and fluorometrically determined by using a commercially available fiber optic-based instrument (34). Under fluorometric monitoring, riboflavin shows native fluorescence, but nicotinic acid and thiamine had to be prechromatographically converted to fluorescent derivatives. A new fluorescent tracer, fluoresceinamine, isomer II, was used to label the nicotinic acid. Thiamine was converted to fluorescent thiochrome by oxidation with potassium ferricyanide solution in aqueous sodium hydroxide. The vitamins were separated by HPTLC on silica gel plates using methanol–water (70:30) as the mobile phase. In these conditions, the R_f values for the thiamine, riboflavin, and nicotinic acid derivatives were 0.73, 0.86, and 0.91, respectively. Calibration curves for the determination of 300–750 ng thiamine, 48–320 ng riboflavin, and 10–100 ng nicotinic acid were established.

An overpressured layer chromatographic procedure with photodensitometric detection for the simultaneous determination of water-soluble vitamins in multivitamin pharmaceutical preparations was developed and evaluated (35).

VII. PANTOTHENIC ACID

The biologically active forms of pantothenic acid, a member of the vitamin B complex, are coenzyme A and acyl-carrier protein (Fig. 7). In solid Pharmaceuticals and foods, the pantothenic

Table 5 R_f Values and Retention Times of the Purified Spirulina B₁₂ Compounds, Authentic B₁₂, and Cyanocobamides on TLC and HPLC

	Spirulina compd I	Spirulina compd II	B ₁₂	Benzimidazolyl cyanocobamide	5-Hydroxybenzimidazolyl cyanocobamide	Pseudo vitamin B ₁₂	<i>p</i> -Cresolyl cyanocobamide
Silica gel 60 TLC ^a							
Solvent I	0.14	0.23	0.23	0.18	0.20	0.14	0.38
Solvent II	0.42	0.56	0.56	0.52	0.47	0.42	0.62
C ₁₈ reversed- phase HPLC ^b							
Isocratic	12.8	15.6	15.6	12.2	11.8	12.8	>30
Gradient	18.4	19.0	19.0	18.3	18.2	18.4	26.1

^aSolvent I: 1-Butanol–2-propanol–water (10:7:10). Solvent II: 2-propanol–NH₄OH (28%)–water (7:1:2).

^bIsocratic: 20% (v/v) methanol solution containing 1% (v/v) acetic acid. Gradient: a linear gradient of methanol (5–70%, v/v) in 1% (v/v) acetic acid solution.

Source: Ref. 22.

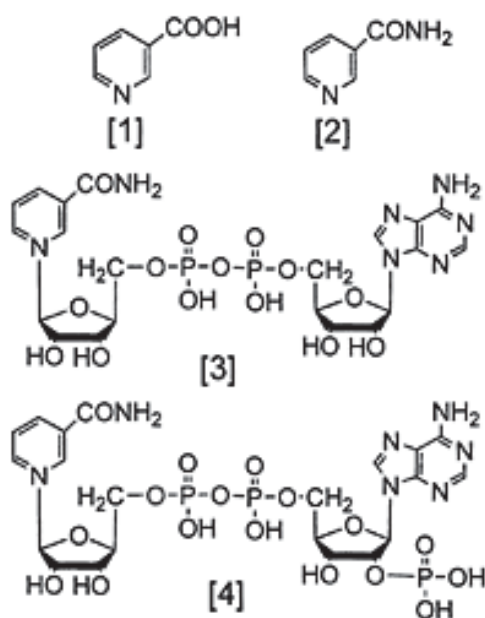


Figure 6 Structural formulas of (1) nicotinic acid, (2) nicotinamide, and related compounds 3, nicotinamide adenine dinucleotide (NAD⁺) and 4, nicotinamide adenine dinucleotide phosphate (NADP⁺).

Table 6 TLC of Nicotinic Acid and Nicotinamide and Their Metabolites

Compound	Layer	I	I	I	II
	Solvent	A	B	C	D
NADP ⁺		0.03	0.50	0.70	—
NAD ⁺		0.13	0.61	0.58	—
Nicotinic acid adenine dinucleotide		0.15	0.52	0.57	—
Nicotinamide mononucleotide		0.11	0.63	0.73	—
Nicotinic acid mononucleotide		0.13	0.47	0.75	—
Nicotinamide		0.87	0.88	0.45	0.32
Nicotinic acid		0.77	0.82	0.55	—

Layer: I, MN 300G cellulose plate, ascending chromatography; II, silica gel 60 F₂₅₄, ascending chromatography. Solvent: A, 1 M ammonium acetate—95% ethanol (3:7), pH 5.0; B, 2-butyric acid—ammonia—water (66:1.7:33); C, 600 g ammonium sulfate in 0.1 M sodium phosphate; D, 2-propanol—conc. HCl—water (70:15:15).

Source: Refs. 32 and 33.

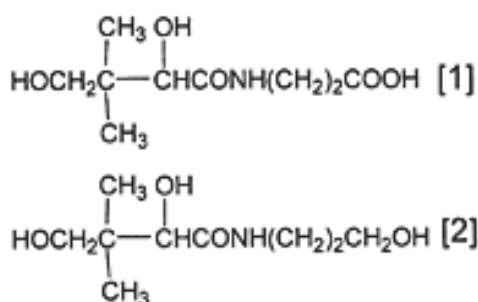


Figure 7 Structural formulas of (1) pantothenic acid and (2) panthenol.

acid sodium and calcium salts are often used as additives because they are easily handled and stable. Panthenol is usually used in liquid pharmaceutical preparations and in cosmetics.

A rapid, simple, and specific TLC method was developed for estimation of panthenol and pantothenic acid in pharmaceutical preparations containing other vitamins, amino acids, syrups, and enzymes (36). Panthenol and pantothenic acid were extracted with ethanol (tablets and capsules) or benzyl alcohol (liquid oral preparations) and isolated from other ingredients by TLC on silica gel 60 plates with 2-propanol–water (85:15) as solvent. β -Alanine (pantothenate) or β -alanol (panthenol) was liberated by heating for 20 min at 160°C. The liberated amines were visualized by ninhydrin reaction and estimated by spectrodensitometry at 490 nm. Recoveries for panthenol and pantothenic acid were 99.8±2.25% and 100.2±1.7%, respectively.

An overpressured layer chromatographic procedure with photodensitometric detection for the simultaneous determination of water-soluble vitamins in multivitamin pharmaceutical preparations was developed and evaluated (35). HPTLC on silica gel plates with 1-butanol–pyridine–water (50:35:15) as mobile phase was used. The quantification was carried out without derivatization (vitamin B₁, vitamin B₂, vitamin B₆, folic acid, nicotinamide, vitamin C) or after spraying ninhydrin reagent (calcium pantothenate) or 4-demethylaminocinnamaldehyde (vitamin B₁₂, biotin). This method was applied to the analysis of multivitamin solutions (Table 7).

VIII. BIOTIN

Biotin, hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole-4-pentanoic acid, has been isolated from egg yolk (Fig. 8). Biotin is required by all living cells but is biosynthesized only by plant, fungi, and most microorganisms. Sources of exogenous biotin for animals are found in yeast extracts, liver, kidneys, egg yolks, milk, and cereals. Biotin is very stable and can be autoclaved without being affected. However, biotin can be easily oxidized into biotin sulfoxides and biotin sulfone in very dilute solution.

Several unidentified avidin-binding substances in human urine were analyzed and identified by TLC (37). Urine was collected before and after intravenous administration of 18.5 μ mol biotin to healthy adults. As shown in Table 8, unknown substances 1, 3, and 6 were identified as biotin sulfone, bisnorbiotin methyl ketone, and tetranorbiotin-*l*-sulfoxide, respectively, by derivatization with *p*-demethylaminocinnamaldehyde after TLC separation. Recently, biotin and metabolites were accurately assayed in urine from six healthy adults (38); of that total, biotin accounted for 32± 12%, bisnorbiotin for 52±15%, bisnorbiotin methyl ketone for 7.9±5.8%, biotin-*d,l*-sulfoxide

Table 7 Resolution of Water-Soluble Vitamins by Overpressured Liquid Chromatography (OPLC) Versus Conventional HPTLC

Vitamin	Resolution	
	OPLC	HPTLC
Vitamin B ₁	6.16	
Vitamin B ₂	1.44	0.30

Folic acid	0.37	0.37
Vitamin B ₁₂	3.37	0.84
Nicotinamide	1.00	0.8
Vitamin C	1.86	1.02
Calcium pantothenate	0	0.72
Biotin	0.71	0
Vitamin B ₆	—	0.64

Eluant used was 1-butanol–pyridine–water (50:35:15).

Source: Ref. 35.

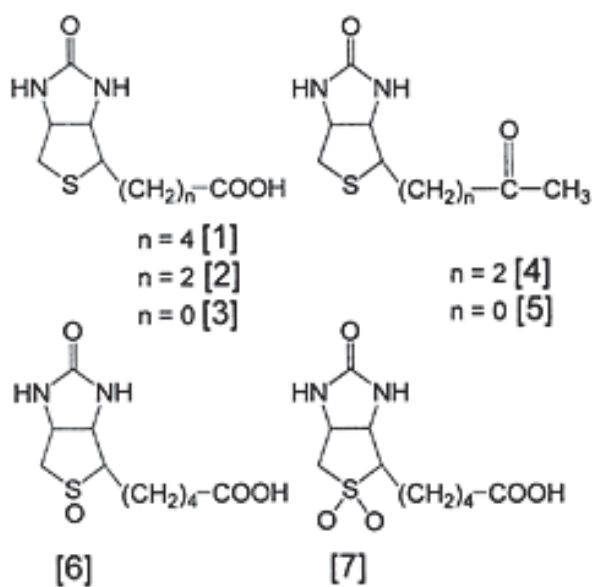


Figure 8 Structural formulas of (1) biotin and related compounds: 2, disnorbiotin; 3, tetranorbiotin; 4, bisnorbiotin methyl ketone; 5, tetranorbiotin methyl ketone; 6, biotin sulfoxide; and 7, biotin sulfone.

for $4.0 \pm 3.2\%$, and biotin sulfone for $3.6 \pm 1.9\%$. After intravenous administration of $18.4 \mu\text{mol}$ of biotin, the urinary excretion of biotin metabolites increased 21–130-fold above baseline values.

IX. FOLIC ACID

The main compound of folates, members of the vitamin B complex, is pteroyl-L-glutamic acid (folic acid). Folic acid does not occur naturally but is used to fortify food because it is more stable than naturally occurring folates; tetrahydrofolates usually exist in the polyglutamate form with up to 12 molecules of glutamic acid (Fig. 9).

A TLC system comprising cellulose powder (MN300 UV₂₅₄), 3.0% (w/v) NH₄Cl, and 0.5% (v/v) 2-mercaptoethanol gives good resolution of pteroylmonoglutamates and several related compounds (Table 9) (39). This TLC system is used to evaluate the transport and metabolism of reduced folates in blood.

Table 8 R_f Values of Authentic Standards of Biotin Metabolites and Putative Biotin Metabolites on TLC

Compound	Solvent I	Solvent II
Unknown 1	0.49	0.17
Biotin sulfone	0.49	0.17
Unknown 3	0.78	0.29
Bisnorbiotin methyl ketone	0.78	0.29
Tetranorbiotin	0.57	0.21
Tetranorbiotin methyl ketone	0.74	—
Tetranorbiotine- <i>d</i> -sulfoxide	0.29	0.05
Unknown 6	0.22	0.01
Tetranorbiotin- <i>l</i> -sulfoxide	0.22	0.01

Separation was on microcellulose plates. Solvent I, 1-butanol–acetic acid–water (4:1:1); solvent II, 1-butanol.

Source: Ref. 37.

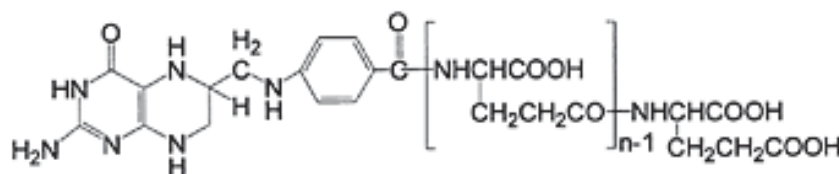


Figure 9 Structural formula of reduced polyglutamyltetrahydrofolate.

A TLC-densitometric method was used to evaluate the purity of folic acid preparations for the purpose of determining the *N*-(4-aminobenzoyl)-L-glutamic acid content as an

impurity (Fig. 10) (40). The main advantage of this method was that it ensured achieving reasonable and credible results, and the second feature was its speed.

X. ASCORBIC ACID (VITAMIN C)

Ascorbic acid (L-ascorbic acid), 2,3-endiol-L-gulonic acid- γ -lactone, is biosynthesized in all chlorophyll-containing plants and in the liver and kidney of most mammals, amphibians, reptiles, and

Table 9 TLC of Naturally Occurring Folates (Pteroylmonoglutamates) and Pteroate on MN 300 UV₂₅₄ Powder

Compound ^a	NH ₄ Cl ^b	Sodium phosphate ^c		Detection ^d
		A	B	
Pte	0 ^f	0	0	Q/A
H ₂ —PteGlu	10	26	24	BF/BF
PteGlu ^e	24	56	40	Q/A
5,10-CH=H₄—PteGlu	32 ^g	79 ^g	45 ^g	Q-BF/BF
H ₄ —PteGlu ^e	56	70	59	BF/BF
5-CHO—H ₂ —PteGlu ^e	72	85	76	Q/YBF
5-HCNH—H ₄ —PteGlu	72	85		Q/
5,10-CH ₂ —H ₄ —PteGlu	75 ^g		82 ^g	Q/
5-CH ₃ —H ₄ —PteGlu ^e	80	86	79	Q/
10-CHO—H ₄ —PteGlu	82	81	82	Q/
5-CH ₃ —H ₄ —PteGlu	87	58	84	Q/
10-CHO—PteGlu	70	58		Q/
10-CHO—H ₂ —PteGlu	73	72		Q/BF

^aAll tetrahydro compounds and 5-CH₃— and 5,6-H₂—PteGlu are racemic mixtures with the exception of *l*-5-formimino-tetrahydropteroylglutamate.

^b3.0% (w/v) NH₄Cl containing 0.5% (v/v) 2-mercaptoethanol as antioxidant (pH 6.2).

^c(A) 0.1 M sodium phosphate buffer, pH 7.0, and (B) 0.1 M sodium phosphate buffer, pH 6.0, both containing 0.5% (v/v) 2-mercaptoethanol.

^dExposure to UV light at 254 and 366 nm. Abbreviations: A, absorption; B, blue; F, fluorescence; Q, quench; and Y, yellow.

^eCompounds also applied in their radioactive forms.

^fMean R_f values $\times 100$ of 2–11 determinations.

^gElongated spot.

Source: Ref. 39.

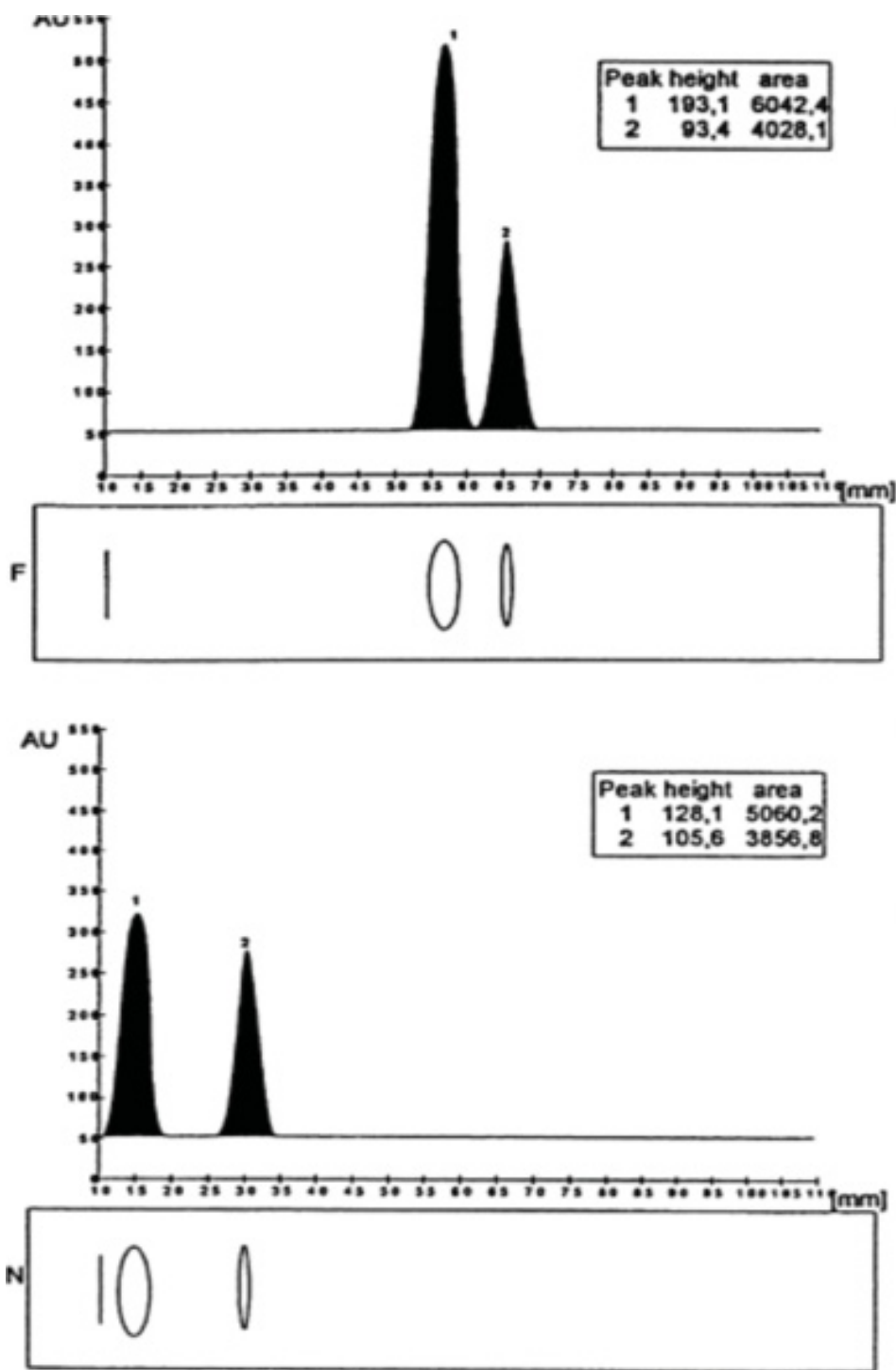


Figure 10 Densitogram and chromatograms of folic acid preparations containing (1) folic acid and (2) *N*-(4-aminobenzoyl)-L-glutamic acid. The separation was performed in 1-propanol–ammonia

(25%)–ethanol (2:2:1) and toluene–methanol–glacial acetic acid–acetone (14:4:1:1) mobile phases while taking measurements in UV light at 278 nm. (Adapted from Ref. 40.)

birds. The ability to synthesize ascorbic acid is lacking in insects, invertebrates, most fish, and humans.

L-Ascorbic acid is the naturally occurring form of ascorbic acid and has the most biological activity. D-Ascorbic acid as well as D- and L-isoascorbic acids (erythorbic acid) have only marginal vitamin C activity. The oxidized form of L-ascorbic acid is dehydroascorbic acid (Fig. 11), which is very unstable in aqueous solutions and is degraded by hydrolysis to 2,3-diketo-L-gulonic acid and further transformed and degraded to several compounds.

Thin-layer chromatography has been widely used to determine ascorbic acid concentrations in foodstuffs (41–43), pharmaceutical preparations (43–45), and biological materials (43, 46, 47). Mushrooms contain reducing substances with chemical properties similar to those of ascorbic acid, and osazones were formed from the reducing substances in 19 kinds of edible mushrooms (42). Using TLC (Wakogel FM) with toluene–acetone–acetic acid (2:1:1) or chloroform–ethyl acetate–acetic acid (60:35:5) as solvents, these substances were developed to examine their distribution and that of ascorbic acid.

A TLC method has been described for isomers of ascorbic acid and their oxidation product, dehydroascorbic acid, on sodium borate–impregnated silica gel and cellulose plates (Table 10) (43). This procedure has been adopted to separate and identify ascorbic acid and dehydroascorbic acid in fresh orange and lime juices, pharmaceutical preparations (ascorbic acid), and guinea pig tissues (liver, kidney, and eye lens) and fluids (plasma and urine).

El Sadek et al. (44) describe an HPTLC method for the determination of the components of an analgesic mixture and used silica gel plates with methylene chloride–ethyl acetate–ethanol–formic acid (3.5:2:4:0.5) as mobile phase for separating paracetamol, ascorbic acid, caffeine, and phenylephrine. The plates were scanned with a scanning spectrophotometer at 264 nm for ascorbic

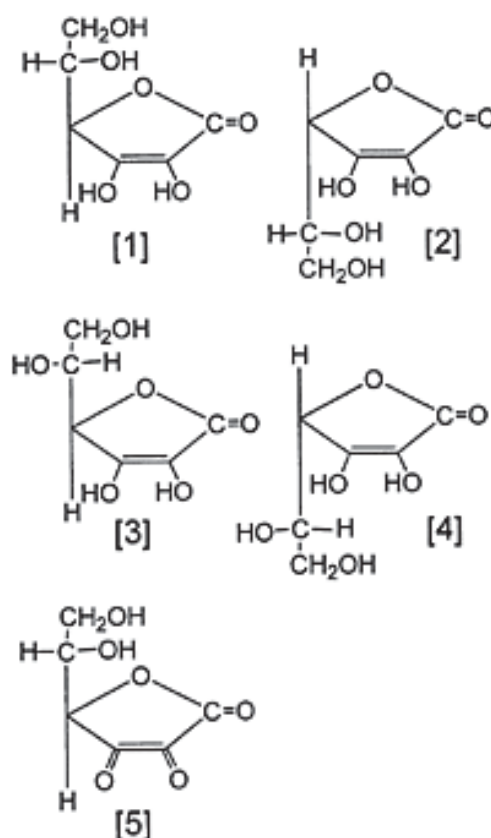


Figure 11 Structural formulas of ascorbic acid and related compounds.

1, L-Ascorbic acid (L-AsA); 2, D-ascorbic acid (D-AsA); 3, L-isoascorbic acid (L-IAsA); 4, D-isoascorbic acid (D-IAsA); 5, Ldehydroascorbic acid (L-DHAsA).

Table 10 TLC of Stereoisomers of AsA and DHAsA on Metaphosphoric Acid-Impregnated Plates (R_f Values×100)

Compound	Silica				Cellulose			
	D	B	RP	RP-B	D	B	RP	RP-B
L-AsA	31	13	32	15	42	12	43	13
D-AsA	34	13	34	16	40	11	37	12
D-IAsA	37	13	36	17	50	13	43	13
L-DHAsA	62	7	40	9	55	0	41	0
L-DHAsA ^a	62	7	40	9	55	0	41	0

D-DHAsA ^a	60	7	32	9	52	0	39	0
D-Dehydroisoascorbic acid ³	59	7	24	9	47	0	37	0

^aNorit- treated. D, direct; B, sodium borate; RP, re versed-phase; RP-B, reversed-phase sodium borate. Solvent: Acetonitrile-acetone-water-acetic acid (80:5:15:2). R_f values are the means of three determinations. The coefficient of variation is 5%.

Source: Ref. 43.

acid (*R_f* value 0.53), 254 nm for paracetamol (*R_f* value 0.87), and 274 nm for phenylephrine (*R_f* value 0.22) and caffeine (*R_f* value 0.69).

Ascorbic acid and dipyrone (metamizole) are sometimes combined in pharmaceutical dosage forms to relieve pain and fever. Simultaneous determination of ascorbic acid and dipyrone by silica gel 60 TLC was achieved by using water-methanol (95:5) as the developing system (45). The developed plates were scanned directly at 260 nm. The *R_f* values for ascorbic acid and dipyrone were 0.96 and 0.65, respectively.

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Inorganic and Organometallic Compounds**Ali Mohammad***Aligarh Muslim University, Aligarh, India***I. INTRODUCTION**

Thin-layer chromatography (TLC), discovered in 1938 by Izmailov and Schraiber and standardized by Stahl, is still regarded as one of the most effective techniques for isolation, identification, and quantitative analyses of inorganic and organic compounds. In addition to being an off-line technique in which the various procedural steps can be carried out independently, TLC offers several other advantages such as minimal sample cleanup, wide choice of mobile phases, flexibility in sample detection, high sample-loading capacity, easy accessibility, open and disposable nature of TLC plates, low solvent consumption, comparatively low operational cost, and relatively little need for modern laboratory facilities. The poorer separation efficiency and the influence of environmental conditions on the reproducibility of R_f values have, however, been major disadvantages of TLC compared to high-performance liquid chromatography (HPLC) and gas chromatography (GC).

Izmailov and Schraiber introduced “drop chromatography” and separated certain medicinal compounds on horizontal binder-free alumina spread on a glass plate, with the development done by placing solvent drops on the adsorbent. Meinhard and Hall, 10 years later, used a mixture of alumina and Celite binder to separate Fe^{2+} from Zn^{2+} . This was the first application of planar chromatography to the separation of inorganic ions.

The importance of inorganic TLC received recognition in the 1960s when Seiler separated inorganic substances. After the work of Seiler, the TLC of metal ions received a great impetus. The most important fields of inorganic TLC applications have been the analysis of rock, biological, food, pharmaceutical, industrial, soil, water, and industrial wastewater samples. The work on TLC of inorganics published up to the end of 1972 was reviewed by Brinkman et al. (1), and that appearing in the years 1973–1994 was documented by Mohammad et al. (2–5). An exhaustive review describing the historical background, principle, mechanism involved, and applications of salting-out planar chromatography appeared in 1967 (6). In 1999, Masami (7) reviewed inorganic TLC, presenting the procedural details related to TLC plates, development devices, detection and quantification. The purpose of this chapter is to review the salient work that appeared in the literature on TLC of inorganics and organometallics (8–95) during 1995–2001.

II. METHODOLOGY

Thin-layer chromatography is an off-line process in which various steps are carried out independently. Most workers have used one-dimensional ascending techniques for the development of TLC plates in a presaturated closed chamber at room temperature ($20 \pm 2^\circ\text{C}$). Two-dimensional and reversed-phase partition development techniques have also been used. TLC/HPTLC in combination with spectrophotometry, atomic absorption spectrometry, photodensitometry, stripping voltammetry, and square-wave anodic stripping voltammetry has been used for quantification of inorganic species. Techniques such as ion-exchange column chromatography for preconcentration followed by TLC separation of inorganics (30, 91) and consecutive TLC (50) for the separation of hafnium (Hf) (low R_f) from zirconium (Zr) (high R_f) have also been reported. Rozylo and coworkers (96) suggested the use of filter paper as a concentrating medium in TLC.

The basic TLC procedure involves the spotting of sample mixture ($5\text{--}10\ \mu\text{L}$ for conventional TLC and $1\text{--}2\ \mu\text{L}$ for HPTLC) about $1.5\text{--}2\ \text{cm}$ above the lower edge of the layer; drying the spot completely at room temperature or at an elevated temperature; developing the plate, usually by one-dimensional ascending technique in a closed chamber (cylindrical or rectangular) to a distance of $8\text{--}10\ \text{cm}$; removing the plate from the developing chamber; removing mobile phase from the layer by drying; detecting spots on the plate using a suitable detection reagent and procedure; measuring the R_f values of resolved spots; and determining the separated analyte. The differential migration of components in a mixture is due to varying degrees of affinity of the components for the stationary and mobile phases.

In a TLC system, the R_f coefficient is a basic quantity used to express the exact position of the solute on the developed chromatogram. It is calculated as the ratio

$$R_f \text{ (retardation factor)} = \frac{\text{distance of chromatographic spot center from the start}}{\text{distance traveled by the solvent from the start}}$$

The R_f value varies from 0 (solute remains on the point of application) to 0.999 (solute migrates up with the solvent front). Resolution (R_s), which determines the separation efficiency of ions, is defined as the ratio of the center-to-center distance (x) between the two components (A and B) and the average diameter of the two spots (Fig. 1):

$$R_s = \frac{x}{0.5(d_1 + d_2)}$$

The value of R_s serves to define the separation of components from the mixture. For $R_s=1$, the two components are reasonably well separated; for $R_s>1$ there is better separation, and for $R_s<1$ there is poorer or no separation. Improved resolution can thus be achieved either by decreasing the average diameter of the two spots or by increasing the distance between them. The sensitivity,

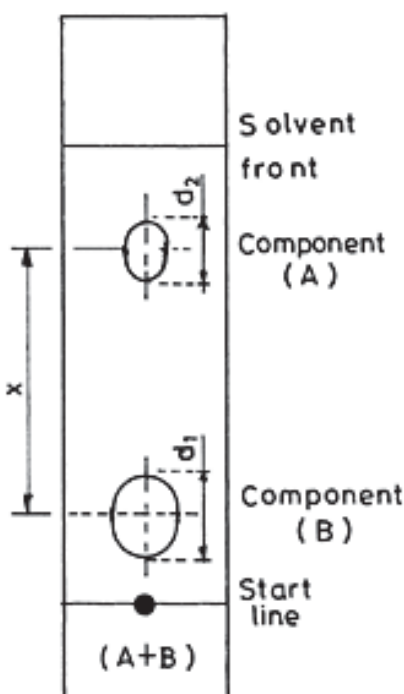


Figure 1 Illustration of resolution of a two-component mixture on a chromatogram.

which is related to the number of molecules of the components per unit area on a TLC plate, will be higher for more compact spots.

III. SAMPLE PREPARATION

The sample solution to be analyzed must be sufficiently concentrated to provide clear detection and/or be pure enough that it can be separated as a discrete and compact zone or spot. For low concentrations of analyte in a complex sample, preconcentration and cleanup procedures must frequently precede TLC.

Metal solutions are generally prepared by dissolving their corresponding salts (nitrates or chlorides of analytical grade quality) in distilled water, 0.1 M HNO_3 , or 0.1 M HCl to a final metal concentration of 0.05–0.2 M. Solutions of rare earth elements are prepared by dissolving their nitrates in 0.1 M HNO_3 or by fusing their oxides followed by their dissolution in 0.5–6 M HNO_3 . Anion standard solutions are prepared from sodium, potassium, or ammonium salts of the corresponding acids using distilled water, dilute acid, or alkali.

Metal complexes are generally taken as freshly prepared solutions in ethanol, acetone, chloroform, or distilled water. The complexes are occasionally dissolved in the corresponding solvent systems being used as mobile phase. In most cases, the complexes are produced by direct synthesis and their purity is checked by microanalysis prior to TLC. In a few cases, the metal complexes are either obtained by extraction or developed

directly in the starting zone of the chromatoplate by mixing the metal salt and the complexing agent. Specific standard methods are followed for sample preparation to identify and determine metal ions in various samples (e.g., biological, water, alloy, geological, and fly ash samples).

IV. CHROMATOGRAPHIC SYSTEMS

A combination of stationary and mobile phases constitutes a chromatographic system. The proper selection of stationary- and mobile-phase conditions determines the effectiveness of a separation.

A. Stationary Phase

A large number of layer materials have been used as the stationary phase in inorganic TLC, but silica gel, as usual, has been the much favored layer material. Thin layers of silica gels G (gypsum binder) and S (starch binder) with or without "fluorescent indicator" have been used most frequently. Cellulose, an organic material, is used as a sorbent to perform separations with better sensitivity of detection and less development time than paper chromatography. The layer materials used so far in inorganic TLC/HPTLC may be categorized as follows.

1. *Non-Surface-Modified or Untreated Layers.* These include silica gel, alumina, cellulose, polyamide, polyacrylonitrile, Sephadex, and kieselguhr. Layers of chitin and its diacetylated derivative, chitosan, have also been used to separate metal ions.
2. *Impregnated or Treated Sorbents.* In general, silica gel impregnated with aqueous salt solutions, EDTA, high molecular weight amines, organophosphorus compounds, sodium salt of chondroitin sulfate, piperazine, and other organic chelating agents has been widely used for the separation of metal ions and rare earth elements. Metal complexes have been separated on silica gel impregnated with chlorobenzene, *p*-toluidine, or surfactants and on layers of egg shell powder impregnated with Triton X-100.
3. *Bonded or Chemically Modified Sorbents.* Lipophilic C₁₈ bonded silica gel has been used for rare earth elements (REEs) and metal complexes, aminopropyl silica gel (NH₂) and octadecyl silica gel (C₁₈) for lanthanide complexes of tetraphenylporphine, silica gel modified with mercapto propyltrimethoxysilane for toxic cations, and surface-modified cellulose as well as cellulose derivatives for several metal ions.
4. *Mixed Sorbents.* Combinations of silica gel and microcrystalline cellulose (MC) has been used for noble metal ions and transition metal chlorosulfates; silica gel and MC cellulose containing ammonium nitrate for REEs; and silica gel and inorganic ion-exchanger gel mixtures for transition metal ions. Chicken eggshell powder mixed with MC for the analysis of transition metals and rare earth chlorosulfates and MC plus kieselguhr for TLC separation and colorimetric determination of thiocyanate in water and wastewater have also been used.
5. *Impregnated Mixed Sorbents.* Mixtures of silica gel and stannic arsenate or stannic arsenosilicate gel impregnated with tributyl phosphate and tributylamine have been used for detection, identification, and quantitative separation of heavy metal cations.

6. *Other Sorbents.* Synthetic inorganic ion exchangers, porous glass sheets, soil and soil-fly ash mixture, polychrome A, carbamide-formaldehyde copolymer, and an immobilized analog of dibenzo-18-crown-6 on silica support for metal ions, diatomite for REE, polyacrylonitrile for Co(III) complexes, and silufol as well as plazmachrom for metal 1,3-diketonates have also been used, but to a lesser extent.

B. Mobile Phase

For inorganic TLC, a large number of mobile phases (aqueous, nonaqueous, and mixed aqueous-organic) are reported in the literature. In general, mixtures of organic solvents containing some aqueous acid, base, or a buffer are well suited for the separation of ionic species, whereas for nonionic species, anhydrous organic solvents and water-containing mobile phases are most useful. The water solutions of mono- or polybasic acids or their alkali metal salts are usually selected as aqueous mobile phases. These developers may be put in the following groups:

1. *Inorganic Solvents.* These include solutions of mineral acids, bases, salts, and mixtures of acids, bases, and/or salts.
2. *Organic Solvents.* This group includes acids, bases, hydrocarbons, alcohols, amines, ketones, aldehydes, esters, phosphates, and their mixtures in various proportions.
3. *Mixed Aqueous-Organic Solvents.* These are organic solvents mixed with water, mineral acids, inorganic bases, or dimethylsulfoxide and buffered salt solutions.
4. *Surfactant-Mediated Solvents.* Surfactant-mediated systems (micellar solutions or microemulsions) consisting of anionic (sodium dodecyl sulfate), cationic (cetyl trimethylammonium bromide), and nonionic (Triton-X 100) surfactants make up this group. Micellar mobile phases with added organic or inorganic substances are more efficient. The multifunctional properties of micelles are responsible for providing novel separations of inorganics.

V. DETECTION AND IDENTIFICATION

After development, TLC plates are dried at or above room temperature to completely remove the mobile phase. The resolved ions on the plate are detected by their original color, natural fluorescence, or quenching of fluorescence or as colored zones produced after chemical reaction with an appropriate reagent.

The majority of detection procedures used for inorganic ions involve the spraying of dried TLC plates with a chromogenic reagent capable of forming colored products with the separated species. Typical chromogenic reagents have detection limits ranging from 1 ng to several micrograms. In some cases the detection is completed by inspecting the chromatoplates, after spraying with a suitable reagent, under UV light or by exposing the plates to ammonia vapors or hydrogen chloride.

The detection reagents most useful for inorganic ions are discussed in the following subsections.

A. Metal Ions

In addition to using conventional detection reagents such as dithizone, dimethylglyoxime, potassium ferrocyanide, and 8-hydroxyquinoline for metal ions, new reagents such as sulfochloro-phenolazorhodanine, pyridine-2-aldehyde-2-furoylhydrazone, phenolazotriaminorhodanine, and benzolazobenzolazorhodanine have been proposed for selective detection of toxic heavy metals at nanogram levels. Radiometry is used to detect Pr(III), Pr(IV), and Tb(III). Aluminum ions with high sensitivity at the ppb level have been selectively detected by measuring the fluorescence of complexed Al. The use of square-wave anodic stripping voltammetry as a direct on-plate detection method for Cd^{2+} , Cu^{2+} , and Pb^{2+} (detection limit of each cation, less than 10 ng) was reported by Dewald et al. (56).

B. Anions

For the detection of anions, saturated silver nitrate solution in methanol, 0.2–0.5% diphenylamine solution in 4 mol/L H_2SO_4 , 1% aqueous solution of potassium ferrocyanide, 0.5% alcoholic solution of pyrogallol, 10% FeCl_3 solution in 2 mol/L HCl , 1% KI in 1.0 mol/L HCl , and a mixture of aqueous KSCN and SnCl_2 in 1.0 mol/L HCl , ammonical AgNO_3 , aqueous bromocresol green, $\text{FeSO}_4 + \text{FeCl}_3$, alizarin, benzidine solution, $(\text{NH}_4)_2 \text{MoO}_4 + \text{SnCl}_2$ and 0.1% bromocresol purple containing diluted NH_4OH have been used. Autoradiography, scintillation counting, and radiometric detection methods have also been applied.

C. Rare Earth Elements

The rare earth elements (REEs) have been detected by first spraying the plate with 0.1% arsenazo(III) solution and then with aqueous ammonia followed by gentle heating; second, heating at 70°C for 10 min after spraying with 0.02% chlorophosphonazo solution and then 0.5 mol L^{-1} HCl ; and third, exposure of the plate to NH_3 after spraying with tribromochlorophosphonazo or xylenol orange solution. Saturated ethanolic solution of alizarin and dilute solutions of tribromoarsenazo (0.2–1%) have been used to detect REEs.

D. Metal Complexes

Most metal complexes, being colored, are visible without further treatment. Metal oxinates; some geometrical isomeric complexes of Rh, Pt, and Co methylbenzylthiocarbamate metal chelates; Cu(II) carboxylates; and Co (glycine) are self-colored but can be located under ultraviolet light. β -Diketonates of Fe, Cr, and Co; organotin compounds; alkali metal xanthates; and piperidine dithiocarbamate complexes can be detected with iodine vapor. The colorless diethyldithiocarbamate complexes of Cd^{2+} , Hg^{2+} , Pb^{2+} , and Zn^{2+} are visualized as yellow-green chelates after spraying the TLC plates with 5% CuSO_4 solution. A fluorometric method has been used for the detection of heavy metal complexes with pyrene-substituted *N*-acylthiourea. Sometimes spots are

detected by spraying colored reagents such as pyrocatechol violet or copper sulfate or by immersing the TLC plates in a dilute solution (0.05%) of phenylfluorone reagent. *N,N*-Diethyl-*N'*-benzoylthiourea-metal chelates have been detected by graphite furnace atomic absorption spectrometry and by UV detection.

VI. QUANTIFICATION

The three main approaches related to quantitative TLC are (a) visual estimation and spot-size measurement, (b) zone elution and spectrophotometry, and (c) in situ densitometry.

A. Visual Estimation and Spot-Size Measurement

Visual estimation and spot-size measurement is the simplest form of semiquantitative analysis; its accuracy and reproducibility fall in the range of 10–30%. A definite volume of sample is chromatographed alongside standards containing known amounts of analyte. After detection, the weight of analyte in the sample is estimated by visually comparing the size and intensity of the sample zone with the standards. The visual comparison works well if the applied amounts of sample are kept close to the detection limit and the sample is accurately bracketed with standards.

B. Zone Elution and Spectrophotometry

Zone elution involves drying the layer, locating the resolved analyte zones, scraping the separated zones of sample and standards, and eluting the analyte from the layer material with a suitable volatile solvent. The eluates are then concentrated and analyzed by any independent microanalytical method. The scraping and elution processes are usually performed manually. Spectrophotometry has been the most widely used technique for quantification of eluted inorganic species.

C. In Situ Densitometry

In situ densitometry is the preferred technique for quantitative TLC analysis of separated substances. The substances are quantified by in situ measurement of absorbed visible or UV light or by measurement of emitted fluorescence after scanning with an optical densitometer with a fixed sample light beam in the form of a rectangular slit. The absorption of UV light is measured either on regular layers or on layers with incorporated phosphor. The modern scanner with a computer-controlled motor-driven monochromator allows automatic recording of in situ absorption and fluorescence excitation spectra. Methods based on fluorescence are preferred over absorption for quantitative densitometric analysis because of their higher sensitivity, the wider linear range of the calibration curve (peak height vs. concentration), and better selectivity. Transmission or reflectance scanning can also be used for photometric evaluation of substances.

D. Typical Results

It is clear from the foregoing that TLC has experienced tremendous development in the study of inorganics and organometallics. However, a very selective approach has been adopted here, and R_f values with adequate details are presented in condensed form in Tables 1–28 (arranged in chronological order), which appear at the end of the chapter.

VII. CONCLUSIONS

This chapter demonstrates the utility of TLC and HPTLC in the analysis of inorganics and organometallics. I have synopsized information that has been scattered in the TLC literature on inorganics. TLC has made considerable progress in the analysis of inorganic substances within the past few years. However, there is a considerable need to develop forced-flow planar chromatographic techniques for the analysis of inorganic species present in environmental and pharmaceutical samples. There is great room for improvement in the quantitative nature of TLC by coupling it with more complex instrumentation-based detection methods. Thus, the emphasis in the future will be on so-called joint techniques. The combination of relatively nontoxic and thermally stable micellar mobile phases and layers with an additional concentration zone seems to open new possibilities for TLC applications in environmental analysis for in situ cleanup of sample solutions that contain matrices of complex composition.

Table 1 R_f , Resoluton Factor (R), and Limit of Detection Data for Cu and Co Dioximato Complexes Obtained on Silica Gel 60 F₂₅₄ HPTLC Plates

Parameter	Chelating agent					
	α -Furyldioxime M ₁		α -Benzyldioxime M ₂		Dimethylglyoxime M ₃	
	Cu	Co	Cu	Co	Cu	Co
R_f	0.90	0.81	0.84	0.76	0.87	0.73
Limit of detection ^a (ng)	0.30	0.20	0.40	0.25	0.25	0.13
R	1.21	—	1.55	—	1.87	—

^aDetection limits in *n*-pentanol were measured as a signal that is twice the noise level.

Mobile phase: Benzene–2-propanol–ethyl acetate (60:38:2), for α -furyldioxime complexes; benzene–2-propanol–diethyl ether (60:10:30), for benzyldioxime complexes; benzene–2-propanol–ethyl acetate (80:15:5) for dimethylglyoxime complexes.

Remark: This is a simple HPTLC method for the separation and quantification of dioximate complexes of Cu(II) and Co(II). Dimethylglyoxime was the best complexing agent for separation.

Source: Ref. 97.

Table 2 hR_f Values of Rare Earth Elements
Realized on Different Types of Silica Gels with 1.0
M NH_4Cl Solution as Mobile Phase

Rare earth element	$hR_f (R_f \times 100)$ value ^a			
	S ₁	S ₂	S ₃	S ₄
Sc	1.3	1.1	1.1	0.8
Y	51.0	23.3	36.9	42.9
La	93.8	85.9	95.7	88.9
Ce	85.9	71.3	88.8	78.3
Pr	80.2	59.5	81.4	71.3
Nd	79.7	75.5	80.8	70.6
Sm	61.0	32.8	56.1	50.4
Eu	55.3	28.5	50.6	46.0
Gd	60.3	32.3	55.4	50.6
Tb	50.1	24.0	42.1	41.1
Dy	44.6	20.1	34.3	35.3
Ho	45.8	20.2	32.9	36.5
Er	38.0	15.4	22.1	32.5
Tm	21.3	8.0	11.1	19.3
Yb	11.4	4.7	6.3	10.4
Lu	9.5	3.6	5.4	8.8

^aStationary phases: S₁ and S₂=Wako gel B-O pretreated with 0.1 M tris(hydroxymethyl)aminomethane (THAM) and 0.001 M NH_4Cl solutions, respectively; S₃=Merck 60HR pretreated with 0.1 M THAM solution (pH 7); S₄=Merck 60H pretreated with 0.1 M THAM solution (pH 7).

Remarks: Useful separations of two- or multicomponent mixtures of rare earths were achieved on silica gels pretreated with 0.1 M amine–0.1 M HCl (pH 7.0) solutions using 1.0 M NH_4Cl as mobile phase. The value of each metal was found to decrease with increases in pK_a value of the amines as well as with increases in concentration and pH of the amine solutions used for the pretreatment.

Source: Ref. 24.

Table 3 Effect of Additives on Binary Separation of IO_4^- from NO_2^- and Br^- on Kieselguhr G Layers Developed with a Water-in-Oil Microemulsion System^a

Additives	Separations ($R_f \times 100$)
1. Amines	
(a) β -Naphthylamine	IO_4^- (05)– NO_2^- (85) IO_4^- (06)– Br^- (ND)
(b) Diphenylamine	IO_4^- (21)– NO_2^- (88) IO_4^- (21)– Br^- (74)
(c) 2-Nitroaniline	IO_4^- (20)– NO_2^- (65) IO_4^- (17)– Br^- (78)
2. Phenols	
(a) Phenol	IO_4^- (03)– NO_2^- (82) IO_4^- (10)– Br^- (85)
(b) Resorcinol	IO_4^- (07)– NO_2^- (90) IO_4^- (07)– Br^- (81)
(c) Pyrogallol	IO_4^- (ND)– NO_2^- (80) IO_4^- (ND)– Br^- (81)
3. Heavy metals	
(a) Hg^{2+}	IO_4^- (05)– NO_2^- (92) IO_4^- (08)– Br^- (83)
(b) Pb^{2+}	IO_4^- (03)– NO_2^- (90) IO_4^- (01)– Br^- (ND)
(c) Ag^+	IO_4^- (05)– NO_2^- (94) IO_4^- (05)– Br^- (ND)
(d) Bi^{3+}	IO_4^- (04)– NO_2^- (94) IO_4^- (06)– Br^- (ND)

ND: Not detected.

^aSDS (8 g)–1-pentanol (24 mL)–water (8 mL)–heptane (160 mL).

Remarks: Layers of kieselguhr were found most useful compared to other adsorbents for differential migration (hR_f values are given in parentheses) of anions. Quantitative separation of IO_4^- from accompanying ions, limits of identification, and dilution of a few anions are reported.

Source: Ref. 23.

Table 4 hR_f Values of Cations on Various Stationary Phases with 0.1 M Ethylenediamine–0.1 M Tartaric Acid (pH 4.0) as Mobile Phase

Stationary phase ^a	hR_f		
	Co ²⁺	Cu ²⁺	Fe ²⁺
IP plate	85	86	80
PPPA-coated plate	85	81	88
CS plate	51	60	40
CS-PPPA plate	44	54	56

^aIntact plate (IP); TLC plate coated with a thin film of plasma-polymerized propargyl alcohol (PPPA) for 10 min (PPPA-coated plate); chondroitin sulfate (CS)–impregnated plate using 3% CS–Na solution (CS-plate); and CS-impregnated (CS–Na, 3%) plus PPPA-coated (10 min) plate (CS-PPPA plate).

Remarks: CS=immobilized TLC plate displays cation-capture ability largely affected by the presence of PPPA film, which contributes to immobilization of CS as well as to the capture of metal cations.

Source: Ref. 27.

Table 5 hR_f Values of Toxic Cations Chromatographed on Cellulose DEAE-Silica Gel R (1:1, w/w) Layers Developed with Isopropanol–Ethanol–5 N HCl (5:5:1, v/v)

Cation	$hR_f (R_f \times 100)$ value ^a
Fe ²⁺	98
Co ²⁺	33
Ni ²⁺	24
Cd ²⁺	19
Cu ²⁺	43
Pb ²⁺	04
Hg ²⁺	—
Hg²⁺	07
Cr ³⁺	0
Zn ²⁺	38
Sb ³⁺	62
Mn ²⁺	48

^aVisualization with ninhydrin–2-aldehyde–2-furoylhydrazide and examination in UV light at $\lambda=366$

nm.

Remark: This method can be applied to the separation and identification of toxic cations from industrial wastewaters or from other waters polluted with these metal ions.

Source: Ref. 31.

Table 6 TLC System Showing hR_f Values of Rare Earth Benzoates in Decreasing as Well as Increasing Order of Atomic Number

Stationary phase	Mobile phase ^a	Metal ions (in order of increasing at. no.)	Respective hR_f values
Silica gel	IBMK–acetone(1:1)	Ce, Eu, Dy, Yb	88, 76, 71, 70
	IBMK–benzene(1:1)	Eu, Gd, Dy, Yb	98, 76, 51, 35
	IBMK–methanol(1:1)	Nd, Sm, Eu	96, 94, 71
	1,4-Dioxane–water (2:1)	Sm, Eu, Gd, Yb	87, 85, 70, 43
Silica gel–cellulose (9:1)	IBMK–benzene(1:1)	Sm, Eu, Gd, Yb	79, 70, 59, 53
	1,4-Dioxane–propanol (2:1)	La, Nd, Sm, Eu, Gd	63, 64, 73, 81, 82
	1,4-Dioxane–methanol (2:1)	La, Nd, Sm, Eu, Gd	65, 71, 76, 80, 82
Silica gel–alumina (9:1)	Benzene–methanol (1:2)	Nd, Eu, Gd, Dy	48, 52, 63, 84
	Benzene–methanol (2:1)	La, Nd, Eu, Yb	35, 43, 60, 63
Silica gel–cellulose (2:1)	Benzene–propanol (2:1)	Ce, Sm, Eu, Gd, Dy	37, 34, 38, 47, 56
	IBMK–methanol(1:1)	La, Eu, Gd, Dy	56, 72, 89, 95
Silica gel impregnated with 10% aq. aniline	5% Aq. KBr	La, Nd, Eu	45, 48, 53

^aIBMK=Isobutyl methyl ketone.

Remarks: Chromatography of seven rare earth (La, Ce, Nd, Sm, Eu, Dy, and Yb) benzoates was performed on single, mixed, and impregnated adsorbent layers employing 125 solvent systems. Differential migration of lanthanide benzoates was observed on silica gel and alumina mixed with silica gel or cellulose layers developed with some organic and nonacidic solvent systems. The best separation conditions were noted with mixed silica gel–alumina (9:1) as stationary phase and 0.5 M aqueous ammonia as mobile phase.

Source: Ref. 38.

Table 7 hR_f Value ($R_f \times 100$) of Metal Ions Obtained on Cellulose, Ionex 25SA-Na and Ionex 25SB-Ac Layers Developed with Selected Mobile Phases^a

Adsorbent	Mobile phase	Zn	Mn	Cd	Pb	Cu	Ag	Hg	Co	Ni	Fe	Cr	Sn	Bi
Cellulose	2 M HNO ₃ –methanol (1:1)	88	76	85	62	93	—	—	83	85	80	—	55	50
	2 M HNO ₃ –methanol (1:2)	86	79	85	55	87	—	—	85	83	71	—	60	46
	2 M HNO ₃ – <i>n</i> -propanol (1:2)	41	30	63	27	10	63	40	56	54	25	36	64	00
Ionex 25SA-Na	3 M HCl	49	37	—	—	43	—	—	34	37	34	14	72	60
	2 M HNO ₃	67	63	90	76	65	—	—	62	61	50	—	97	90
	2 M HNO ₃ (developed twice)	73	68	80	75	75	73	56	73	74	57	27	79	76
Ionex 25SB-Ac	2 M HNO ₃	68	92	—	56	89	—	11	92	89	86	00	04	09
	0.1 M HNO ₃	60	78	—	50	50	—	00	85	83	39	00	08	07

^aComplexes of above metals with dithizone, 4-(2-pyridylazo)resorcinol, and EDTA were also chromatographed on cellulose, Florisil, and Ionex 25SA-Na using aqueous organic or mixed organic mobile phases.

Remarks

1. Ion-exchange TLC of both metal cations and metal complexes.
2. Separation and identification of metals in human bones, placenta, and milk.
3. Good separations of metals present in human tissues were obtained on cellulose [acetone, acetone–methanol (1:2)] or Ionex 25SA-Na [acetone–methanol–0.1 M HNO₃ (5:35:1)]. Air samples were also analyzed for the presence of heavy metals using the same chromatographic system.

Source: Ref. 30.

Table 8 hR_f ($R_f \times 100$) Values of Metal Ions^a Achieved on Stannic Selenite Silicate Layer Developed with Pure DMSO, 1.0 M HNO₃, and DMSO–1.0 M HNO₃ Mixtures

Metal ion	Mobile phase ^b						
	M ₁	M ₂	M ₃	M ₄	M ₅	M ₆	M ₇
Be ²⁺	00	90	70	45	35	45	70
Al ³⁺	00	80	60	40	50	60	70

Cr ³⁺	05	70	85	90	85	30	40
Mn ²⁺	15	70	05	00	25	35	40
Fe ²⁺	10	40	00	10	40	25	70
Fe ³⁺	30	05	30	20	10	05	15
Co ²⁺	20	80	90	15	05	50	60
Ni ²⁺	10	95	15	50	55	60	00
Cu ²⁺	15	40	40	10	30	30	20
Zn ²⁺	30	35	20	20	45	60	35
Cd ²⁺	05	20	30	50	40	30	35
Sb ⁵⁺	00	15	00	05	10	65	00
Ce ³⁺	30	40	10	05	50	45	65
Ce ⁴⁺	25	25	50	30	15	25	30
Hg ₂ ²⁺	75	40	30	70	55	65	75
Hg ²⁺	85	65	70	70	65	70	85
Pb ²⁺	10	30	15	00	00	15	15
Bi ³⁺	35	15	35	10	05	15	15

^aAs³⁺, W⁶⁺, Mo⁶⁺, Ag⁺, Sb³⁺, and Ti⁺ remain at the point of application in all mobile phases.

^bM₁=Pure DMSO; M₂=1 M HNO₃; M₃-M₇ are mixtures of DMSO and 1 M HNO₃ in 9:1, 7:3, 1:1, 3:7, and 1:9 ratios, respectively.

Remarks: Stannic selenite silicate layers were found highly selective for As³⁺, Mo⁶⁺, and W⁶⁺. The mechanism of retention was explained in terms of adsorption, precipitation, and ion exchange. The metal ions were also chromatographed on silica layers.

Source: Ref. 52.

Table 9 hR_f Values of Certain Anions Obtained on Cellulose, Kieselguhr, and Their Mixtures with Mixed Solvent Systems Containing NH₄OH and CH₃COCH₃

Mobile phase ^a	Stationary phase ^b	$hR_f (R_f \times 100)$								
		SCN ⁻	NO ₂ ⁻	MnO ₄ ⁻	Fe(CN) ₆ ³⁻	Fe(CN) ₆ ⁴⁻	CrO ₄ ²⁻	Cr ₂ O ₇ ²⁻	MoO ₄ ²⁻	Mo ₇ O ₂₄ ⁶⁻
M ₁	S ₁	49	21	0	6	0	10	8	9	12
	S ₂	79	21	0	6	0	20	19	11	15
	S ₃	80	32	0	7	0	24	19	17	15
	S ₄	85	86	0	12	0	28	25	21	23
	S ₅	85	88	0	83	0	41T	43	36T	38

M ₂	S ₁	91	79	0	54	6	23	20	24	30
	S ₅	90	89	0	90	17	91	89	88	90
M ₃	S ₁	91	82	0	89	78	70	78	83	80
	S ₅	90	90	0	90	80	91	90	90	91
M ₄	S ₁	93	91	0	92	90	90	88	90	92
	S ₅	90	90	0	92	91	93	90	91	95
M ₅	S ₁	95	93	0	95	96	95	92	93	92
	S ₅	92	93	0	93	93	94	94	96	95

T=Tailed spot ($R_L - R_T > 0.3$).

^aMobile phase: Mixtures of 0.1 M NH₄OH and CH₃COCH₃ in ratios of 1:9 (M₁), 3:7 (M₂), 5:5 (M₃), 7:3 (M₄), and 9:1 (M₅).

^bStationary phase: Microcrystalline cellulose (S₁), kieselguhr G (S₅), and their mixtures in the ratio of 4:1 (S₂), 3:2 (S₃), and 1:1 (S₄).

Remarks: SCN⁻ was separated from other ions and determined by spectrophotometry at 460 nm using acidic FeCl₃ solution as chromogenic reagent. Beer's law was followed up to 11.84 ppm of SCN⁻. The proposed TLC=colorimetric method was applied to fortified samples of photogenic waste, river, and sea waters.

Source: Ref. 53.

Table 10 Separation of Metal Complexes by TLC^a

Complex ^b	Mobile phase	$hR_f (R_f \times 100)$	Color of the resolved spot
Co(INTC) ₃	Benzene	12	Pink
		08	Pink
Co(INAP) ₃	Chloroform	27	Yellow
		16	Yellow orange
Pt(INAP) ₂	Benzene	50	Yellowish green
		33	Reddish brown
		16	Violet
		08	Brownish green
Pt(INAA) ₂	Ethyl acetate– acetone (9:1)	93	Violet
		75	Green
		42	Brown
Ni(INAAI) ₂	Ethyl acetate	93	Orange
		86	Orange yellow
		76	Yellow orange

65

Yellow

^aStationary phase: Silica gel 60 containing CaSO₄ as binder.^bCo(INTC)₃=Co-isonitrosothiocamphor; Co(INAP)₃=Co-isonitrosoacetophenone; Pt(INAP)₂=Pt-isonitrosoacetophenone; Pt(INAA)₂=Pt-isonitrosoacetylacetone; Ni(INAAI)₂=Ni-isonitrosoacetylacetone.*Remarks:* TLC and column chromatographic techniques were used for separation and detection of isomers of metal complexes of isonitrosothiocamphor with Co(III), isonitrosoacetophenone with Co(III) and Pt(III), and isonitrosoacetylacetone with Pt(II) and an imine derivative of its Ni(II) complex.*Source:* Ref. 49.**Table 11** *R_f* Values of β -Diketones and Their Chelates with Transition Metal Cations in Micellar Mobile Phases Containing Sodium Dodecyl Sulfate (SDS) (2.5×10^{-2} M)

Chelate	Zone detected	<i>R_f</i> × 100	
		S ₁	S ₂
BA	BA	68	60
DBM	DBM	82	71
Cu(BA) ₂ ·2H ₂ O	Cu ²⁺ and chelate	52	16
Cu(DBM) ₂ ·2H ₂ O	Cu ²⁺	00	00
Ni(BA) ₂ ·2H ₂ O	Ni ²⁺	56	27
Ni(DBM) ₂ ·2H ₂ O	Ni ²⁺	57	26
Co(BA) ₂ ·2H ₂ O	Co ²⁺	69	37
Co(DBM) ₂ ·2H ₂ O	Co ²⁺	50	30
Co(BA) ₃	Chelate	00	00
Co(DBM) ₃	Chelate	00	00
Fe(BA) ₃	Fe ³⁺	00	00

BA=Benzoylacetone; DBM=dibenzoylmethane; S₁ and S₂ are silufol and plasmachrom plates, respectively.*Remarks:* The reversed phase (plasmachrom) exhibited stronger retention of both metal ions and 1,3-diketones than normal phase (silufol). Separations of transition metals as 1,3-diketones were more efficient with BA than with DBM.*Source:* Ref. 98.

Table 12 hR_f Values of Metal Ions Obtained on Silica Gel Layers Prepared in 0.5% Sodium Carboxymethyl Cellulose Solution at Various Formic Acid Concentrations

Metal ion	$hR_f(R_f \times 100)$						
	100 (V_{FA}/V_{MIBK})						
	0.0	1.25	2.5	5.0	12.5	25.0	50
Be	03	10	06	10	37	43	47
Ga	26	42	44	59	72	71	79
In	25	31	40	58	60	60	65
Tl	00	00	00	00	31	48	62
Bi	53	53	53	57	75	83	81
Sc	T	T	09	12	32	47	59
Cu	04	13	05	13	36	51	68
Au	69	81	85	85	85	84	83
Zn	11	19	21	27	58	54	51
Hg	66	75	79	78	87	92	94
Ti	03	13	13	10	28	39	49
Zr	02	08	06	11	40	46	55
V	09	11	13	28	31	39	41
Nb	02	03	06	11	27	43	51
Ta	02	03	06	11	28	41	55
Mo	13	11	15	17	31	45	54
Fe	70	72	83	85	83	82	83
Co	01	03	06	17	30	48	63
Ni	02	05	05	11	40	44	54
Rh	00	00	00	03	14	22	30
Pd	03	06	12	15	30	39	54
Os	12	18	18	28	53	59	74
Ir	04	16	18	26	45	54	67
Pt	03	12	18	31	38	48	57
Cr	00	00	00	00	04	19	39

Mn	02	01	06	15	43	49	60

Ag^+ and Al^{3+} remain near the point of application at all FA concentration levels. All the metals are taken in their valence states.

T=Tailed spot; V_{FA} =volume of formic acid; V_{MIBK} =volume of methyl isobutyl ketone.

Remarks: A simple theoretical model has been developed to examine thin-layer chromatographic behavior of metal ions. The model is represented by the equation $R_f/(1-R_f)=(b+aC_p)/(1-C_p)$. R_f is the retention factor of a metal ion, C_p is the concentration of competing ion, and a and b are constants.

Source: Ref. 57.

Table 13 R_f Values of Separated Cu^{2+} and Zn^{2+} Ions from Synthetic Alloys on Stannic Phosphate Silicate Layers in Buffered EDTA Media (pH 2.56)

Synthetic alloy	Composition Cu/Zn	R_f	
		Zn	Cu
Cartridge brass	70:30	0.10	0.55
Commercial bronze	90:10	0.10	0.45
Red brass	85:15	0.05	0.40
Muntz metal	60:40	0.10	0.60
High brass	65:35	0.10	0.60

Remarks: The R_f values of metal ions on stannic phosphate silicate layers were measured using buffered EDTA systems as mobile phase. In addition to some binary separations, quantitative determination of Zr^{4+} was reported. The method was applied to detect Zn and Cu in synthetic alloys.

Source: Ref. 60.

Table 14 hR_f ($R_f \times 100$) Values of Metal Cations Obtained with Selected Mobile Phases on Cellulose Layer

Mobile phase ^a	hR_f values												
	Fe^{3+}	Cu^{2+}	Ni^{2+}	Co^{2+}	UO_2^{2+}	VO^{2+}	Zn^{2+}	Cd^{2+}	Pb^{2+}	Ti^+	Bi^{3+}	Hg^{2+}	Ag^+
M_1	35T	20T	50T	40T	10	25	50T	50T	35	25T	95	92	50
M_2	10	55T	70T	70T	35	45	ND	ND	ND	ND	95	90	75
M_3	15	20T	50T	50T	10	30T	50T	50T	78	ND	ND	90	30T
M_4	15	65	75T	75T	30T	45	95	98	95	95	90	87	71T

T=Tailed spot; ND=not detected.

^a M_1 =Water; M_2 =3% aqueous Brij-35; M_3 =water-acetone (9:1, v/v); M_4 =3% Brij-35-acetone (9:1, v/v).

Remarks: The migration behavior of heavy metal cations on cellulose layers was investigated

using aqueous micellar, hydro-organic, and water–organic surfactant mobile phases. Acetone was found to be the most effective additive at 10% concentration level with 3% Brij.

Quantitative determination of UO_2^{2+} by spectrophotometry after preliminary TLC separation from Fe^{3+} and Hg^{2+} was also performed.

Source: Ref. 71.

Table 15 hR_f Values ($R_f \times 100$) of Metal Ions on Plain and Impregnated Silica Gel G Layers Developed with Mixtures of NaCl and HCOOH^a

Metal ion	hR_f value ^b				
	M ₁	M ₂	M ₃	M ₄	M ₅
Fe^{3+}	75	T	0	35(0)	30
Cu^{2+}	100	80	100	95 (100)	20
UO_2^{2+}	100	80	0	95 (60)	25
Tl^+	100	100	ND	ND (100)	T
Cd^{2+}	100	100	0	90 (100)	100
Zn^{2+}	100	70	0	90 (100)	80
Pb^{2+}	0	0	ND	80 (100)	0
Ag^+	0	T	0	T (100)	0
Ni^{2+}	100	100	100	100 (100)	100
Co^{2+}	100	100	100	100 (100)	100
Bi^{3+}	100	90	0	100 (90)	100
Hg^{2+}	100	100	0	100 (100)	100
Al^{3+}	70	T	T	T (T)	T
Zr^{4+}	0	0	0	9(0)	0
Th^{4+}	100	50	40	T (40)	25

T=Tailed spot; ND=not detected.

^aMobile phase: M₁=1.0 M NaCl–1.0 M HCOOH (1:1, v/v); M₂=1.0 M NaCl–0.1 M HCOOH (1:1); M₃=0.1 M NaCl–1.0 M HCOOH (1:1); M₄=0.1 M NaCl–0.1 M HCOOH (1:1); M₅=0.1 M NaCl–0.01 M HCOOH (1:1).

^b hR_f values given in parentheses are for metal ions chromatographed on silica layers impregnated with 0.1 M aq. KSCN and developed in M₄.

Remarks: The chromatographic system comprising M₄ as mobile phase and silica gel impregnated with 0.1 M KSCN as stationary phase was used for identification and separation of Hg^{2+} , UO_2^{2+} , and Fe^{3+} from spiked samples of distilled water, seawater, and industrial wastewater.

Source: Ref. 72.

Table 16 Separation of Individual Metal Cations
Obtained on Layered Double Hydroxide Plates

Stationary phase ^a	Mobile phase	Separation (R_f)	Interfering ions	Develop, time (min)
S ₁	1.0 M NH ₄ OH	Mo ⁶⁺ (0.90) from 23 cations	—	30
	3.0 M NH ₄ SCN	Cd ²⁺ (0.95) from 18 cations	Tl ⁺ , Ag ⁺ , Hg ²⁺ , Cu ²⁺ , Ni ²⁺ , Co ²⁺	60
S ₂	1.0 M KBr	Hg ²⁺ (0.91) from 20 cations	Au ³⁺ , Cd ²⁺ , Zr ⁴⁺ , Mo ⁶⁺	26
	1.0 M NaF	W ⁶⁺ (0.71) from 18 cations	Tl ⁺ , Au ³⁺ , Ce ⁴⁺ , Se ⁴⁺ , W ⁶⁺	32
		Se ⁴⁺ (0.51) from 18 cations	Tl ⁺ , Au ³⁺ , Ce ⁴⁺ , Mo ⁶⁺ , W ⁶⁺	32
	1.0 M KCl	As ²⁺ (0.66) from 20 cations	Tl ⁺ , Cd ²⁺ , Ce ⁴⁺ , Mo ⁶⁺	28
		Au ³⁺ (0.88) from 20 cations	Tl ⁺ , Hg ²⁺ , Ce ⁴⁺ , Mo ⁶⁺	28
	1.0 M (NH ₄) ₂ SO ₄	Tl ⁺ (0.85) from 20 cations	Hg ²⁺ , Mo ⁶⁺	37
		As ³⁺ (0.58) from 10 cations	—	47
		IO ₃ ⁻ (0.02) from 16 cations	—	37
	1.0 M NR ₄ SCN	Ba ²⁺ (0.90) from 16 cations	Ag ⁺ , Tl ⁺ , Sr ²⁺ , Cd ²⁺ , Hg ²⁺ , Cu ²⁺ , Ni ²⁺	40
		Sr ²⁺ (0.90) from 16 cations	Ag ⁺ , Tl ⁺ , Cd ²⁺ , Ba ²⁺ , Ni ²⁺ , Hg ²⁺ , Cu ²⁺	40
	5.0 M NH ₄ Br	As ³⁺ (0.57) from 7 cations	—	47
	0.05 M EDTA (pH 5)	Co ²⁺ (0.70) from 2 cations	—	30
	0.05 M EDTA (pH 10)	Tl ⁺ (0.25) from 5 cations	—	40
	5 M Lactic acid	Au ³⁺ (0.89) from 3 cations	—	40
S ₃	1.0 M NH ₄ Cl (pH 10)	Cu ²⁺ (0.55) from 16 cations	Tl ⁺ , Ag ⁺ , Cd ²⁺ , Hg ²⁺ , Ni ²⁺ , Mo ⁶⁺	155
	0.05 M EDTA	Pb ²⁺ (0.76) from 21 cations	—	140

cations			
1.0 M NH ₄ SCN	Ag ⁺ (0.95) from 18	Hg ²⁺ , Tl ⁺	180
cations			

^aMixture of layered double hydroxide with silica gel 60 G as binder in the ratios 1:1 (S₁), 1:2 (S₂), and 3:1 (S₃) (w/w).

Remarks: Chromatography of 26 inorganic cations and 17 anions was performed; cations Sr²⁺, Bi³⁺, Pb²⁺, Fe²⁺, **UO₂²⁺**, Cr³⁺, W⁶⁺, V⁶⁺, Zr⁴⁺, and Zn²⁺ remained near the point of application at all concentrations of (NH₄)₂SO₄ in the mobile phase because of strong interactions with the adsorbent.

Source: Ref. 77.

Table 17 hR_f Values of Some d-Block Metal Ions Observed on 20% TBA-Impregnated Sorbent Layers

Metal ion	$hR_f (R_f \times 100)$ value															
	S ₁				S ₂				S ₃				S ₄			
	M ₁	M ₂	M ₃	M ₄	M ₁	M ₂	M ₃	M ₄	M ₁	M ₂	M ₃	M ₄	M ₁	M ₂	M ₃	M ₄
VO ²⁺	72	80T	75	45T	20	18	40	25	52	15	37T	40	15	37	15	08
Cr ³⁺	75	95	96	95	13	10	22	45	36	32	32	70	10	05	30	14
Mn ²⁺	85	88	98	95	08	37	22	55	62	34	30	75	05	15	30	48
Fe ²⁺	75T	90T	90	75	22	07	10	15	15	15	15	87	05	15	40	05
Fe ³⁺	77	80T	95	15	20	05	10	05	16	12	10	55T	05	12	35	02
Co ²⁺	85	77	65	40	52	25	30	90	45	40	40	60	15	15	45	90
Ni ²⁺	72	87	95	92	48	30	30	60	30	24	27	80	15	22	29	—
Cu ²⁺	72	80	90	70	08	16	37	50	15	17	35	50	15	40	40	00
Zn ²⁺	87	95	98	92	08	22	16	60T	36	21	41	77	05	15	27	37
Zr ⁴⁺	85	90	90	15	05	12	13	00	57	38	40	05	05	00	00	00
MoO₄²⁺	80	90	92	90	20	08	05	18	40	33	30	05	10	43	50	10
WO₄²⁻	75	92	95	80	00	00	00	00	04	00	05	05	52	00	00	00
PtCl₆²⁺	25	05	08	10	05	10	32	05	13	12	08	10	ND	ND	ND	ND
Hg ⁺	35	30	55	32	08	30	05	35	15	10	16	08	27	15	30	40
Cd ²⁺	75	82	88	75	42	42	30	77	51	48	50	95	13	35	18	35

T=Tailing peak; ND=not detected.

Stationary phase: S₁=starch; S₂=alumina; S₃=starch–alumina (1:1, w/w); S₄=alumina–talc (1:1, w/w).

Mobile phase: M₁, M₂, M₃, and M₄ are aqueous solutions (0.1 M) of oxalic, tartaric, citric and succinic acids, respectively.

Remarks: The mechanism of migration is explained in terms of adsorption and complex formation

Ti, Ag, Hg, Pu, and Pd remain near the point of application on all layers irrespective of the mobile phase used. The addition of talc to alumina has a significant effect on the migration behavior of metal ions when oxalic or succinic acid media are used as mobile phase.

Source: Ref. 99.

Table 18 hR_M ($R_M \times 100$) Values of Co(III) Complexes Investigated at Selected Concentrations of $(\text{NH}_4)_2\text{SO}_4$ on Silica and Other Layers

Complex	Isomer	$R_M \times 100$				
		Concentration of $(\text{NH}_4)_2\text{SO}_4$ (mol %)				
		3.3	4.4	5.5	6.6	7.7
1. $[\text{CoNH}_3\text{gly}_2\text{NO}_2]$	Cis (O), trans (NH ₂)	-112	-106	-95 (-63) ^c	-75 (-50) ^d	-69 (-37) ^e
2. $[\beta\text{-ala}]_2$	Cis (O), trans (NH ₂)	-72	-60	-48 (-45)	-37 (-35)	-29 (-19)
3. $[\text{Co}(\text{NH}_3)_2\text{gly}(\text{NO}_2)_2]$	Cis (NO ₂), trans (NH ₂ NH ₃)	-128	-112	-95 (-60)	-75 (-50)	-58 (-35)
4. $[\beta\text{-ala}]$	Cis (NO ₂), trans (NH ₂ , NH ₃)	-112	-95	-75 (-55)	-60 (-43)	-41 (-31)
5. $[\text{Cotngly}(\text{NO}_2)_2]$	Cis (NO ₂), trans (NH ₂)	-29 (-02) ^a	-19 (43) ^b	-09 (-43)	-05 (-33)	14 (-21)
6. $(\beta\text{-ala})$	Cis (NO ₂), trans (NH ₂)	05	-09	25 (-27)	37 (-23)	43—
7. $[\text{Coen}_2(\text{NO}_2)_2]^+$	Trans (NO ₂)	-31 (-41)	-23 (-18)	-10 (-95)	-02 (-83)	-09—
8. ent	Trans (NO ₂)	-07 (-23)	-02 (-03)	14 (-75)	23 (60)	33 (41)
9. $[\text{Cooxygly}_2]^+$	Trans (N)	-138	-112	-106 (-50)	-91 (-43)	-79 (-31)
10. $(\beta\text{-ala})_2$	Trans (N)	-95	-87	-72 (-31)	-58 (-23)	-50 (-14)
11. $[\text{Coengly}(\text{NO}_2)_2]$	Cis (NO ₂), trans (NH ₂)	No salting out (31)	(14)			
12. ala	Cis (NO ₂), trans (NH ₂)	No salting out (12)	(35)			

The values in parentheses were obtained on ^apolyacrylonitrile, ^bCN-modified silica gel, and ^{c,d,e}cellulose layers.

Key: gly H=glycine; ala H=alanine; en=1,2-diaminoethane; tn=1,3-diaminopropane, and ox=oxalato dianion.

Remarks: Enlargement of the chelating ring in Co(III) complexes containing bidentate

aminocarboxylato and/or diamine ligands leads to increase in R_M values.

Source: Ref. 78.

Table 19 hR_f ($R_f \times 100$) Values of Metal Ions Observed on Plain and Mixed Layers Developed with Mixture of Acid and Alcohol

Cation	hR_f					
	M_1		M_2		M_3	
	S_1	S_2	S_1	S_2	S_1	S_2
Cu	94	86	65	97	58	75
Ni	97	86	00	75	00	67
Co	91	80	50	98	37	69
Cd	00	98	00	96	24	62
Hg	96	95	21	66	69	92
Cr ³⁺	46	81	55	91	50	44
Zn	96	79	00	00	00	34
Mn	93	76	56	28	32	40
Fe ²⁺	83	50	65	95	35	72
Fe ³⁺	79	84	85	98	35	92
Pb	00	00	00	00	00	00

Mobile phase: $M_1=2$ M HCl–C₂H₅OH–H₂O (1:1:1, v/v); $M_2=4$ M HCl–C₂H₅OH (4:1, v/v); $M_3=2$ M HCl–isoamyl alcohol (4:1, v/v).

Stationary phase: S_1 =Silica gel+naturally occurring mixed oxide exchanger; S_2 =silica gel.

Remarks: Combined ion exchange and solvent extraction techniques were used for the separation of metal ions using synthetic organic ion-exchange papers and inorganic ion exchanger.

Source: Ref. 79.

Table 20 hR_f ($R_f \times 100$) Values of Separated Cations Under Different Experimental Conditions

Stationary phase	Mobile phase	Separation (hR_f)
Ion-exchange gel– silica gel G (1:1)	Water–HCl–acetone (8:2:90)	Tl ⁺ (00)–Co ²⁺ or Cd ²⁺ (80)
	Water–formic acid–acetone (8:2:90)	Co ²⁺ (100)–Tl ⁺ (00), Cd ²⁺ (100)–Ag ⁺ (25), Co ²⁺ (100)–Pb ²⁺ or Bi ³⁺ (00)

	Water-formic acid-acetone (2:8:90)	Cd^{2+} (100)– Co^{2+} (10), Cd^{2+} (100)– Tl^+ or UO_2^{2+} (00), Cd^{2+} (100)– Ag^+ (20)
Ion-exchange gel– silica gel G (1:1) impregnated by 1 M TBP in acetone	Distilled water	Ni^{2+} (76)– Fe^{3+} (10)
	1 M Formic acid	Cu^{2+} (80)– Pb^{2+} (10), UO_2^{2+} (80)– Tl^+ (10)
	10 M Formic acid	Co^{2+} (80)– Pb^{2+} (0), Cd^{2+} (75)– Ag^+ (15)
	Pure formic acid	Tl^+ (65)– Pb^{2+} (00)
	Water–HCl–acetone (8:2:90)	Cu^{2+} (90)– Pb^{2+} (10), UO_2^{2+} (90)– Tl^+ (10), Cd^{2+} (90)– Ag^+ (10)
	Water–formic acid–acetone (2:8:90)	UO_2^{2+} (00)– Pb^{2+} (00), Co^{2+} (65)– Ag^+ (00)
Ion-exchange gel– silica gel G (3:1) impregnated by 1 M TBP in acetone	1 M Sodium formate–1 M formic acid (1:1)	Zn^{2+} (90)– Bi^{3+} or Pb^{2+} (15)
	1 M Sodium formate–1 M KI (2:8)	Ni^{2+} (80)– Cu^{2+} or Zn^{2+} (10)
	1 M Sodium formate–1 M KI (8:2)	Ni^{2+} (75)– Cu^{2+} or Pb^{2+} (00)
Ion-exchange gel– silica gel G (1:3) impregnated by 1 M TBP in acetone	1 M Sodium formate–1 M KI (8:2)	Ni^{2+} (72)– Fe^{3+} or Cu^{2+} (00)

Remarks: Tributyl phosphate (TBP), when used as impregnant in reversed-phase TLC, yields better separations of metal ions than when used as the mobile phase in normal-phase TLC. The optimum impregnated concentration level was 1.0 M TBP.

Source: Ref. 84.

Table 21 hR_f of Mixtures of Zn^{2+} , Cd^{2+} , and Hg^{2+}
Chromatographed on Silica Gel G Layers
Developed with Cetyl Trimethylammonium
Bromide (CTAB) Containing Mobile Phase

Mobile phase (M + 99 mL of aqueous electrolyte solution) ^a	$hR_f (R_f \times 100)$		
	Zn^{2+}	Cd^{2+}	Hg^{2+}
0.01 M NaCl	05	22	92
0.05 M NaCl	03	34	95
0.10 M NaCl	02	56	98
0.20 M NaCl	02	68	98
0.01 M HCl	02	25	82

0.05 M HCl	15	40	92
0.10 M HCl	22	43	95
0.20 M HCl	30	48	95
0.01 M CaCl ₂	05	32	87
0.05 M CaCl ₂	02	37	92
0.10 M CaCl ₂	03	50	87
0.20 M CaCl ₂	05	87	90
0.01 M LiCl	05	27	85
0.05 M LiCl	05	37	95
0.10 M LiCl	05	47	97
0.20 M LiCl	04	57	95
0.01 M KCl	05	32	76
0.05 M KCl	02	50	90
0.10 M KCl	02	67	95
0.20 M KCl	02	76	92
0.10 M Br	02	16	95
0.05 M Br	02	35	97
0.10 M Br	02	55	95
0.20 M Br	02	70	97
0.10 M urea	05	48	97
0.10 M NaNO ₃	10	42	92
0.10 M oxalic acid	52	92	95
0.10 M tartaric acid	58	90	95
0.10 M KOH	05	25	81
0.10 M NaOH	05	25	85

^aM=1.0 mL CTAB–ethanol (1:2 w/v). Salt solutions were prepared in distilled water.

Remarks: A novel mobile phase comprising (1:2 w/v CTAB+ethanol)–water, 1:99, was identified for rapid separation of mixtures of Zn²⁺, Cd²⁺, Hg²⁺, and Co²⁺. The substitution of water by aqueous solutions of NaCl, HCl, CaCl₂, LiCl, KCl, NaBr, NaNO₃, NaOH, or KOH, oxalic acid, or tartaric acid influences the mutual separation of Zn²⁺, Cd²⁺, and Hg²⁺. The method was applied for identification and separation of Zn²⁺, Cd²⁺, Co²⁺, and Hg²⁺ in spiked industrial wastewater, in metal sulfide ores, and in metal hydroxide sludge samples.

Source: Ref. 80.

Table 22 hR_f ($R_f \times 100$) Values of I^- , IO_3^- , and IO_4^- Chromatographed on Single-Adsorbent Layers with Mixtures of 0.1 M NH_4OH and Acetone as Mobile Phase

Anion	Mobile phase ^a	Stationary phase			
		Aluminum oxide G	Microcrystalline cellulose	Kieselguhr G	Silica gel G
I^-	M ₁	65	56	95	90
	M ₂	70	91	96	92
	M ₃	86	92	97	97
	M ₄	90	92	97	98
	M ₅	97	94	98	98
IO_3^-	M ₁	00	00	89	00
	M ₂	00	55	90	75
	M ₃	08	72	90	92
	M ₄	24	85	92	90
	M ₅	30	94	94	95
IO_4^-	M ₁	00	00	T ^b	00
	M ₂	00	60	T	00
	M ₃	00	74	T	00
	M ₄	00	85	T	00
	M ₅	00	95	T	00

^aMixtures of 0.1 M NH_4OH and acetone in volume ratios of 1:9 (M₁); 3:7 (M₂); 5:5 (M₃); 7:3 (M₄); and 9:1 (M₅).

^bBadly tailed spot.

Remarks: The chromatographic system comprising alumina–M₅ (0.1 M NH_4OH –acetone, 9:1) was best because it furnished more compact and better resolved spots for iodide and its oxyanions. TLC coupled with iodometry was used to determine I^- , IO_3^- , and IO_4^- in fortified distilled water.

Source: Ref. 88.

Table 23 TLC Systems Used for Analysis of Co, Ni, and Cu from Their Mixture

Stationary phase		Mobile phase ^a	$hR_f (R_f \times 100)$		
Adsorbent	Impregnant		Co	Ni	Cu
Cellulose	—	M ₁	34	09	60
Chitin	—	M ₂	27	22	05
Silica gel	1% NH ₄ SCN	M ₃	90	95	00, 1.0 DS ^b
RP-18	—	M ₄	63	38	28
Silica gel	0.3 M sodium molybdate	M ₅	84	80	30
Silica gel	40% Tributylamine	M ₆	90	94	30
Silica gel	Dibenzoylmethane	M ₇	34	73	81
Stannic arsenate ion-exchange (SAIE) gel– silica gel (10:1, w/w)	0.2 M Tributyl phosphate (TBP)	M ₈	52	90	10

^aM₁=Acetone–conc. HCl–water (86:8:7); M₂=water–methanol (3:7); M₃=1.0 M formic acid–1.0 M sodium formate (3:7); M₄=MeOH–water–acetic acid (50:30:4); M₅=1.0 M sodium formate; M₆=1.0 M sodium formate–1.0 M formic acid (8:2); M₇=ethyl acetate– formic acid–H₂O–pyridine (3:1:1:05); M₈=0.10 M KSCN.

^bDS=Double spot.

Remarks: The TLC system comprising SAIE gel–silica gel (10:1, w/w) impregnated with 0.2 M TBP as stationary phase and 0.1 M KSCN as mobile phase was found to be the best for resolving Co, Ni, and Cu from their mixture. TBP was more effective as impregnant than as mobile phase. The method was applied for identification of Ni and Cu in vegetable and fruit juice samples.

Source: Ref. 87.

Table 24 hR_f Values Obtained on Alumina Layer for Metal Ion Complexes with Sodium Diethyldithiocarbamate Using Mixtures of Benzene and Chloroform as Mobile Phases

% CHCl ₃ in CHCl ₃ –C ₆ H ₆ mobile phase	$hR_f (R_f \times 100)$ ^a							
	Metal complex							
	Cd	Co	Cu	Fe	Hg	Mn	Pb	Zn
0	05	65	51	52	66	59	54	09
10	05	68	53	60	69	60	59	09
20	09	78	64	70	80	74	72	12
30	10	83	73	75	84	78	79	17

40	15	90	86	84	91	86	85	19
50	19	94	90	87	91	91	93	26
60	23	95	92	89	95	92	94	27
70	34	95	94	92	97	94	94	30
80	39	95	96	95	97	95	95	32
90	46	99	99	98	99	96	98	36
100	57	100	100	100	100	100	100	44

^a*hR_f* values are drawn from the published figure.

Remarks: Chromatography of dithiocarbamate complexes of metals (Cd²⁺, Co²⁺, Cu²⁺, Fe²⁺, Hg²⁺, Mn²⁺, Pb²⁺, and Hg²⁺) was also performed on silica layers using the same mobile phases. The polarity of the mobile phase influences the retention behavior of the metal complexes.

Source: Ref. 100.

Table 25 Separation of Mn from Ni, Fe, Cu, and Zn as Their Chlorosulfates on Mixed Layer Consisting of Silica Gel and Cellulose (2:1 w/w) in the Presence of Selected Anions Using Distilled Water as Mobile Phase

Anion	Separation (<i>R_f</i> ×100)
IO₄⁻	Mn (76)–Ni (55)–Cu (32)–Fe (09)
	Mn (73)–Ni (60)–Zn (26)–Fe (09)
IO₃⁻	Mn (76)–Ni (59)–Cu (25)–Fe (09)
	Mn (74)–Ni (57)–Zn (29)–Fe (07)
BrO₃⁻	Mn (74)–Ni (57)–Cu (31)–Fe (08)
	Mn (71)–Ni (57)–Zn (27)–Fe (09)
NO₃⁻	Mn (76)–Ni (55)–Cu (27)–Fe (09)
	Mn (73)–Ni (51)–Zn (35)–Fe (07)
NO₂⁻	Mn (77)–Ni (62)–Cu (27)–Fe (08)
	Mn (76)–Ni (55)–Zn (35)–Fe (07)
F ⁻	Mn (68)–Ni (55)–Cu (30)–Fe (13)
	Mn (72)–Ni (52)–Zn (19)–Fe (09)
PO₄³⁻	Mn (73)–Ni (57)–Cu (24)–Fe (02)
	Mn (75)–Ni (57)–Zn (22)–Fe (07)
SCN ⁻	Mn (74)–Ni (55)–Cu (24)–Fe (17)
	Mn (77)–Ni (59)–Zn (21)–Fe (06)

Remarks: The separation efficiency of water is increased when the mixed adsorbent layer is used instead of single adsorbents.

Source: Ref. 92.

Table 26 Separation of Anions Achieved Experimentally on Selected Blended Adsorbent Layers Using Tributylphosphate–Formic Acid (2:8) as Mobile Phase

Stationary phase	Separation ($R_f \times 100$)
Stannic arsenate–silica gel (1:9)	MnO_4^- (05)– CrO_4^{2-} (55)/ $\text{Cr}_2\text{O}_7^{2-}$ (80)/ IO_3^- (65)/ IO_4^- (60)/ BrO_3^- (95)/ SCN^- (92)/ $\text{Fe}(\text{CN})_6^{3-}$ (91)
Stannic arsenate–alumina (1:9)	CrO_4^{2-} (09)– I^- (59)/ NO_2^- (56)/ BrO_3^- (48)/ SCN^- (62) $\text{Cr}_2\text{O}_7^{2-}$ (08)– I^- (59)/ NO_2^- (62)/ BrO_3^- (47)/ SCN^- (70) IO_3^- (07)– IO_3^- (78)/ NO_2^- (69)/ BrO_3^- (53)/ SCN^- (80) MnO_4^- (00)– SCN^- (97) $\text{Fe}(\text{CN})_6^{3-}$ (05)– SCN^- (95)
Stannic arsenate–cellulose (1:9)	MnO_4^- (00)– BrO_3^- / SCN^- / $\text{Fe}(\text{CN})_6^{3-}$ (95)
Tin(IV) molybdosilicate–silica gel (1:9)	MnO_4^- (09)– BrO_4^{2-} (76)/ $\text{Cr}_2\text{O}_7^{2-}$ (98)/ NO_2^- (95)/ BrO_3^- (92)/ SCN^- (97)
Tin(IV) molybdosilicate–alumina (1:9)	CrO_4^{2-} (10)– NO_2^- (70)/ BrO_3^- (67)/ SCN^- (74) $\text{Cr}_2\text{O}_7^{2-}$ (11)– NO_2^- (66)/ BrO_3^- (62)/ SCN^- (89) IO_3^- (10)– NO_2^- (85)/ BrO_3^- (65)/ SCN^- (71)

Remarks: Mixed stannic arsenate–alumina (1:9, w/w) layers were found most useful for selective separation and quantitation of IO_3^- with tributylphosphate-formic acid eluent.

Source: Ref. 95.

Table 27 hR_f Values of Ag^+ , Cu^{2+} , Hg^{2+} , Zn^{2+} , Cd^{2+} , and Cr^{6+} on Alumina Layer as a Function of $(\text{NH}_4)_2\text{SO}_4$ Concentration^a

Metal ion	$hR_f (R_f \times 100)$				
	M_1	M_2	M_3	M_4	M_5
Ag^+	06	12	62	68	71
Cu^{2+}	00	00	00	00	00
Hg^{2+}	00	00	09	32	28

Zn ²⁺	00	00	00	00	00
Cd ²⁺	00	00	00	05	15
Cr ⁶⁺	00	00	09	32	32

^aMobile phase: Molar concentration of (NH₄)₂SO₄: M₁=0.01, M₂=0.10, M₃=0.50, M₄=1.00, and M₅=2.50 M.

Remarks: Alumina G layers developed with aq. (NH₄)₂SO₄ solution (2.5 M) were found most suitable for selective separation of Ag⁺. The selective separation (*hR_f* value given in parentheses) of Au³⁺ (100) from Cu²⁺ (00) and Ni²⁺ (42) was also achieved on silica layer developed with aq. cetyl trimethylammonium bromide solution (1.2 mm). These methods have been applied to recover gold and silver from secondary sources.

Source: Ref. 93.

Table 28 *hR_f* Values of Rare Earth Elements (REEs) on Silica Gel (pH 7.0) Layer Treated with 0.1 M Tris(hydroxymethyl)aminomethane and 0.1 M HCl Using Aqueous Alkaline Earth Metal Nitrate Solution as Mobile Phase

Rare earth	Mobile phase (mol/L)								
	Mg(NO ₃) ₂				Ca(NO ₃) ₂		Sr(NO ₃) ₂		Ba(NO ₃) ₂
	0.2	0.4	1.0	3.0	0.4	1.0	0.4	1.0	0.4
Sc	2	1	2	1	2	2	2	2	2
Y	17	26	42	45	22	31	21	28	19
La	77	83	91	96	86	90	82	87	80
Ce	57	71	84	91	69	79	66	74	62
Pr	47	63	79	87	60	72	56	66	52
Nd	47	64	80	88	61	73	56	67	52
Sm	28	44	65	77	39	56	36	49	31
Eu	24	40	61	71	35	49	32	43	27
Gd	26	42	63	73	37	52	34	45	29
Tb	19	29	46	56	25	35	24	32	20
Dy	14	23	38	44	20	27	19	25	16
Ho	15	23	38	43	20	27	19	24	16
Er	12	18	29	32	14	19	13	18	11
Tm	7	10	17	19	8	12	8	11	7
Yb	5	7	10	12	6	7	5	7	5
Lu	5	6	8	10	5	7	5	6	5

Remarks: To demonstrate the effectiveness of the silica gel-alkaline earth metal nitrate systems for the separation of REEs, typical chromatograms showing the separation of multicomponent mixtures containing adjacent lanthanides are presented in the published paper.

Source: Ref. 94.

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22

Lipids

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1. INTRODUCTION AND ADVANTAGES

Thin-layer chromatography (TLC) is used extensively for lipid analysis and is a valuable tool for the separation and tentative identification of neutral and complex lipid classes. TLC can achieve separations of complex mixtures comparable to other types of liquid column chromatography. Although numerous sorbents are available for lipid TLC, silica gel is the one used most frequently. In recent years, lipid TLC has made use of the newer, finer grades of silica gel, which are also used in high-performance liquid chromatography (HPLC) columns. TLC has the distinct advantage of allowing for the separation of lipids of different polarities on the same plate with a single solvent system.

Silica gel plates can be sprayed or dipped with general or specific detection reagents for the identification of numerous lipophilic compounds. Moreover, prior to sample spotting, a TLC plate can be treated with chemicals that alter its properties, e.g., borate and silver ions. Such alterations can be made on either homemade or commercial plates, thus increasing the versatility of TLC in the separation of additional compounds. TLC allows for the removal of lipids by scraping and elution of compounds from plates [preparative layer chromatography (PLC)]. It also allows for the quantification of lipid classes by in situ densitometry. TLC has been used as a rapid, convenient, and inexpensive method for testing (scouting) solvent systems for HPLC. The development of high-performance TLC (HPTLC) has enhanced the versatility of lipid TLC. HPTLC allows for the use of less sample and development solvent and shorter development times. The use of two-dimensional (2-D) TLC is also valuable for the separation of multicomponent samples, particularly those with complex lipid mixtures. TLC interfaces with numerous other analytical techniques, most notably gas-liquid chromatography (GLC) and mass spectrometry. Although lipid TLC is currently used mainly in basic research and in the pharmaceutical industry, in the future it will probably be used more in clinical work, particularly with the advent of automated TLC procedures.

II. DEFINITION, STRUCTURE, AND OCCURRENCE

There is no universal definition of the term "lipids." Lipidologists have provided useful definitions for workers interested in the chromatographic analysis of these compounds. Thus, Kates (1) considered lipids as compounds generally insoluble in water but soluble in a variety of organic solvents, e.g., ether, hexane, chloroform. He recognized various classes of lipids, including hydrocarbons, alcohols, aldehydes, fatty acids, and derivatives such as glycerides, wax esters, phospholipids, glycolipids, and sulfolipids. His consideration of lipids also included the fat-soluble vitamins and their derivatives, carotenoids, sterols, and their fatty acids. Chapter 1 in Kates (1) provides a comprehensive treatment of the classification and structure of lipids. Chapter 1 in Hammond (1a) provides concise coverage of lipid structure. Gunstone and Herslöf (1b) considered "that lipids are compounds based on fatty acids or closely related compounds such as the corresponding alcohols or the sphingosine bases." These authors also published a lipid glossary (1b) that included the names of fatty acids, lipids, and major fats and oils and terms related to their analyses; also included in their glossary are the major journals and societies that deal with lipid chemistry. Gunstone and Herslöf (1c) revised and extended the earlier edition of their lipid glossary. They added new entries, extended existing entries, and added new key references. They used more graphics to particularly depict molecular structures. The number of glossary entries increased from 900 to more than 1200, graphics from about 61 to more than 180, and the text from 100 to 237 pages. This is a very useful book on all aspects of lipid chemistry.

Christie (2) noted that a variety of diverse compounds generally soluble in organic solvents are usually classified as lipids, i.e., fatty acids and their derivatives, steroids, terpenes, carotenoids, and bile acids. He suggested that many of these diverse compounds have little in the way of structure or function to make them related and that many substances regarded as lipids, e.g., glycolipids, gangliosides, may be more soluble in water than in organic solvents. Table 1 provides a list of diverse lipophilic substances that have been examined by TLC. It includes typical sorbents and solvent systems and references for these lipophilic substances.

This chapter is concerned mainly with the more restrictive definition of lipids following Christie (46). A convenient system of classification of lipids based on this schema considers the simple lipids (compounds that upon hydrolysis yield no more than two types of primary products per mole), also referred to as neutral or apolar lipids, and polar or complex lipids (compounds that upon hydrolysis yield three or more primary products per mole). Complex lipids are the glycerophospholipids (or simply phospholipids) and the glycolipids (also termed glyceroglycolipids and glycosphingolipids), including gangliosides. Tables 2 and 3 provide lists of neutral and complex lipids along with their major chemical and physical properties and common sources of these compounds. This chapter is concerned mainly with the thin-layer chromatographic analysis of these compounds.

Gunstone and Padley (46a) edited a book on lipid technologies and applications that contains 31 chapters in six parts: Part I, Introduction; Part II, Processing; Part III, Food Emulsions; Part IV, Non-Aqueous Foods; Part V, Special Food Applications; Part VI,

Nonfood Uses. Although not directly related to TLC, the book provides information on lipid structure and will be of interest to lipid chromatographers.

III. FUNCTIONS

Lipids are involved in many biological functions associated with plants, animals, and microbial organisms. The numerous functions of lipids have been studied in part with TLC and PLC methods. In the discussion that follows, significant references are given whenever possible to work that used TLC as a method of analysis.

Lipids are important as storage materials for energy reserve (47). In mammals and birds the storage depot is usually in the form of adipose tissue (48) and contains mainly triacylglycerols along with lesser amounts of free fatty acids and mixed glycerides. In sharks, skates, and rays (the elasmobranchs or cartilaginous fishes), the fat depots consist mainly of squalenes and glyceryl ethers, which are of a lower density than triacylglycerols and contribute to the buoyancy of these fishes (49). In the teleosts (bony fishes), lipids are deposited mainly in the liver, bone marrow, and muscles (50). The presence of lipids in the bone of the teleost *Helicolenus dactylopterus lahiller* (blackbelly rosefish) was studied (50a), and the lipid composition of the bone was determined by TLC with scanning densitometry; the study also used histological sections of bone to clarify the sites of lipid deposition in bone. Findings from the study suggest that lipid functions as both a hydrostatic agent and energy reserve in the blackbelly rosefish (50a). Less information is available on the storage sites of lipids in invertebrates (51). Mermithid nematodes have specialized organs called trophosomes that are used to store lipids. TLC separations of three species of mermithids showed that the trophosomes contained a similar array and distribution of lipid fractions. Triacylglycerols constituted the most abundant type of lipid, with phospholipids the next most abundant. Sterol esters and free sterols were less abundant (51a). During starvation, both vertebrates and invertebrates use lipids as an energy reserve. A TLC study (52) showed that

Table 1 Selected Examples of TLC Separations of Various Lipoidal Substances

Compounds separated	Stationary phase ^a	Mobile phase ^b	Ref.
Carotene and canthaxanthin	KC	MeOH-AC (1:1)	3
Carotenoid aldehydes	PO	MeOH	4
Carotenoids and aldehydes	KC	Petroleum ether-acetonitrile-methanol (2:4:4)	4a
Ceramides	SG+2% Na arsenite	CHCl ₃ -MeOH (95:5)	5
Ceramide acetates	AgNO ₃ ^c	CHCl ₃ -B-AC (80:20:10)	5
Chlorophylls	KC	MeOH-THF-H ₂ O (75:20:5)	6
Chloroplast pigments	SG	IO-AC-E(3:1:1)	7

	Sucrose	PE-AC (95:5)	8
	Ca(OH) ₂	PE-MeCl ₂ (95:5)	9
	KC	MeOH-AC-H ₂ O (20:4:3)	10
	KC	BuOH-MeOH (8:7)	10
Chloroplast pigments	KC	PE-AN-MeOH (2:4:4)	4a
Cholesteryl esters	PO	MEK-AN (7:3)	11
	KC	AN-MEK-CHCl ₃ (50:35:15)	3
Cholesteryl esters	KC	MeOH-AC (1:1)	3a
Conjugated bile acids	KC	EtOH-0.3 M CaCl ₂ -DMSO (25:25:2)	12
	KC	EtOH-KH ₂ PO ₄ (pH 2.5 (1:1)	13
Cortico steroids	SG	CHCl ₃ -MeOH-H ₂ O (188:12:1)	14
	F	BuAc or BuAc-H ₂ O (300:15)	15
Diglyceride acetates	AgNO ₃ ^c	CHCl ₃ -MeOH-H ₂ O (65:25:4)	16
Fat- soluble dyes	AgNO ₃ ^c	B-E-HAc (90:10:0.1)	17
Fat-soluble food colors	SG	CHCl ₃ or H-EA (90:10)	17
Fatty acids	AgNO ₃ ^c	PE-E (40:10)	18
	SG	H-HAc-H ₂ O (100:5:2.5)	18a
Fatty acid methyl esters	SO	AN-HAc-H ₂ O (70:10:25)	19
	KC	HAc-AN-THF (45:45:10)	20
	KC	MeOH-HAc-HOCOOH (80:10:10)	20
	KC	AN-5% NH ₄ OH (95:5)	20
Gangliosides	SG	CHCl ₃ -MeOH-2.5 N NH ₄ OH (60:40:9)	21
Glycerides	BA ^c	CHCl ₃ -AC (96:4)	22
Glyceryl ethers	SG	H-E (95:5)	23
Glycosphingolipids	SG	CHCl ₃ -MeOH-H ₂ O (65:25:4)	24
	SG	CHCl ₃ -MeOH-0.02% CaCl ₂ (60:40:9)	24a
Higher alcohols and glycols	Al	H-AC (3 or 4:1), H ₂ O sat.	25
Isomers of hydroxy acids, esters, and alcohols	BA ^c	PE-E (6:4)	26
Lipids	SG	PE-E-HAc (70:40:2 to 80:20:1) or PE-E (95:5 to 80:20)	27 27
	SG	CHCl ₃ -MeOH-H ₂ O (65:25:4)	28

Phospholipids	SG	CHCl ₃ -MeOH-H ₂ O (25:10:1)	29
	SG	CHCl ₃ -MeOH-HAc-H ₂ O (25:15:4:2)	29
	SG	AC-HAc-H ₂ O (100:2:1)	30
	SG	DBK-HAc-H ₂ O (40:25:3.7)	31
	(NH ₄) ₂ SO ₄ ^c	CHCl ₃ -MeOH-AC-H ₂ O (40:25:7:4:2)	31a
Phosphonolipids	SG	CHCl ₃ -HAc-H ₂ O (60:34:6)	32
Serum lipids	SG	PE-IPA(99:1)	33
Simple lipids	SG	H-E-HAc (80:20:2)	34
Compounds separated	Stationary phase ^a	Mobile phase ^b	Ref.
Steroids	SG	CH-EA(70:3 or 85:15)	35
	F	H-B (1:1)	36
Steroid glycosides	SG	MeCl ₂ -MeOH-FA (80:15:1)	37
Sterols	SG	H-EA (80:20)	38
	Al	IO-EA (80:20)	38
	AgNO ₃ ^c	CHCl ₃ -AC (95:5)	39
	KC	AN-CHCl ₃ (40:35)	3
	KC	AN-CHCl ₃ -MAc (55:25:15)	3
Sterols and sterol acetates	Un	HAc-AN (1:3)	40
Triacylglycerols	TD	AC-AN (8:2)	41
	AgNO ₃ ^c	CH-B mixtures	42
	AgNO ₃ ^c	IPA-CHCl ₃ (1.5:98.5)	42a
Ubiquinones	PO	MeOH-IPa(90:10)	9
Vitamin A isomers	SG	PE-methylheptanone (11:2)	43
Vitamins D ₂ and D ₃	SG	CHCl ₃ or H-EA (90:20)	44
Wax esters	SG	CCl ₄ or H-E (98:2)	45

^aStationary phases: Al, aluminum oxide; BA, boric acid; F, formamide; KC, Whatman KC₁₈ reversed-phase plates; PO, paraffin oil; SG, silica gel; SO, silicone oil; TD, tetradecane; Un, undecane.

^bMobile phases (all solvents given in volume proportions unless otherwise noted): AC, acetone; AN, acetonitrile; B, benzene; BuAc, butyl acetate; CH, cyclohexane; ChCl₃, chloroform; DBK, diisobutyl ketone; DMSO, dimethylsulfoxide; E, ethyl ether, EA, ethyl acetate; EtOH, ethanol; FA, formic acid; H, hexane, HAc, acetic acid; IO, isooctane; IPa, isopropyl alcohol; MAC, methyl acetate; MeCl₂, methylene chloride; MEK, methyl ethyl ketone; MeOH, methanol; PE, petroleum ether; THF, tetrahydrofuran.

^cImpregnating agent used on silica gel.

the medically important planorbid snail, *Biomphalaria glabrata*, depleted mainly triacylglycerols and free fatty acids during starvation. Fried and Sherma (52a) reviewed TLC methods used to analyze lipids in gastropod molluscs. Fried et al. (52b) used TLC and transmission electron microscopy to show that lipid storage and accumulation occurred in the digestive gland cells of this snail. TLC was used extensively to study the effects of a high fat diet on the lipid composition of *Biomphalaria glabrata* snails (52c). Reed et al. (52d) used TLC and flame ionization detection (FID) to show that neutral lipids, particularly triacylglycerols, play an important role as a fuel source to support the swimming behavior of macroalgal spores of palm kelp in the genus *Pterygophora*.

Lipids are important in the structural integrity of cells and comprise the major components of all membranes (53). Lipids of particular importance in these roles are sterols, phosphoglycerides, glycolipids, and sphingolipids.

Complex lipids in the membranes of neuronal tissues are involved in the transmission of electrical signals; phosphoinositides and their metabolic products play a role in cellular chemical communication. The use of TLC to study phosphoinositide metabolism in resting and stimulated cells has been reviewed (54).

Lipids are important as pheromones, precursors of pheromones, or carriers of pheromones in plants and animals. This topic was reviewed for vertebrates and insects by Shorey (55) and for invertebrates (mainly helminths) other than insects by Haseeb and Fried (56). Lipophilic pheromones or their carriers are mainly glycerides, free fatty acids, and sterols. Insects excrete long-chain alcohols, alkyl acetates, aldehydes, and ketones that serve as intraspecific pheromones (57). Shanas et al. (57a) used TLC to analyze lipids in the Harderian gland of the mole rat. Greater

Table 2 Lipids Frequently Separated by TLC

Compound	Chemical and physical properties	Sources
<i>Neutral lipids^a</i>		
Triacylglycerols (triglycerides)	Glycerol moiety with each hydroxyl group esterified to a fatty acid	Most fats and oils of plant and animal origin
Diacylglycerols (diglycerides)	2 mol fatty acid per mole glycerol	Trace levels in animal and plant tissues
Monoacylglycerols (monoglycerides)	1 mol fatty acid per mole glycerol	Trace levels in animal and plant tissues
Cholesterol	Tetracyclic ring with double bond in one ring and one free hydroxyl group	Vital role in maintaining membrane fluidity; principal sterol of high animals
Cholesteryl esters	Esterified form of the above	In animal tissues
Wax esters	Fatty acids esterified to long-chain alcohols with aliphatic chains similar to	Ubiquitous in animal, plant, and microbial tissues; various functions

	the acids	
Free fatty acids	Long-chain carboxylic acids; variation in branching, occurrence of other functional groups, and number of double bonds; atypical as the free form and usually found esterified as waxes or glycerides	Usually occur in small amounts in plant and animal tissues; free fatty acid turnover is very rapid in plasma; free fatty acids may serve as indicators of spoilage in food
	<i>Complex lipids</i>	
1,2-Diacyl- <i>sn</i> -glycerol-3-phosphate (phosphatidic acid)	Strongly acidic; typically isolated as a mixed salt; water-soluble, and care is needed to extract from tissues	Traces in tissues; precursor to most other glycerophospholipids
1,2-Diacyl- <i>sn</i> -glycerol-3-phosphoryl-1'- <i>sn</i> -glycerol (phosphatidylglycerol)	Acidic lipid	Traces in tissues; functions as lung surfactant and in chloroplast of plants
Diphosphatidylglycerol (cardiolipin)	Acidic lipid	Constituent of mitochondrial lipids; found in heart muscle
Phosphatidylcholine (lecithin)	Two fatty acid moieties in each molecule	Abundant lipid in membranes
Lysophosphatidylcholine	One fatty acid moiety in each molecule; quite soluble in H ₂ O and easily lost	Minor component of tissues
Phosphatidylethanolamine (cephalin)	Amine group can be methylated to form other compounds	Very abundant lipid in animals, plants, and microorganisms
Lysophosphatidylethanolamine	1 mol fatty acid per mole lipid	Trace lipid in animals, plants, and microorganisms
Phosphatidylserine	Weakly acidic lipid; usually isolated from tissues as a salt	Present in animals, plants, and microorganisms
Phosphatidylinositol	Contains myoinositol, the optically inactive form of inositol	Common in animals, plants, and microorganisms; regulator of vital cell processes
Phosphonolipids (glycerophosphonolipids)	Lipids with a phosphoric acid derivative esterified to glycerol; carbon-phosphorus bond not easily hydrolyzed by reagents	Found mainly in protozoa and marine invertebrates

^aFor information on the TLC separation of less commonly occurring neutral lipids, see p. 327 in Ref. 1.

Table 3 Glycolipids and Gangliosides Frequently Separated by TLC

Types	Examples	Chemical and physical properties
Plant and bacterial glycolipids	A. Monogalactosyldiacylglycerol B. Digalactosyldiacylglycerol C. Sulfoquinovosyldiacylglycerol	1,2-Diacyl- <i>sn</i> -glycerols joined by glycosidic linkage at position <i>sn</i> -3 to the carbohydrate; present in plant and bacterial tissues; soluble in acetone as distinguished from phospholipids, which are not
Sphingolipids	A. Ceramides; sphingosine is the best known B. Sphingomyelin C. Glycosylceramides="cerebrosides" D. Fucolipids="globosides" E. Sulfoglycosylsphingolipids="sulfatides"	Characterized by long-chain bases; the bases are long aliphatic amines with two or three hydroxyl groups, often with a unique trans double bond in position 4; in tissues as part of a complex lipid; major constituent of plasma membranes in animal, plant, and microbial cells
Gangliosides (monosialogangliosides; disialogangliosides; trisialogangliosides; tetrasialogangliosides)	Ceramide polyhexosides with sialic acid groups; contain glucose, galactose, and <i>n</i> -acetylgalactosamine units	Found mainly in nervous tissues; also in lower concentrations in non-neural tissues; soluble in polar organic solvents and in water

than 50% of the fresh weight of the gland was lipid. Male and female lipid components differed considerably, and this sexual dimorphism suggested that the gland function is sex-specific, supporting earlier reports that the mole rat Harderian gland is a source of pheromone. Lipids also function as antimicrobial agents in the skin of mammals (58), antidesiccants in the cuticle of insects (59), and bioacoustic lenses in dolphins (cetaceans) (60). A special structure (the elaisome) located in the seeds of numerous species of plants from various taxa release lipids, particularly diacylglycerols, that are attractive to ants (61). A study (62) used TLC to show that ants responded rapidly to the elaisomes or the diacylglycerol fraction of the elaisomes of seeds of the perennial herb *Hepatica americana*. Morrone et al. (62a) examined the anatomy and chemical composition of elaisomes of *Urochloa paucispicata* in the family Panicea. TLC analyses of the elaisomes showed that they contain triacylglycerols, free fatty acids, and diacylglycerols. Field observations showed that three ant species in the family Formicidae responded to these elaisomes and carried them to their nests.

Christie (2) discussed various functions of lipids: their involvement in disturbances of lipid metabolism associated with specific lipidoses and gangliosidoses; accumulation of various neutral, complex, and conjugated lipids in arteriosclerosis and atherosclerosis (coronary blood vessel and heart disease); the role of lipids in nutrition, disease, and human welfare; the importance of fats and oils as agricultural products and as major items in international trade; the role of fats as a major dietary component and supplier of calories for humans in developed countries; and the contribution that fats make to the taste and structure of foods.

Ando and Saito (63) contributed an excellent review on TLC and HPTLC lipid analysis of normal and pathological tissues associated with various lipid disorders.

Glycolipids play a vital role in cellular metabolism, and TLC has been used in part to elucidate their functions (64). Located mainly at the external surfaces of cell membranes, these lipids help to regulate cell growth and serve as receptors for toxins, hormones, viruses, and other substances. They serve as differentiation markers and cofactors for ion transport (65), and they serve to modulate immune responses (66) or possibly as antigens *per se* (67).

Browsers et al. (67a) discussed functions of lipids in parasitic worms, including work amenable to TLC. For instance, the surface of adult human blood flukes, *Schistosoma mansoni*, consists of two closely opposed lipid bilayers, a probable morphological and functional adaptation to parasitism. This membrane complex provides an effective tool for defeating the host's immune system. The authors presented an overview of lipid metabolism in *S.mansoni* adults of interest because schistosome adults and other helminths cannot synthesize fatty acids or cholesterol and must obtain these lipids from the host.

IV. SAMPLE PREPARATION

Lipid analysis should be done as soon as possible after samples are obtained from plant and animal tissues. If analysis is delayed, samples should be refrigerated overnight at 4°C or for longer periods in a freezer at -20°C or colder. Formalin or other fixatives used in histology laboratories should not be added to tissues, because they may produce spurious results during subsequent TLC analysis. Reddy et al. (67b) tested the effects of freezing and thawing and of formalin and ethanol fixation on HPTLC analysis of neutral lipids in *Biomphalaria glabrata* snails. Their study showed that these procedures produced spurious results in subsequent TLC analysis of neutral lipids in snail tissues. For lipid work, glassware should be chemically cleaned in dichromate or sulfuric acid and then further cleaned in chloroform-methanol (2:1) prior to use. Glass vials, jars, and bottles are recommended for lipid analysis, along with aluminum foil or Teflon-lined lids.

Because lipids are easily auto-oxidized, samples should be handled and processed under nitrogen whenever possible. Samples should not be stored dry but preferably in a small volume of suitable solvent, e.g., chloroform or hexane, under nitrogen and maintained at -20°C. The presence of significant amounts of methyl esters in animal tissues may indicate a preparation artifact, because this lipid is usually not a significant component of animal tissue (68). The neutral lipid mixture 18-4A supplied by Nu-Chek (Elysian, MN) contains methyl oleate. A similar lipid mixture containing methyl oleate

along with other neutral lipids is supplied by Matreya (Pleasant Gap, PA) under the name Non-Polar Lipid Mix-B. The use of either standard during TLC, along with the sample, allows for a check on the presence of methyl esters in the sample. An extract of a blank can be used to determine the validity of a TLC observation. For example, if saline is supplemented with human serum and the mixture is extracted with chloroform and spotted on a TLC plate, neutral lipid TLC procedures should detect mainly cholesterol and cholesteryl esters along with lesser amounts of free fatty acids and triacylglycerols. If the saline alone is treated in an identical manner, it should be lipid-negative.

If the lipids of interest are major constituents, the sample may be applied directly to the plate with no further cleanup (see first method in Table 4). Samples used may be blood, urine, saliva, or tissue homogenates. Kupke and Zeugner (69) directly applied a 0.5 μL sample of plasma to an HPTLC silica gel plate. The sample was applied to the origin over a 15 μL spot of methanol, and the spot was covered immediately with an additional 1–3 μL of methanol. The plate was air-dried and then developed with chloroform–methanol–water (65:30:5) twice, each time for a distance of 3.7 cm, then developed in hexane–diethyl ether–acetic acid (80:20:1:5) to within 1 cm of the top of the plate. The plate was treated with NH_4HCO_3 , then heated for 10 min at 150°C, and sharp fluorescent lipid spots were detected. Excellent separations of the major neutral lipid and phospholipid fractions were obtained.

If the lipids of interest are minor constituents of the sample or present in relatively low concentrations in a complex biological sample, extraction, isolation, and concentration steps usually precede TLC. Numerous sample preparation methods are available, and personal choice often dictates which one will be used. Of all the procedures, that of Folch et al. (70), or a modification of the original procedure, is used more frequently than any other. Table 4 lists numerous procedures used for the sample preparation of lipids.

Reviews on sample preparation include that of Christie (77a) on obtaining lipid extracts from tissues and that of Fried (77b) on obtaining and handling biological materials and prefractionating extracts for lipid analyses. Chapter 2 in Hammond (1a) also provides detailed descriptions on lipid extraction of photosynthetic tissue, oilseeds, tiger prawns (crustaceans), coffee whitener, wheat flour, spores, and volatile fatty acids from cells grown in culture media.

Table 4 Methods Used for Sample Preparation of Lipids

Sample	Lipid extraction technique	Comment	Ref.
Serum and tissue homogenates	Samples applied directly to TLC plates; plates developed with organic solvents.	Direct application of sample; detection of major classes of serum and tissue lipids.	69
Biological samples	Chloroform–methanol (2:1); typically 1 part of tissue or fluid to 20 parts of the solvent.	Most widely used method of lipid extraction; useful for TLC of neutral and complex lipids.	70
Biological samples	Chloroform–methanol– H_2O (1:2:0.8); following extraction, dilute the sample with 1 vol chloroform and 1 vol water to get a biphasic system.	Particularly useful for extraction of more polar lipids such as gangliosides.	71

Blood	Chloroform–isopropanol–water (7:11:2).	Extracts lipids but not pigments; lipids are not contaminated with blood pigments; neutral and complex lipids are quantitatively extracted.	72
Brain tissue	Preextraction of tissue with 0.25% acetic acid followed by chloroform–methanol (2:1).	Nonlipid material first removed with the acetic acid; relatively pure lipid fraction then obtained by treatment with chloroform–methanol.	73
Viruses	Ethanol–ether (3:1); solvent-to-tissue ratio is 30:1.	Useful prior to TLC analysis of strains of influenza viruses; TLC analysis then separates cholesterol from neutral and complex lipids.	74
Biological tissues	Chloroform–methanol (1:2); typically 1 part tissue to 3 parts solvent mixture.	Good for large amounts of tissue where complete recovery of lipid is not needed; does not use as much solvent as many of the previous extraction techniques.	71
Amniotic fluid or blood plasma	Amniotic fluid or blood plasma (about 1 mL) is added directly to a Spice C ₁₈ solid-phase extraction cartridge (Analtech, Newark, DE); the analyte eluted with chloroform–methanol.	Good separation of phospholipids achieved, because most extraneous material is removed; technique also useful for separating neutral lipids from steryl esters and phospholipids.	75
Plant tissues	Tissues first treated with isopropanol, then chloroform–isopropanol (1:1) prior to usual chloroform–methanol (2:1) extraction procedure.	Isopropanol inhibits the action of plant Upases.	76
Biological samples	Samples passed through columns packed with silica gel or Florisil.	Elution with chloroform separates simple from complex lipids; complex lipids removed by elution with methanol.	46
Biological samples	DEAE-Sephadex column chromatography.	Enrichment of acidic phospholipids and gangliosides from the sample; allows for the detection of minor membrane lipids.	77
Glycolipids from neural and nonneural tissues	Samples applied to a DEAE column.	Enrichment of gangliosides and acidic lipids prior to TLC.	63
Glycolipids from biological samples	Elute glycolipids from a silica gel H column with tetrahydrofuran–methanol–methyl–water.	Glycolipids elute from the column ahead of the phospholipids.	63

Biological samples	Elute lipids from a silica gel column with chloroform, chloroform–acetone, then chloroform–methanol.	Good subfractionation of neural lipids from glycolipids and phospholipids.	63
Biological samples	Derivatize the lipids; serine and ethanolamine phosphatides are trifluoroacetylated; the derivatized forms are isolated by argentation TLC.	Useful when these lipids cannot be obtained intact.	63

V. CHROMATOGRAPHIC SYSTEMS

A. General

This section considers the various chromatographic systems, i.e., combinations of sample mixture, stationary phase, and mobile phase, used during TLC of lipids. Various types of development, i.e., one-dimensional (1-D), multiple developments in the same direction, and two-dimensional (2-D), are considered here.

B. Stationary Phase

Silica gel is the most widely used stationary phase (sorbent) for lipid analysis. Many types of precoated silica gel plates are available from numerous manufacturers in different sizes and varying layer thicknesses. Commercial plates are available with glass, plastic, or aluminum foil backings, and general texts on TLC should be examined for details along with the literature from commercial suppliers of TLC plates. Whereas most workers in the United States use commercially prepared plates, some European and other workers make use of homemade plates. The use of homemade plates in lipid analysis has been discussed by Kates (1) and Christie (46).

Commercial plates often contain binders that may alter the properties of the plate. Trial and error may be needed to determine the suitability of a particular plate for a given analysis. Silica gel plates, both commercial and homemade, may be altered by spraying, dipping, or treating with various reagents that modify the properties of the plate. Various examples follow: silver nitrate plates (1–5% AgNO₃) used to separate *cis*-enoic compounds based on unsaturation (78); plates impregnated with borate used to differentiate *cis*–*cis* and *cis*–*trans* vicinal diols (79); glucocerebroside and galactocerebroside separated on borate-impregnated silica gel plates (63). Addition of EDTA (ethylenediamine tetraacetate) (0.09% w/w) to silica gel allows for clear-cut separation of phospholipids developed in chloroform–methanol–acetic acid–water (75:45:3:1) (80). EDTA-silica gel is useful in separating acidic phospholipids (63). Silica gel impregnated with ammonium sulfate improves the resolution of phosphatidylserine from phosphatidylinositol (81).

High-performance thin-layer (HPTLC) plates are very useful for lipid analysis (82). Such plates are made of fine particles of narrow size distribution and have excellent resolving power. The amount of sample can be reduced considerably from that applied to conventional TLC plates, and numerous samples can be used with minimal amounts of

the mobile phase. HPTLC plates are also used frequently in densitometric studies on lipids. Commercially prepared reversed-phase TLC plates are also available but have had limited use in lipid chromatography. Retention data obtained by reversed-phase TLC are somewhat comparable to those obtained by HPLC (63).

Precoated plates should be protected from laboratory fumes or stored in vacuo. Plates left at room temperature for long periods may turn yellow. Washing the plates (predevelopment) in development solvents or in chloroform-methanol (1:1) may reduce the stained background and allow for separations with less interference.

C. Mobile Phase

Numerous mobile phases (development solvents) are available for lipid work (see Table 1). They often consist of solvent mixtures that vary in polarity, along with small amounts of salts or acids. Because a mixed solvent system allows for an undefined gradient in solvent composition during movement on the silica gel layer, samples of different polarities can be developed on a single plate; in TLC the velocity of the solvent movement is reduced as the solvent front nears the top of the plate; optimal separation is obtained with bands or spots with R_f values between 0.1 and 0.6 (63).

D. One-Dimensional Solvent Systems

1. Neutral Lipids

Numerous solvent systems are available for one-dimensional (1-D) TLC of lipids. For neutral lipid separation, the system of Mangold (83) or numerous modifications of it are used; this system consists of petroleum ether (or *n*-hexane), diethyl ether, and acetic acid in various combinations, i.e., 80:20:1 or 70:30:1 or others. The presence of the acetic acid prevents tailing of the spots. This system provides separation of glycerides, free sterols, steryl esters, free fatty acids, and other neutral lipid classes and leaves the phospholipids at the origin. Higgs et al. (86a) designed a new one-dimensional system for neutral lipid separation on silica gel that provided good separation of diacylglycerols from both free fatty acids and free sterols. Plates were developed first in toluene followed by a mobile phase of hexane-chloroform-methanol (30:18:2) in the same direction. Table 5 shows R_f values of neutral lipids in the Mangold system and in various other related systems used for neutral lipid separations. More esoteric lipids separated in neutral lipid solvent systems are described in Kates (1), and his table on p. 327 lists R_f values for the minor lipid components. Figure 1 shows a typical separation of neutral lipids from snail tissues in the Mangold system.

Because the Mangold system does not provide unequivocal separation of all neutral lipids found in animal and plant material, the dual-solvent system of Skipski et al. (85) is often used. In this system, glycerides and free fatty acids are clearly separated from free sterols by double development in the same direction with two different mobile phases. The first phase consists of isopropyl ether-acetic acid (96:4). Following development in this solvent system, the plate is dried and then developed in the same direction in the second mobile phase, which consists of petroleum ether-diethyl ether-acetic acid (90:10:1). A separation of snail liver tissue using the Skipski system is shown in Fig. 2.

In addition to the value of this dual system in TLC, it can be used in PLC to isolate the free sterol fraction (87, 87a).

2. Phospholipids

One-dimensional solvent systems for phospholipids often contain mixtures of chloroform, methanol, and water. The system of Wagner et al. (88) is frequently used to separate the more common phospholipids of animal and plant tissues. Chloroform-methanol-water (65:25:4) is used to move the neutral lipids mainly as a single band at or near the solvent front and for good separation of most of the common phospholipids (see Table 6 for R_f values of phospholipids in this and related solvent systems). Another one-dimensional system devised by Skipski et al. (92) consists of chlo-

Table 5 R_f Values of Common Neutral Lipids Separated in Various Solvent Systems^a on Silica Gel

Compound	$R_f \times 100$						
	S_1	S_2	S_3	S_4	S_5	S_6	S_7
Cholesteryl esters	90	97	97	85	94	90	67
Triacylglycerols	35	63	79	70	60	82	57
Free fatty acids	18	42	21	62	39	50	16
Cholesterol	10	28	42	38	19	30	27
1,3-Diacylglycerols	8	24	66	46	21	40	36
1,2-Diacylglycerols	8	21	53	41	15	25	36
Monoacylglycerols	0	8	11	10	2	5	7

^a S_1 =petroleum ether-diethyl ether-acetic acid (90:10:1) (84). S_2 =hexane-diethyl ether-formic acid (80:20:2) (34). S_3 =toluene-diethyl ether-ethyl acetate-acetic acid (80:10:10:0.2) (34). S_4 =isopropyl ether-acetic acid (96:4) followed by petroleum ether-diethyl ether-acetic acid (90:10:1) in the same direction (85). S_5 =petroleum ether-diethyl ether-acetic acid (80:20:1) (83). S_6 =heptane-isopropyl ether-acetic acid (60:40:4) (86). S_7 =toluene followed by hexane-chloroform-methanol (30:18:2) in the same direction (86a).

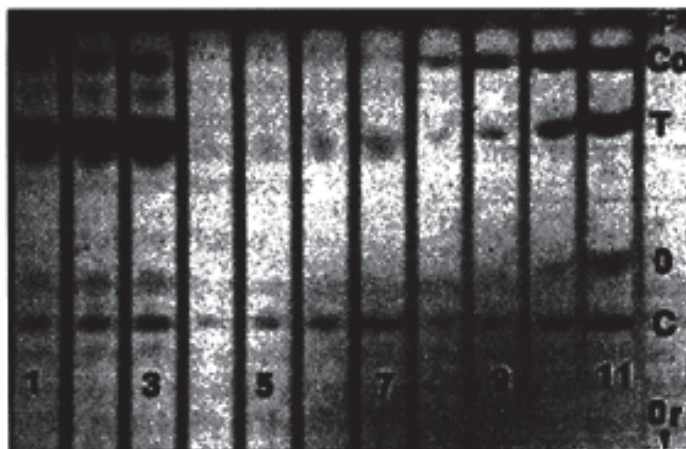


Figure 1 Photograph of a chromatogram of whole snail extracts (extraction done in chloroform–methanol, 2:1) of *Biomphalaria glabrata* maintained on either a yolk–lettuce diet (lanes 1–3) or a lettuce–Tetramin diet (lanes 4–7). Various aliquots of samples were applied to the preadsorbent area (not shown in the figure) of this Whatman high-performance preadsorbent silica gel plate. Lanes 8–11 contain various amounts of the mixed neutral lipid standard 18–5a. The plate was developed in petroleum ether–diethyl ether–acetic acid (80:20:2), and spots were detected by spraying the plate with 5% ethanolic phosphomolybdic acid and heating for 15 min at 115°C. Abbreviations: C, cholesterol; Co, cholesteryl oleate; F, solvent front; O, oleic acid; Or, origin. The major neutral lipids in the snails fed on the yolk–lettuce diet (lanes 1–3) are triacylglycerols, sterol esters, and free sterols. Neutral lipids are sparser in snails fed on the lettuce–Tetramin diet (lanes 4–7), and free sterols and

triacylglycerols are the major neutral lipids in that population.

reform-methanol-acetic acid-water (50:25:7:3). This system is usually suitable for the separation of most major phospholipids. A system devised by Vitiello and Zanetta (93) consisting of methyl acetate-*n*-propanol-chloroform-methanol-0.25% KCl (25:25:28:10:7) not only resolves phospholipids but also separates neutral lipids, cerebroside, sulfatides, and gangliosides. Bradova et al. (93a) separated phospholipids from human brain and liver on silica gel by four or five one-dimensional developments with chloroform-methanol-2-propanol-0.25% KCl-ethyl acetate (30:9:25:6:18). Wang and Gustafson (93b) described a one-dimensional TLC method for the separation of phospholipids and lysophospholipids from tissue lipid extracts. They used a layer of 0.4% ammonium sulfate in silica gel H and a mobile phase of chloroform-methanol-acetic acid-acetone-water (40:25:7:4:2). Figure 3 shows a line drawing of a one-dimensional separation of cellular phospholipids (54).

It is often desirable to separate both phospholipids and neutral lipids on the same silica gel plate. This can be done using the double development procedure of Johnston (94) or any one of various minor modifications. In this technique, phospholipids are first separated in chloroform-methanol-water (65:25:4). After the plate is dried, it is developed in the same direction in the second solvent, which consists of hexane-diethyl ether (4:1). For detailed procedures of this double development technique, see Fried and Shapiro (95). Aloisi et al. (95a) compared a total of 24 solvent systems for the one-dimensional separation of neutral lipids and phospholipids on preadsorbent silica gel plates. They found that the best overall separation was achieved by consecutive development with chloroform-methanol-water (65:24:4), chloroform-hexane (3:1), and carbon tetrachloride; the system of choice for quantification by scanning densitometry was hexane-diethyl ether-formic acid (80:20:2).

3. Glycolipids

For the one-dimensional separation of glycolipids, various combinations of chloroform-methanol-water or chloroform-acetone-methanol-acetic acid-water can be used (24, 96). Glycolipid separation in a single dimension is best done in the absence of phospholipids (46). The four main

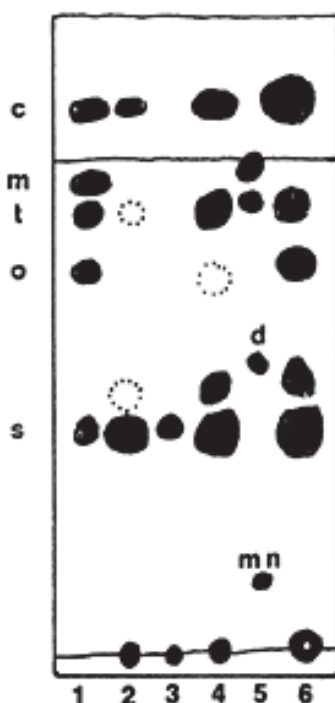


Figure 2 Separation of snail liver tissue and neutral lipid standards on a silica gel sheet using the dual solvent system of Skipski et al. (85). See text for a description of the mobile phases used. The sheet was sprayed with 10% phosphomolybdic acid in ethanol to detect the lipids. Lane 1 shows separation of a mixed neutral lipid standard containing equal amounts of cholesterol (s), oleic acid (o), triolein (t), methyl oleate (m), and cholesteryl oleate (c). Lane 5 contains equal amounts of a different neutral lipid standard consisting of mono-olein (mn), diolein (d), triolein (t), and methyl oleate (m). Lanes 2, 4, and 6 show separations of neutral lipids in the snail liver. Note the abundance of free sterols (s) in this tissue. The material at the origin is phospholipid. Lane 3 is not relevant to this

discussion. (Redrawn from Ref. 106
with permission of Marcel Dekker,
Inc.)

Table 6 R_f Values of Common Phospholipids
Separated in Various Solvent Systems^a on Silica
Gel

Compound	$R_f \times 100$					
	S_1	S_2	S_3	S_4	S_5	S_6
Diphosphatidylglycerol	91	94	—	—	—	—
Phosphatidylglycerol	—	—	90	78	—	45
Phosphatidylethanolamine	65	56	81	59	40	70
Phosphatidylserine	—	47	51	47	13	44
Phosphatidylinositol	—	34	34	52	13	38
Phosphatidylcholine	24	21	90	30	20	36
Sphingomyelin	25	12	8	23	12	26
Lysophosphatidylcholine	6	6	—	—	6	20

^a S_1 =chloroform-methanol-water (25:10:1) (89). S_2 =chloroform-methanol-acetic acid-water (25:15:4:2) (89). S_3 =chloroform-light petroleum-methanol-acetic acid (50:3:16:1) (90). S_4 =chloroform-ethanol-triethylamine-water (30:34:30:8) (91). S_5 =chloroform-methanol-water (65:25:4) (88). S_6 =chloroform-methanol-2-propanol-0.25% KCl-ethyl acetate (30:9:25:6:18) (93a).

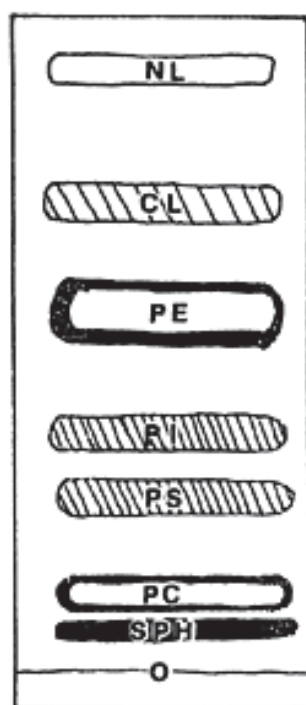


Figure 3 Separation of the major cellular and tissue phospholipids of animal tissues by one-dimensional TLC on a silica gel H plate. The mobile phase used was chloroform–methanol–acetic acid–water (50:37.5:3.5:2). See Ref. 54 for additional details. Abbreviations: O, origin; SPH, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; CL, cardiolipin; NL, neutral lipids. (Redrawn from Ref. 54 with permission of Elsevier Press, Inc.)

classes of glycolipids, i.e., cerebrosides, di- and triglycosylceramides, and ceramide trihexoside-*N*-acetylgalactosamine, can be separated on silica gel layers with the mobile phase chloroform–methanol–water (65:25:4) (24). Expected R_f values of these compounds would be, approximately, ceramide trihexoside-*N*-acetylgalactosamine, 0.16; ceramide trihexosides, 0.31 and 0.36; ceramide dihexosides, 0.55 and 0.62; ceramide monohexosides, 0.78 and 0.86.

4. Gangliosides

One-dimensional TLC can be used to separate gangliosides with various combinations of chloroform–methanol–water or *n*-propanol–water as solvent systems (63). The mobilities of acidic gangliosides as well as the compactness of bands are influenced by the presence of salts or ammonia, but this is not the case for neutral glycolipids (63). For examples of one-dimensional separation of gangliosides, see Ando and Saito (63) and Fig. 4. Ledeen (21) provided an example of a ganglioside separation on silica gel using the basic solvent system chloroform–methanol– 2.5 M aqueous ammonia (60:40:9). The approximate R_f values were as follows: trisialogangliosides, 0.14 and 0.28; disialogangliosides, 0.41, 0.57, and 0.69; monosialogangliosides, 0.85 and 0.97. Rementzis et al. (21a) used one-dimensional silica gel TLC to identify gangliosides from the muscle of the common Atlantic mackerel, *Scomber scomborus*. They identified monosialogangliosides and disialogangliosides with the mobile phase propanol–water (7:3); the compounds were detected by spraying with resorcinol reagent.

E. Two-Dimensional Solvent Systems

1. Neutral Lipids

Two-dimensional solvent systems have been helpful in resolving some complex lipid mixtures. Although not used frequently for neutral lipid separations, they can be helpful in resolving non-

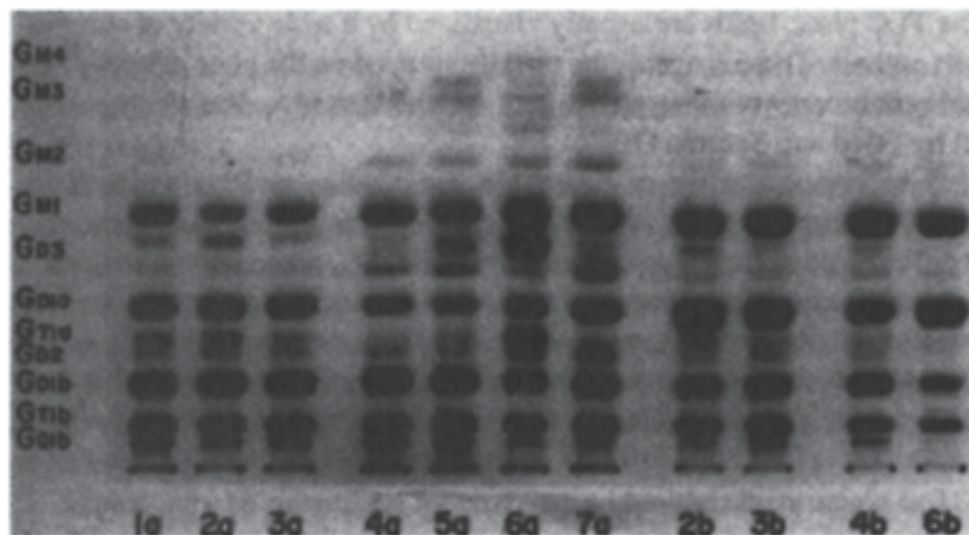


Figure 4 Separation of brain gangliosides on an HPTLC plate developed one-dimensionally with the mobile phase chloroform–methanol–0.22% aqueous CaCl_2 (55:45:10). The gangliosides were detected with the

resorcinol–HCl reagent. Lanes 1a–3a show brain gangliosides from control cases; lanes 6a, 7a show brain ganglioside patterns from patients with known gangliosidosis diseases. Other lanes are not relevant to this discussion. (Reproduced from Ref. 63 with permission of Elsevier Press, Inc.)

polar lipids such as hydrocarbons, steryl esters, methyl esters, and mixed glycerides that migrate close to each other in one-dimensional TLC. Thompson (97) used a 2-D system to separate the neutral lipids of the digestive gland-gonad (DGG) complex of the medically important snail *Biomphalaria glabrata*. The first development was in hexane–diethyl ether (80:20); after the plate was dried, it was turned 90° and developed in the second direction in hexanediethyl ether methanol (70:20:10). Figure 5 shows the results of this separation.

2. Phospholipids

Two-dimensional systems are often used to separate complex phospholipid mixtures in plant and animal tissues. See reviews in Mangold (98) and Rouser et al. (99) for details. The first development is typically in chloroform–methanol–water (65:25:4), and development in the second direction is often in either *n*-butanol–acetic acid–water (60:20:20) or chloroform–acetone–methanol–acetic acid–water (5:2.1:1:0.5). Although 2-D procedures may increase the resolution of some spots, they often result in large spots with tails. Figure 6 shows a 2-D separation of phospholipids from snail tissue, and Fig. 7 shows a 2-D separation of serum lipids. Table 7 lists frequently used 2-D solvent systems for complex lipid mixtures.

3. Glycolipids

Glycolipids are often difficult to separate completely by one-dimensional TLC because of their complex array of oligosaccharides. Therefore, a variety of 2-D TLC procedures have been devised to separate individual glycolipids and phospholipids on the same plate. Glycolipids of plant and bacterial origin are usually separated on silica gel G using the 2-D system first described by Nichols (100).

The solvent system in the first direction is chloroform–methanol–7 N ammonium hydroxide (65:30:4), with chloroform–methanol–acetic acid–water (170:25:25:6) in the second direction. Excellent separation is obtained for neutral lipids, monogalactosyldiacylglycerols, cardiolipin, phosphatidic acid, sterol glycosides, ceramide monohexosides, phosphatidylethanolamine; phosphatidylglycerol, digalactosyldiacylglycerols, sulfoquinovosyldiacylglycerols, phosphatidylcholine, and phosphatidylinositol. An additional 2-D system used for the separation of glycolipids

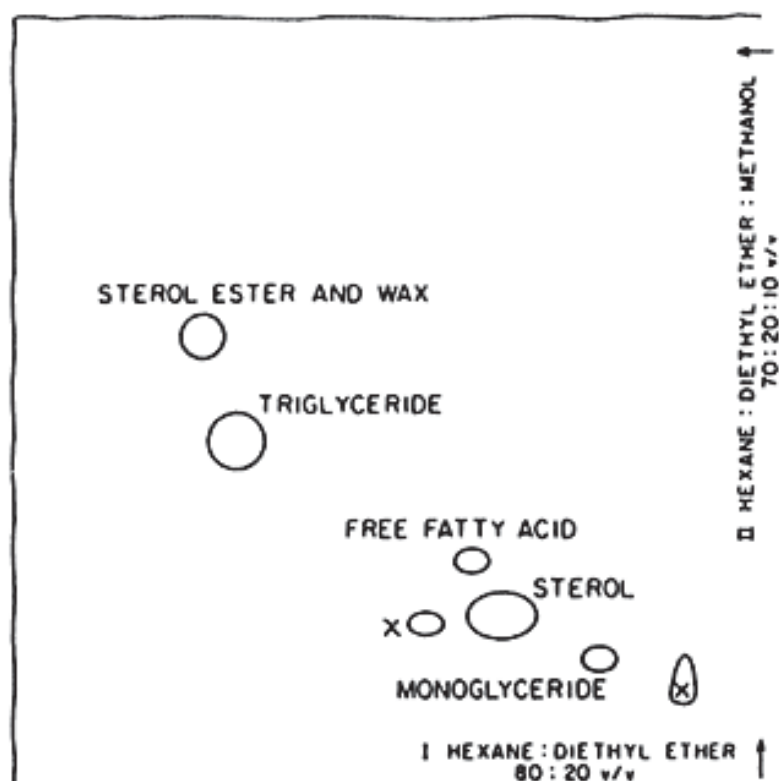


Figure 5 Drawing of a thin-layer chromatogram of the neutral lipids from an extract of the digestive gland–gonad (DGG) complex of *Biomphalaria glabrata* snails. The silica gel G plate was developed in the first direction in hexane–diethyl ether (80:20) and in the second direction in hexane–diethyl ether–methane (70:20:10). Lipids were detected by spraying the plate with 50% H_2SO_4 and then charring it at 150°C for 5 min. (Reproduced from Ref. 97 with the permission of Pergamon Press, Ltd.)

and phospholipids on silica gel H plates is that by Gray (102), which uses chloroform–methanol–water (56:25:4) in the first direction and tetrahydrofuran–methylal–methanol–water (10:6:4:1) in the second direction.

4. Gangliosides

As noted for glycolipids, because of the diversity of oligosaccharides associated with gangliosides, it is usually difficult to achieve complete separation of these complex lipids using one-dimensional TLC. Various maps have been prepared in which ganglioside species have been separated using the 2-D system of Ohashi (101). She used HPTLC plates and the solvent system chloroform-methanol-0.2% aqueous CaCl_2 (60:35:8) in the first direction and *n*-propanol-water-28% aqueous ammonia (75:25:5) in the second direction (Fig. 8). Her system allowed for separating gangliosides with elongated saccharide chains. Combining 2-D TLC with autoradiography also allows for the resolution and detection of numerous minor gangliosides (103).

Authors who provide 2-D maps of gangliosides typically use the nomenclature of Svennerholm (104). In this system, the subscript M, D, or T is used to indicate mono, di, or trisialogangliosides, respectively. Additionally, a numerical subscript is used to indicate the structure of the oligosaccharide backbone to which the sialic acid residues are attached. For further discussion of this topic, see Fishman et al. (105).

VI. DETECTION

Following development of the TLC plate, lipids can be visualized by a wide variety of chromogenic or fluorescent detection reagents. These reagents are usually sprayed on the plate or applied

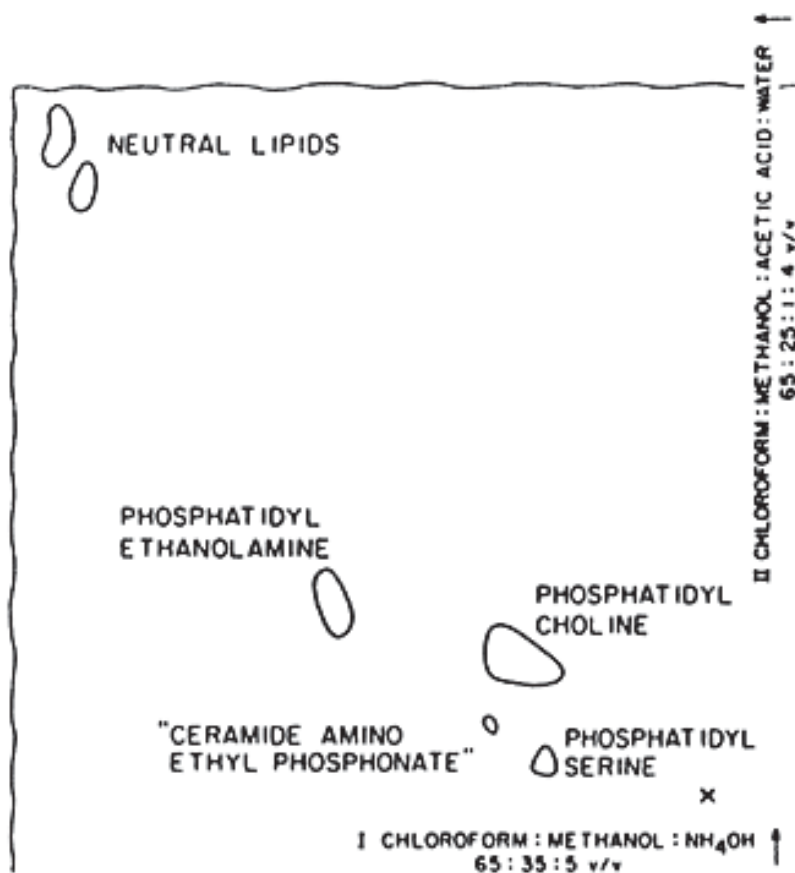


Figure 6 Drawing of a thin-layer chromatogram of phospholipids separated from an extract of the digestive gland–gonad (DGG) complex of *Biomphalaria glabrata* snails. The silica gel G plate was developed in the first direction in chloroform–methanol–NH₄OH (65:35:5) and in the second direction in chloroform–methanol–water–acetic acid (65:25:4:1). Phospholipids were detected by using a variety of specific phospholipid detection reagents. (Reproduced from Ref. 97 with the permission of Pergamon Press, Ltd.)

by dipping the plate into a suitable chamber containing the reagent. Methods used to detect compounds have been described in detail by Fried and Sherma (106, 106a).

Detection techniques may be nondestructive or reversible, e.g., iodine or Rhodamine B, or destructive, e.g., sulfuric acid. Detection reagents are usually classified as general, i.e., those that react with a wide variety of different compound types, versus specific, i.e., reagents that indicate a particular compound or functional group. Some general reagents are destructive, and others are not; likewise, there are destructive and nondestructive specific chemical detection reagents.

A voluminous literature is available on both general and specific detection reagents. Table 8 on nonspecific detection reagents for lipids and Table 9 on specific detection reagents have been compiled from numerous sources including Stahl (107), Kirchner (108), Zweig and Sherma (109), and Ando and Saito (63).

VII. QUANTIFICATION

Various methods have been described for the quantification of lipids using TLC. The methods can be divided into two categories: (1) scraping and elution of lipids from the TLC plate and (2) direct in situ quantification, usually by densitometric methods. The period 1979–2001 saw considerable activity in the area of in situ densitometry of lipids, and an extensive literature is available. See the latest critical review on thin-layer and paper chromatography by Sherma (110c) for recent literature on this subject.

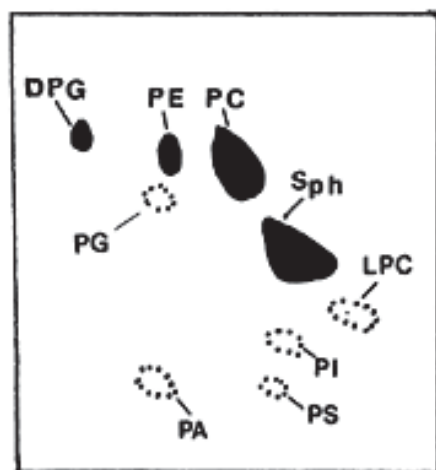


Figure 7 Two-dimensional TLC separation of serum lipids. The TLC plate was developed in the first direction with chloroform–methanol–ammonia (65:25:5) and in the second direction with chloroform–acetone–methanol–acetic acid–water (30:40:10:10:0.5). Abbreviations are as follows: LPC, lysophosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; DPG, diphosphatidylglycerol (cardiolipin); Sph, sphingomyelin. (Redrawn with permission of Supelco, Inc., Bellefonte, PA, from *Supelco Handbook of Lipids and Selected Carbohydrates*, 7th ed., 1981, p. 48.)

Table 7 Recommended Solvent Systems for Two-Dimensional Separations of Complex Lipids^a on Silica Gel

System no.	Direction	Solvent composition and ratio	Comments	Ref.
1	First	Chloroform–methanol–water (65:25:4)	Systems 1 and 2 are good for separation of complex lipids of animal tissue on silica gel H; from 10 to 15 complex lipids are separated.	(99)
	Second	<i>n</i> -Butanol–acetic acid–water (60:20:20)		
2	First	Chloroform–methanol–28% aq. NH ₃ (65:35:5)		(99)
	Second	Chloroform–acetone–methanol–acetic acid–water (10:4:2:2:1)		
3	First	Chloroform–methanol–7 N NH ₄ OH (65:30:4)	Good for separation of plant and bacterial complex lipids on silica gel G; from 10 to 15 complex lipids are separated.	(100)
	Second	Chloroform–methanol–acetic acid–water (170:25:25:6)		
4	First	Chloroform–methanol–0.2% aq. CaCl ₂ (60:35:8)	Excellent for separating a wide variety of ganglioside species on HPTLC plates.	(101)
	Second	<i>n</i> -Propanol–water–28% aq. NH ₃ (75:25:5)		

^a“Complex lipids” refers to phospholipids and glycolipids.

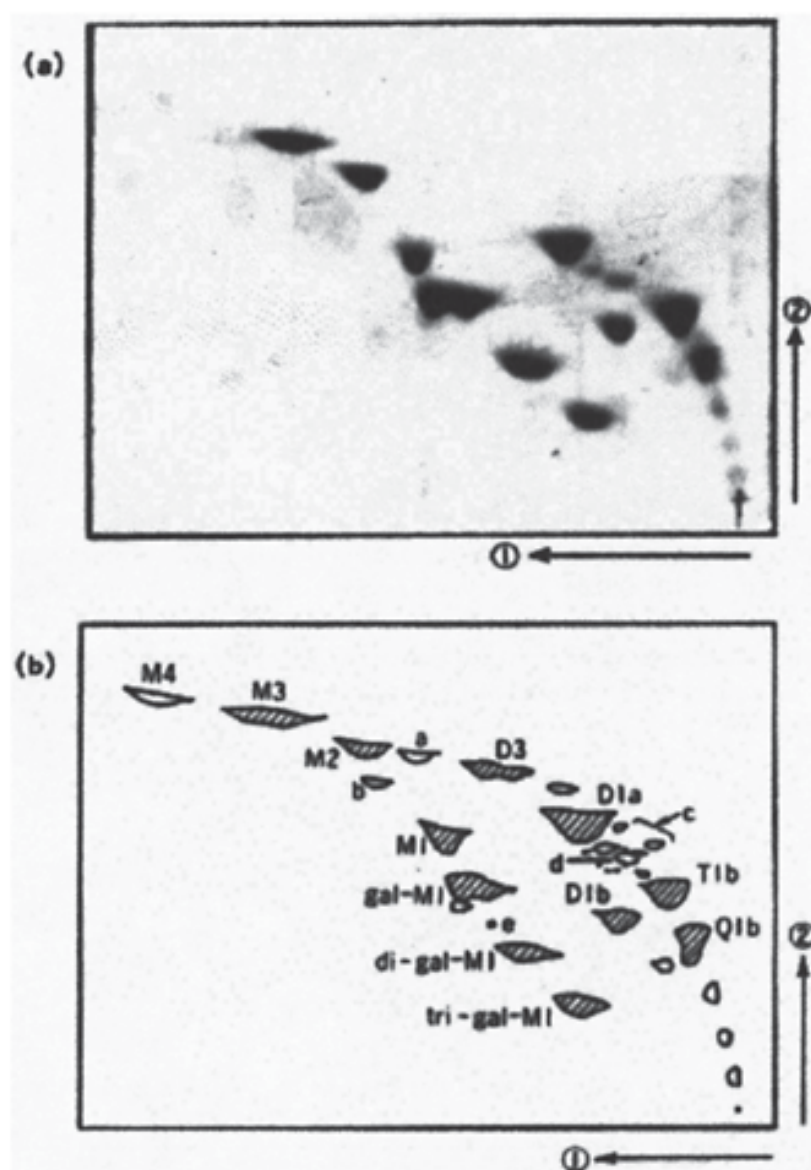


Figure 8 Two-dimensional separation of a mixture of rat brain gangliosides plus authentic standards on an HPTLC plate, (a) Photograph of the chromatogram; (b) tracing. See text for chromatographic conditions and Ref. 63 for a description of the symbols used to designate the major and minor gangliosides separated by TLC. (Reproduced from Ref. 63 with the permission of Elsevier, Inc.)

In method 1 mentioned above, lipids are scraped and eluted from TLC plates and then analyzed by spectrophotometric, gravimetric, chromatographic, or other methods. Scraping and elution procedures are used in preparative layer chromatography (PLC). Because PLC has been reviewed by Sherma and Fried (111), the topic is not considered in this chapter. Prior to scraping and elution of lipids from either an analytical or preparative plate, the bands must be visualized, usually by a nondestructive detection agent such as iodine, water spray, or fluorescent reagent, e.g., fluorescamine or Rhodamine B. Care must be taken to ensure that the detection reagent does not react adversely with the compounds of interest.

Lipids may be quantified by charring with either dichromate or H_2SO_4 and then analyzed by photodensitometry (112). The charring technique may be combined with liquid scintillation, a procedure often used to quantify radiolabeled lipids (113). Phospholipids separated by TLC and then eluted from the plates may be quantified by classical wet chemical determination techniques for phosphorus (114). Similar techniques, although infrequently used, are also available for the

Table 8 Nonspecific Reagents for the Detection of Lipids on TLC Plates or Sheets

Reagent	Procedure	Results
Iodine	Spray as 1% alcoholic solution or place a few crystals in the bottom of a closed tank.	Dark brown spots on a pale yellow or tan background in a few minutes
Rhodamine B	Spray with a 0.05% solution in ethanol.	Violet spots on a pink background
2',7'-Dichlorofluorescein	Spray with a 0.2% solution in 96% ethanol. Observe in UV light.	Saturated and unsaturated polar lipids give green spots on purple background
Coomassie Brilliant Blue	Spray with a 0.03% solution of the Coomassie stain in 20% methanol.	Blue spots on a colorless background
Phosphomolybdic acid	Spray with a 5% solution in ethanol; heat at 100°C for 5–10 min.	Blue-black spots on yellow background
Antimony trichloride	Spray with a 10% solution of SbCl_3 in chloroform; heat at 110°C for 1–2 min.	Various colors on a white background
Potassium dichromate–sulfuric acid	Spray with a 5% solution of potassium dichromate in 40% H_2SO_4 ; heat at 120–180°C for 15–40 min.	Black spots on a colorless background
Sulfuric acid	Spray with 50% aq. H_2SO_4 ; heat as above.	Black spots on a colorless background
Cupric acetate–phosphoric acid	Dissolve 3 g of cupric acetate in 100 mL of an 8% aq. phosphoric acid solution. Heat at 130–180°C for up to 30 min.	Black spots on a colorless background
Sulfuric acid–methanol	Prepare a 50% solution of H_2SO_4 in methanol; spray and heat plates for	Brown-black spots on a colorless background

 several minutes at 100°C.

hydrolysis products of various neutral lipids (46). Gravimetric methods can also be used to quantify lipids separated by TLC, but such methods may be unreliable because small amounts of sorbent, binder, or other impurities may be eluted from the plates and inadvertently weighed (115). Gas-liquid chromatographic procedures can be combined with TLC to estimate natural mixtures of lipids. The samples must first be transesterified along with suitable internal standards (116). Flame ionization detection systems (FID) in combination with TLC have been used in lipid quantification studies (117). The most frequently used system is that of Iatroscan rods or Chromarods from Iatron Laboratories of Japan.

In situ densitometry of lipids is a popular area of research with a large body of literature. Any lipid that can be detected in visible or ultraviolet light is subject to densitometric analysis. Suitable standards are required and should closely match the compounds of interest. The TLC methods for in situ densitometry are similar to those used in conventional TLC, except that more care is needed during sample preparation, spotting and predevelopment of the plate, and choice of mobile phase and detection reagent. The range of weights of the standards spotted on the plate should bracket as closely as possible the weights of the compounds of interest. Densitometry is usually accomplished in the transmittance or reflection mode with a particular commercial den-

Table 9 Specific Chemical Detection Procedures
for Various Lipids

Compound class	Reagent	Procedure	Results
Cholesterol and cholesteryl esters	Ferric chloride	Dissolve 50 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 90 mL H_2O along with 5 mL acetic acid and 5 mL sulfuric acid; spray the plate, then heat it at 90–100°C for 2–3 min.	Cholesterol and cholesteryl esters appear as red-violet spots; cholesterol spot appears before that of the ester.
Free fatty acids	2',7'-Dichlorofluorescein-aluminum chloride-ferric chloride	Prepare three solutions as follows: (1) 0.1% 2',7'-dichlorofluorescein in 95% methanol; (2) 1% aluminum chloride in ethanol; (3) 1% aqueous ferric chloride. Spray the plate in turn with solutions 1, 2, and 3. Warm the plate (about 45°C) briefly between sprays.	Free fatty acids give a rose color,
Lipids containing phosphorus	Molybdic oxide-molybdenum "Zinzadze" reagent	Prepare a 4% solution of molybdic oxide in 70% H_2SO_4 (soln 1); add 0.4 g	Phospholipids appear as blue spots on a white

		powdered molybdenum to 100 mL of solution 1. Add 200 mL H ₂ O and filter. Final spray consists of 100 mL of above +200 mL water and 240 mL acetic acid.	background within 10 min of spraying the plate.
Choline-containing phospholipids (phosphatidylcholine and lysophosphatidylcholine)	Potassium iodide-bismuth subnitrate “Dragendorff” reagent	Prepare a 40% aqueous solution of potassium iodide; prepare a 1.7% solution of bismuth subnitrate in 20% acetic acid; mix 5 mL of the first solution with 20 mL of the second solution and add 75 mL of water; spray the plate, then warm it.	Choline-containing lipids appear in a few minutes as orange-red spots,
Free amino groups (phosphatidylethanolamine and phosphatidylserine)	Ninhydrin	Prepare a 0.2% solution of ninhydrin in <i>n</i> -butanol and add 3 mL acetic acid; spray plate, then heat it in an oven at 100–110°C.	Lipids with free amino groups show as red-violet spots,
Glycolipids	α -Naphthol–sulfuric acid	Prepare a 0.5% solution of α -naphthol in 100 mL methanol–H ₂ SO ₄ (1:1). Prepare a solution of concentrated H ₂ SO ₄ (95:5). Spray plate with the α -naphthol solution; allow to air-dry, then spray with the H ₂ SO ₄ solution. Heat at 120°C until color is maximal.	Glycolipids (cerebrosides, sulfatides, gangliosides, and others) appear as yellow spots; cholesterol appears as a light red spot,
Glycolipids	Orcinol–sulfuric acid	Dissolve 20 mg orcinol in 100 mL of 75% H ₂ SO ₄ . Spray the plate lightly with the reagent, then heat it at 100°C for 15 min.	Glycolipids appear as blue-purple spots against a white background,
Glycolipids versus phospholipids	Iodine	Place iodine crystals in a closed tank; place developed TLC plate in tank until color appears.	Phospholipids stain distinctly, and glycolipids do not.
Gangliosides	Resorcinol	Prepare a 2% aqueous solution of resorcinol. Add 10 mL of this solution to 80 mL of HCl containing 10.5 mL of a 0.1 M CuSO ₄ solution. Spray the plate with this reagent, then heat at 110°C for a few minutes.	Gangliosides appear as a violet-blue color; other glycolipids appear as yellow spots.
Sphingolipids	Sodium hypochlorite–	Add 5 mL of sodium hypochlorite (Clorox) to 50 mL of benzene and	Sphingolipids (ceramides, sphingomyelin

benzidine reagent	dilute with 5 mL of acetic acid. Prepare the benzidine reagent by dissolving 0.5 g of benzidine and 1 crystal of KI in 50 mL ethanol-H ₂ O (1:1). Spray plate with the Clorox reagent and let dry; then spray with the benzidine reagent.	cerebrosides, sulfatides, gangliosides) and lipids with secondary amines produce blue spots on a white background. CAUTION: Benzidine is a carcinogen.

sitometer. Considerable choice exists in the types of densitometers available, from simple models to highly automated instruments coupled to computer systems (118).

By way of an example of in situ densitometry of lipids, the following study is detailed from Morris et al. (87) on the quantification of cholesterol in hen's egg yolk. Because of the availability of hen's egg yolk, this experiment can easily be replicated by chromatographers who seek an introduction to simple in situ lipid densitometric procedures.

A stock cholesterol (99.9% purity) solution was prepared at a concentration of 1 mg/mL in chloroform; the TLC standard was prepared by a 10-fold dilution with chloroform (100 ng/ μ L). Whatman LHPKDF 20 $\times$ 10 cm channeled high-performance silica gel plates with a preadsorbent spotting area were used for quantitative densitometry, and Whatman PK1F 20 $\times$ 20 cm plates with 0.5 mm silica gel layer thickness for preparative TLC. Layers were cleaned by predevelopment with methanol-methylene chloride (1:1) and allowed to air-dry before use. Spots were applied with a Drummond 10 μ L microdispenser. For quantitative TLC, 100–1000 ng of cholesterol standard (1–10 μ L of the 100 ng/ μ L solution) and duplicate 5 μ L portions of the final sample solutions were spotted. Layers were developed in saturated glass tanks and allowed to air-dry. For quantification, lipids were detected on the HPTLC plates with the cupric acetate-H₃PO₄ reagent. Lipid zones were scanned using a Kontes Model 800 densitometer equipped with a Hewlett-Packard Model 3992A integrator/recorder. The sterol fraction from 3 mL of egg yolk (about 12 mg of total lipid) was isolated by preparative TLC and rechromatographed on preadsorbent silica gel plates with chloroform-ethyl acetate (94:6). Cholesterol was detected by the cupric acetate-H₃PO₄ dip reagent as a light brown streak on a faintly blue background with an R_f value of 0.47. Plates were scanned immediately after detection. A typical scan for a series of cholesterol standards in the 100–1000 ng concentration range is shown in Fig. 9. Calibration plots of peak area versus nanograms spotted had a linearity correlation coefficient value of 0.98 or greater. Standards were spotted with samples to provide an individual calibration plot for each plate. Scans of duplicate sample aliquots are also shown in Fig. 9. The cholesterol values of 20 egg yolk samples determined by quantitative TLC ranged from 9.7 to 26.2 mg/g (avg 12 mg/g), which is similar to the values reported in the literature for cholesterol in egg yolk from various birds. See Ref. 87a for an update of this study.

As mentioned previously, an extensive body of literature exists on the quantification of lipids. Table 10 provides selective examples from the 1979–2001 literature on the quantification of both neutral and complex lipids mainly by in situ densitometry.

VIII. SIGNIFICANT STUDIES AND REVIEWS ON THE TLC OF LIPIDS—1990–2001

Pre-1990 reviews on the TLC of lipids were covered by Fried (135) in the first edition of this Handbook. He covered the significant literature through the late 1980s on the TLC and HPTLC

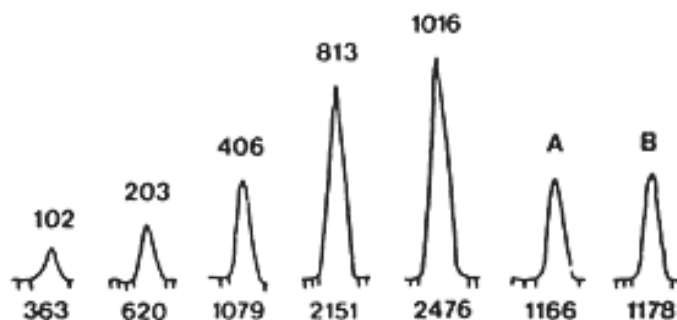


Figure 9 Densitometer scans of 102–1016 ng of cholesterol standards and duplicate 5 μ L aliquots of an egg yolk extract, made at an integrator attenuation setting of $\times 8$. The standard areas (shown below the peaks) had a linearity correlation (R value) of 0.998, and the sample peak areas represent 12.8 (A) and 13.0 (B) mg of cholesterol per gram of yolk. (Reproduced from Ref. 87.)

Table 10 Application of TLC to the Quantitative Analysis of Lipids

Material	Method	Comments	Ref.
<i>Neutral lipids</i>			
Cholesterol in egg yolk	Densitometry	HPTLC silica gel; analysis in 10–100 ng cholesterol range.	87
Neutral lipids in egg yolk	Densitometry	HPTLC silica gel; Mangold solvent system of petroleum ether–diethyl ether–acetic acid (80:20:2) for determination of cholesterol, triacylglycerols, and free fatty acids, and <i>n</i> -hexane–petroleum ether–ethyl ether–acetic acid (50:20:5:1) for cholesterol esters. Linid	

		detection and quantification as described for neutral lipids in <i>Biomphalaria glabrata</i> snails in this table. (Also see Ref. 120a.)	
Free sterols and steryl esters released by parasites	Densitometry	Whatman linear plates with preadsorbent zone; analysis in 50–500 ng range of sterols.	119
Synthetic mixtures of neutral lipids, skin lipids, unsaturated fatty acids, serum lipids	Densitometry	Homemade silica gel plates scored into vertical channels; visualization follows charring at high temperature.	120
Neutral lipids in <i>Biomphalaria glabrata</i>	Densitometry	HPTLC silica gel plate; petroleum ether–diethyl ether–acetic acid (80:20:2) mobile phase; detection by spraying with 5% ethanolic phosphomolybdic acid; lipid zones measured by scanning at 700 nm with a Shimadzu CS 930 TL densitometer operated in the single-beam reflectance mode.	120a
Triacylglycerols in serum	Densitometry	Commercial silica gel plates; visualization follows charring.	121
Natural triacylglycerol and synthetic standards	TLC–flame ionization detection (TLC-FID)	Purified neutral lipids should be used for calibration purposes.	122
Triacylglycerols (medium- and long-chain)	Densitometry–automated multiple development (AMD)	Different classes of lipids separated on a single silica gel 60 HPTLC plate by AMD using a Camag AMD 2.	122a
Fatty acids (dimerized from oils)	TLC-FID	TLC-FID data on the dimerized fatty acids were compared with gas–liquid chromatographic data.	123
Neutral and polar lipid standards	TLC-FID	Alumina (Chromarod A) was as good as silica gel (Chromarod S III) for the separation of neutral lipids, but silica gel produced better separation and higher FID response for polar lipids.	123a
Lipids in cooked beef	TLC-FID	Chromarods S-III (silica gel–coated quartz rods) were used to quantify neutral and polar lipids in cooked beef.	123b
Cholesterol, cholesteryl esters, triacylglycerols, and phospholipids	Densitometry	Nile red solution used for detection of lipids; densitometry by reflectance fluorometry; detection limit of assay was 25–100 ng for each lipid.	124
Triacylglycerols (sunflower oils)	Densitometry	Reversed-phase TLC on silanized kieselguhr layers; identification and quantification of	125

triacylglycerols in sunflower oil.			
Lipids in animal tissue (kidneys and livers of rats)	HPTLC–densitometry	Neutral lipids separated in petroleum ether–diethyl ether–acetic acid; phospholipids in methanol–chloroform– <i>n</i> -propanol–methyl acetate–KCl; visualization by charring with manganese chloride–sulfuric acid; detection limit 20 ng of lipid.	125a
Triacylglycerols in vegetable oils	Densitometry; detection limits of individual components, 0.4 µg	HPTLC of mono-, di-, and triacylglycerols on RP-18 with a mobile phase of dichloromethane–ethyl acetate–methanol–acetic acid (27:22:38:13); detection with PMA.	125b
Material	Method	Comments	Ref.
<i>Phospholipids</i>			
Phospholipids in bile, liver, and plasma	Densitometry	HPTLC plates; detection with an ethanol-modified molybdenum blue reagent.	126
Phospholipid standards	Densitometry	HPTLC plates; quantification used film negatives combined with laser densitometry.	127
Phospholipids in rat brain	Densitometry	HPTLC plates; visualization with molybdenum blue reagent; reflectance densitometric method for simultaneous determination of the individual phospholipids.	128
Phospholipids in human erythrocytes	TLC-FID	Use of Chromarod S-II rods; chloroform–methanol–H ₂ O–acetic acid (45:15:15:0.2) mobile phase; determination made with Iatroscan TH-10 analyzer.	129
Lecithin and sphingomyelin from amniotic fluid	Densitometry	Silica gel plates developed in chloroform–methanol–water (75:25:4); sprayed with phosphomolybdic acid; scanned in densitometer at 450 nm in double-beam transmission mode; detection of each lipid at 0.2 µg level.	130
Rat plasma and liver phospholipids (also neutral lipids)	Densitometry	HPTLC plates; multiple development; detection by charring in dichromate–sulfuric acid; excellent quantitative separation of neutral lipids and phospholipids.	131
Erythrocyte phospholipids	TLC-FID	Multiple development of Chromarods; use of an internal standard, cholesteryl acetate; detector response linear in 0.5–60 µg range; excellent quantitative separation of neutral lipids and phospholipids.	63
Marine bacterial phospholipids	TLC-FID	Elution in chloroform–methanol–water–formic acid (85:30:3:0.2) followed by scanning of the Chromarods.	63a

Phospholipids in <i>Biomphalaria glabrata</i> snails	Densitometry	HPTLC silica gel plates; multiple developments in a chloroform–methanol–isopropanol–0.25% KCl–ethyl acetate (30:9:25:6:18) mobile phase; detection by spraying with 10% cupric sulfate–8% phosphoric acid solution; phospholipids measured by reflectance scanning at 400 nm with a Shimadzu CS-930 densitometer in the single-lane, single-beam mode.	63b
Phospholipids in pharmaceutical drugs (surfactants)	Video densitometry	HPTLC on silica gel F ₂₅₄ plates; chloroform–methanol–acetic acid–H ₂ O mobile phase.	63c
Phospholipids in pharmaceutical products (mammalian brain material)	Densitometry	HPTLC plates; mobile phases optimized using the PRISMA system. Phospholipids separated in methyl acetate–chloroform–1-propanol–methanol–0.25% aqueous KCl.	63d
Nanogram detection of phospholipids in clinical chemistry	Densitometry–automated multiple development (AMD)	HPTLC separation of cardiolipin, phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, and sphingomyelin on silica gel; solvent of chloroform–methanol–2-propanol–triethylamine–0.25% aqueous KCl (60:18:50:31:9). Visualization by spraying with 1,6-diphenyl-1,3,5-hexatriene followed by the molybdenum blue reagent.	63e
Polar and nonpolar lipids (simple method)	Densitometry; reflection mode at 365	HPTLC of nonpolar lipids on silica gel with <i>n</i> -hexane–ether–acetic acid (80:20:1); for polar lipids, chloroform–methanol–water (65:25:4); visualization by exposure to iodine vapor.	63f

Material	Method	Comments	Ref.
Phospholipid products for the pharmaceutical industry	Densitometry at 350 nm; detection limits of 10–100 ng	HPTLC of phospholipids—lysophosphatidylcholine, phosphatidylcholine, phosphatidylglycerine, phosphatidic acid, sphingomyelin, lysophosphatidylethanolamine, phosphatidylinositol, phosphatidylethanolamine—on silica gel with a mobile phase of methyl acetate–chloroform–1-propanol–methanol–0.25% aqueous KCl (25:25:25:10:9). Visualization under UV at 254 and 366 nm and by immersion in an acidic solution of cupric acetate. <i>Glycolipids</i>	63g
Glycolipid standards	Densitometry	HPTLC plates; use of film negatives combined with laser densitometry to analyze complex lipids.	127
Glycosphingolipids	TLC–mass	Complex lipids separated by TLC, permethylated	132

	spectrometry	and analyzed by mass spectrometry; sensitivity allows characterization of 1–5 μg of glycosphingolipid.	
Sphingolipids in the parasitic protozoan <i>Blasto-cystis hominis</i>	Densitometry	Sphingolipids along with neutral lipids and phospholipids were quantified on HPTLC plates; sphingolipids resolved on plates with chloroform–methanol–water (70:22:3) and detected with the orcinol reagent; plates scanned with a Shimadzu Flying Spot densitometer operated in the reflectance mode at 580 nm.	132b
Glycosphingolipids in clinical chemistry research	Densitometry	HPTLC of glycosphingolipids on silica gel with chloroform–methanol–0.2% CaCl_2 (60:35:8). Visualization by spraying with 0.1% 5-hydroxy-1-tetralone in 80% H_2SO_4 .	132c
<i>Gangliosides</i>			
Brain gangliosides	Densitometry	Complex sample preparation; use of HPTLC plates; chloroform–methanol–0.22% CaCl_2 (55:45:10) solvent system; detection by spraying with resorcinol–hydrochloric acid reagent; chromatogram scanned at 580 nm in transmission mode; separation and quantification of 4–8 brain gangliosides.	133
Gangliosides from the cerebral cortex of monkeys	TLC–UV spectrometry	Complex sample preparation; HPTLC silica gel plates; solvent system of acetonitrile–isopropanol–50 mM KCl (10:65:25); gangliosides detected by spraying with a resorcinol–HCl reagent; good resolution of polysialogangliosides.	133a
Brain and extraneural gangliosides	Densitometry	Complex sample preparation; TLC on precoated EM silica gel 60 plates; chloroform–methanol–0.25% CaCl_2 (60:35:8) solvent system; detection with the resorcinol reagent; scanned at 580 nm in the reflectance mode; separation of 4–8 individual gangliosides.	105
Extraneural gangliosides	TLC–enzyme immunostaining	Terminally α -2,3- and α -2,6-sialylated neolacto series gangliosides were selectively detected by immunostaining on TLC plates; procedure useful for detection of amounts down to 10 ng gangliosides.	105a
Gangliosides in cerebrospinal fluid (CSF)	TLC–enzyme immunostaining	Quantitative determination of gangliosides in the CSF of patients with gangliosidosis was accomplished by this combined method.	134

of lipids including studies on neutral lipids, phospholipids, glycolipids, and gangliosides. Coverage included detailed information on sample preparation prior to TLC, selected chromatographic systems for the separation and determination of all major classes of

lipids, and consideration of quantitative TLC of lipids, mainly by use of scanning densitometry. The review has 10 figures, 10 tables, and 135 references. Fried (135a) provided a revised lipid chapter in the second edition of this Handbook to include significant TLC studies from 1990 through 1995; the chapter in the second edition has 173 references.

Fried and Sherma (52a) reviewed TLC methods used to analyze lipids in gastropod molluscs. Wittig and Walker (136) contributed a brief review on the TLC of phospholipids with 19 references. They noted that two-dimensional TLC is a simple, rapid procedure for phospholipid analysis and that the use of markedly different solvent systems in the two dimensions allows for separations that are not readily obtained by HPLC. Ackman (137) included 43 references in a review of newer techniques for the detection of lipids by TLC that supplement the classical scanning methods. These techniques include the use of fiber optics, video imaging, and the Chromarod-Iatroscan TLC-FID approach. Ackman (138) also reviewed the application of TLC to the separation of neutral lipids, citing 47 references. Olsson (139) provided a review with 40 references on recent advances in planar chromatography of food lipids. The review covered densitometry, preparative thin-layer chromatography, and flame ionization detection. Applications were mainly from the areas of dairy, marine, and plant lipids. Nikolova-Damyanova (140) provided a review with 209 references on silver ion chromatography. Silver ion TLC was considered, along with low-pressure silver ion chromatography and silver ion HPLC. Kuksis (141) considered recent advances in TLC in his review on chromatographic analysis of lipids. He noted that HPTLC had emerged as the most satisfactory planar chromatography method for lipids, although Chromarod chromatography was widely used. He also noted that HPTLC is useful for the accurate and sensitive detection of glycolipids by immunoreactivity with appropriate monoclonal antibodies. Fried (77b) contributed a review including 53 references on handling biological materials and prefractionating extracts for lipid analyses. Some TLC systems useful for lipid separations were given, mainly as they related to the efficacy of a particular sample preparation method. Traitler and Jänchen (142) reviewed studies on the analyses of lipids by planar chromatography. Their review has 16 figures, 6 tables, and 43 references. It stresses the usefulness of planar chromatography for analyses of polar and neutral lipids and for the preparative separation of lipid classes for subsequent chromatographic-spectroscopic analyses. The review is particularly pertinent for workers doing lipid analyses in health, food, and cosmetics. Excellent chromatograms and densitograms of representative lipid separations by HPTLC are presented. Fried and Sherma (106) in the third edition of their TLC primer contributed a chapter on lipid planar chromatography including 10 experiments with detailed protocols on various aspects of qualitative and quantitative analyses of lipids by TLC. The chapter has 124 references. Fried and Sherma (106a) included a 38-page chapter on the TLC of lipids in the fourth edition of their introductory primer. In addition to general coverage of the topic, eight experiments on qualitative and quantitative TLC are provided. The chapter has 125 references.

Sherma (110) in his 1994 biennial review on planar chromatography covered the significant TLC literature on lipids from 1991 to 1993. He cited 42 references in the applications section on lipids. His review in 1996 (110a) covered the significant TLC literature on lipids from 1993 to 1995 and included 34 lipid references. His 1998 review

(110b) included the salient literature from 1995 to 1997, also with 34 lipid references. His most recent review (110c) in 2000 covered the literature from 1997 to 1999 and included 29 literature references. All of his biennial reviews have emphasized the fact that lipids continue to be widely analyzed by TLC for a number of reasons, i.e., TLC can easily do class separations of complex mixtures with a wide range of polarities on silica gel and can easily fractionate compounds within classes by using a variety of other types of layers, and compounds are easily detected with a variety of reagents, including phosphomolybdic acid and cupric sulfate.

There have been a number of salient reviews and research articles on TLC of lipids from 1995 to 2001. Tarandjiska et al. (143) separated the molecular species of triacylglycerols from highly saturated plant oils by successive argentation and reversed-phase TLC. Alvarez et al. (144) used HPLC with densitometry to determine dipalmitoylphosphatidylcholine in amniotic fluid as free dipalmitoylglycerol on silver nitrate-modified silica gel HPTLC plates after enzymatic hydrolysis. Bonte et al. (145) separated stratum corneum lipids by automated multiple development (AMD) on HPTLC silica gel plates using an initial isocratic step followed by a 25-step gradient from methanol-water to hexane. Schuerer et al. (146) used densitometric quantification for the separation and analysis of human stratum corneum lipids by sequential one-dimensional TLC with detection by charring. Watanabe and Mizuta (147) detected glycosphingolipids from biological samples by TLC at the 5 pmol level using 5-hydroxy-1-tetralone as fluorescent labeling reagent. Wiesner and Sweeley (148) characterized a complex mixture of gangliosides from human plasma using two-dimensional TLC, resorcinol detection reagent, and computer-assisted image analysis densitometry.

Davani and Olsson (149) developed an HPTLC method for the detection of natural galacto-lipids of oats and wheat origin with 8-anilino-1-naphthalenesulfonate (ANS) as a fluorogenic visualization agent and scanning at an excitation wavelength of 375 nm. Arnsmeier and Puller (150) detected gangliosides by TLC and noted that the process was simplified and made more sensitive by the use of chemoluminescence. Touchstone (151) provided a general review of TLC procedures for lipid separation. His review included 128 references on TLC separation of lipids, including sample preparation and TLC studies with examples of applications indicating the capabilities and practicability of TLC analysis for lipids.

Rabinowitz (152) used silica gel TLC to analyze lipids in the saliva of the medicinal leech *Hirudo medicinalis*. The total lipid content of the saliva was about 3 mg of lipids per 100 mL of saliva. Neutral lipids made up about 67% of the lipids, with polar lipids making up the remainder. TLC was used to determine the profiles of polar and nonpolar lipids. The largest percentages of the identified lipids were phosphatidic acids and free fatty acids. This leech contains a unique lipid distribution in its saliva, and some of these components are important constituents in the anticoagulants present in the saliva.

Conaway et al. (153) used HPTLC with densitometry to examine the effects of restricted food intake versus ad libitum feeding on the neutral lipid content of the medically important planorbid snail *Biomphalaria glabrata*. The results of the study indicated that snails on the re-restricted diet had significant changes in various neutral lipids compared to snails maintained on the ad libitum diet. Rupcic et al. (154) used silica gel TLC to analyze cell lipids of *Candida lipolytica* yeast grown on methanol. The dry cell mass was 5% lipids, 52% of which were polar lipids, mainly phospholipids and

sphingolipids. A high content of the sphingolipid 19-phytosphingosine was found (about 91% of the total long-chain bases). Iwamori et al. (155) described a sensitive method to determine the pulmonary surfactant lecithin/sphingomyelin ratio in human amniotic fluid for the diagnosis of respiratory distress syndrome (RDS) by TLC-immunostaining. The method distinguished the surfactant lecithin/sphingomyelin ratios in normal amniotic fluid from women who delivered babies with RDS syndrome.

Xu et al. (156) described two efficient systems to separate phospholipids and lysophospholipids from the hippocampus region of the rat brain. They found that a chloroform-methanol-acetic acid-acetone-water (35:25:4:14:2) mobile phase was suitable for the separation of 10 phospholipids on a silica gel G plate and that a chloroform-methanol-28% aqueous ammonia (65:35:8) mobile phase also gave good separation of the major phospholipids from their lyso forms. Gennaro et al. (157) used HPTLC to determine phospholipids in snail-conditioned water (SCW) from *Helisoma trivolvis* and *Biomphalaria glabrata* snails. SCW contains pheromones that function to attract larval trematodes to the snails and to facilitate intraspecific attraction and mating behavior. In this study, lipids were extracted from the water in chloroform-methanol (2:1), and extracts and standards were applied to silica gel plates developed in chloroform-methanol-water (65:25:4). Lipids were detected by spraying the plates with 10% cupric sulfate in 8% phosphoric acid and heating, and the zones were quantified by scanning densitometry at 370 nm. The major concentrations of phospholipids in SCW were phosphatidylethanolamine and phosphatidylcholine at concentrations ranging from 0.18 to 0.4 $\mu\text{g/mL}$ per snail.

Maloney (158) reviewed studies on TLC in bacteriology and included methods for sample preparation of lipids and TLC protocols for work on bacteria. TLC is used to determine microbial composition, to study microbial lipases, to identify microbial strains, and to determine host lipids that function as receptors for microbial pathogens. His chapter contains 64 references and two figures. Fried and Haseeb (159) reviewed TLC studies on analysis of neutral lipids, phospholipids, and glycolipids in protozoan and helminthic parasites. They listed selected methods for the TLC analysis of lipids in animal parasites.

Fell (160) reviewed TLC and HPTLC studies in entomology that had been used for the separation and identification of insect lipids and to preparatively isolate lipids for use in other analytical procedures. Lipid analysis of insects includes studies on hemolymph, body tissue, and cuticular lipids. Weldon (161) reviewed TLC studies on skin secretions of vertebrates. The work provides information on obtaining lipid samples from the skin and exocrine glands of vertebrates, on lipid sample preparation, and on chromatographic systems and detection reagents useful for the analysis of nonpolar and polar lipids. Jain (162) reviewed TLC studies on lipids in clinical chemistry, including methods for the analysis of human serum, cutaneous lipids (mainly sebum), and lipids from patients with severe alcoholism. TLC is useful in the analysis of amniotic fluid to determine the lecithin (phosphatidylcholine)/sphingomyelin ratios in children who suffer from respiratory distress syndrome (RDS). Hammond (163) provided an interesting review on all aspects of the analysis of lipids. Pages 45–57 of his review are devoted to qualitative and quantitative aspects of TLC. Muething (164) reviewed TLC analysis of gangliosides, and included 234 references. The review includes basic techniques for the separation of

glycosphingolipids, new approaches for using continuous and multiple development, several preparative TLC methods, and TLC-immunostaining techniques.

Hammond (165) reviewed chromatographic analysis of lipids and covered TLC, GC, and HPLC. The chapter has 184 references, one-third of which are related to TLC. There are three line drawings showing lipid separations in various mobile phases on silica gel plates with and without treatment in silver nitrate. Fried (135a) reviewed TLC studies on neutral lipids, phospholipids, glycolipids, and gangliosides and included coverage on sample preparation, selected chromatographic systems, and densitometric TLC. The review has nine figures and 143 references.

Frazer et al. (166) used silica gel HPTLC to identify neutral lipids and phospholipids in the freshwater snail *Lymnaea elodes*. The mobile phase for neutral lipids was petroleum ether–diethyl ether–acetic acid (40:20:1), and zones were detected with 5% ethanolic phosphomolybdic acid; quantification was by densitometry at 700 nm. The major neutral lipids and their mean percentage weights were triacylglycerols (0.11%), free sterols (0.50%), and free fatty acids (0.18%). The phospholipids were separated in chloroform–methanol–water (65:25:4) and detected by spraying with 10% cupric sulfate in 8% phosphoric acid with quantification by densitometry at 370 nm. The major phospholipids were phosphatidylcholine (0.45%) and phosphatidylethanolamine (0.34%). In a companion study, Frazer et al. (167) used HPTLC to identify neutral lipids in the intestinal trematode *Echinostoma caproni* from experimentally infected ICR mice fed a high fat diet of hen's egg yolk compared with worms from mice fed a standard laboratory diet. Significantly greater amounts of phosphatidylcholine and phosphatidylethanolamine were found in worms from mice on the high fat diet at 2 weeks post-infection. The results of their study suggested that the host diet influenced the lipid content of *E. caproni* adults.

Ruiz and Ochoa (168) described a one-dimensional TLC procedure to quantify phospholipids and neutral lipids in the subnanomolar range. The procedure was used with clinical research samples, and TLC was performed on EDTA-impregnated silica gel plates after preconcentration with chloroform-methanol-water (60:40:10) followed by five stepwise developments: (a) chloroform–methanol–water (65:40:5) to 2 cm; (b) ethyl acetate–2-propanol–ethanol–chloroform–methanol–0.25% KCl (35:5:20:22:15:9) to 5 cm; (c) toluene–diethyl ether–ethanol (60:40:3) to 7.5 cm; (d) *n*-heptane–diethyl ether (94:8) to 10.5 cm; and (e) *n*-heptane to 12.5 cm. The lipids were charred by dipping the plates in a solution of 10% cupric sulfate in 8% phosphoric acid for 10 s and heating at 200°C for 2 min. Lipids were quantified by densitometry with an image analyzer in the transmission mode.

Bodennec et al. (169) described a 2-D TLC procedure for the simultaneous separation of ceramide and diacylglycerol species. Two-dimensional TLC was used to separate 2-diacylglycerol and 1,3-diacylglycerols and ceramide-containing hydroxy and normal fatty acids on silica gel by using chloroform–methanol (10:1) in the first direction and hexane–ethyl ether–acetic acid (80:20:1) in the second direction. The compounds were visualized with the Dittmer and Kaster reagents.

Albrecht et al. (170) used HPTLC with densitometry to study the effects of *Echinostoma caproni* (Trematoda) infection on the polar lipid content of the intestinal mucosa of experimentally infected ICR mice. The major phospholipids detected in both infected and noninfected mucosa were phosphatidylcholine (PC) and

phosphatidylethanolamine (PE). There was a significant decrease in the weight of both PC and PE in the intestinal mucosa of infected mice compared to the uninfected controls. Cerebrosides and sulfatides, but not sphingomyelin, were identified in the intestinal mucosa of both infected and uninfected hosts. The pathobiochemical changes in the polar lipid content of infected hosts probably reflect feeding and behavioral activities of these intestinal parasites in the mouse intestine. Kulkarni et al. (171) used one-dimensional TLC to examine the glycolipid composition of some Indian linseed (*Linum unitatisum* in the family Liliaceae) varieties. The seeds were extracted in chloroform-methanol (2:1) to yield total lipids (42–46%). These were separated into neutral lipids (88–90%), glycolipids (6–7%), and phospholipids (4–6%) by silicic acid column chromatography. The glycolipids were separated by one-dimensional TLC into the following individual components: monogalactosyl-diacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), acylsterylalactoside (ASG), and sterylalactoside.

Young et al. (172) used HPTLC to determine lipids from the cloacal scent gland of the Eastern diamondback rattlesnake, *Crotalus adamentus*, and the Florida cottonmouth, *Agkistrodon piscivorus*. The secretions of both species contained free sterols, triacylglycerols, phosphatidylcholine, and phosphatidylethanolamine; methyl esters were present only in samples from *C. adamentus*, and there was no evidence of monoacylglycerols, diacylglycerols, or alkylglycerols in either species. The possible role of cloacal scent gland lipids in defensive behavior was discussed. Yamashero et al. (173) used HPTLC to analyze lipids in 15 different species of cnidarians, most of which were species of coral from Okinawa, Japan. Neutral lipids consisted of free sterols, sterol esters, triacylglycerols, and monoalkyldiacylglycerols. The authors concluded that sterol composition may be useful in the biochemical classification of cnidarians. Nagyova and Tiffany (174) used TLC to show that polar lipids (mainly phosphatidylcholine and sphingomyelin) are important components responsible for the surface tension of human tears. Lee et al. (175) used HPTLC on silica gel to analyze neutral lipids in the ceca of mice and domestic chicks and in the ceca of chicks infected with a trematode of veterinary and wildlife significance, *Zygocotyle lunata*. The trematode altered the normal lipid pattern of the ceca, suggesting that it had a pathobiochemical effect on the host. The solvent system used was petroleum ether-diethyl ether-acetic acid (80:20:1), and lipids were detected by spraying the plates with 5% phosphomolybdic acid in ethanol and heating for 20 min at 110°C. Quantification was by densitometry at 700 nm.

Muller et al. (176) used HPTLC as described in Lee et al. (175) to examine the pathobiochemical effects of larval trematode parasitism on the marine snails *Ilyanassa obsoletus* and *Littorina littorea*. Parasitism altered the neutral lipid profiles of the snails. Muller et al. (177) used the procedures described in Lee et al. (175) to determine quantitatively by HPTLC various neutral lipids in the cercarial stages of two echinostome (flatworm) parasites. The function of lipids in larval trematodes was discussed.

Snail-conditioned water (SCW) provides a source of pheromones to attract larval trematodes to snails and for intra- and interspecific aggregation and mating responses of snails. Muller et al. (178) used HPTLC to examine the presence of various neutral lipids and phospholipids in snailconditioned water from *Lymnaea elodes*. In addition to the usual chromatographic systems used [see Lee et al. (175)] the mobile phase of *n*-hexane-petroleum ether-diethyl ether-acetic acid, 50:20:5:1, was used to determine the presence

of cholesteryl esters. The paper provided presumptive evidence of lipophilic pheromones released by the *L. elodes* snails.

Nikolova-Damyanova (179) reviewed quantitative TLC studies of triacylglycerols. She discussed the efficiency of silver ion and reversed-phase TLC in the analysis of triacylglycerols, including experimental conditions for the conversion of these techniques into full-scale quantitative analytical methods. The review has 43 references.

Marsit et al. (180) used HPTLC to determine neutral lipids in various larval stages of the paramphistomid trematode *Zygocotyle lunata*. They provided quantitative data on free sterols, triacylglycerols, and free fatty acids on a per organism basis for various larval stages. They found a significant reduction in the quantity of free sterols and free fatty acids in the encysted meta-cercarial stage compared to the cercarial stage, suggesting that these neutral lipids are used in some way during transformation from cercaria to metacercaria. Cline et al. (181) used HPTLC to study neutral lipids and phospholipids in the economically important marine intertidal snail *Cerithidea californica* infected with three species of larval trematodes. Infection altered the lipid patterns of the snail host; TLC analysis of lipids can be useful in chemotaxonomic studies of snails infected with different species of larval trematodes.

Sphingolipids are implicated in various cellular events such as growth, differentiation, and apoptosis. Bodennec et al. (182) described a procedure to fractionate sphingolipid classes from fish gills and human melanoma tissue by solid-phase extraction (SPE) on aminopropyl cartridges. Individual lipids in the SPE fractions were then identified by chromatography in several TLC systems.

Fried (183) provided a brief but concise review of TLC of lipids. It contained four line drawings of typical TLC separations, eight essential tables related to salient features of the topic, and nine selected references.

Fried et al. (184) used HPTLC to study the lipid content in the digestive gland-gonad complex (DGG) of *Biomphalaria glabrata* snails infected with *Schistoma mansoni* and maintained on either a Romaine lettuce diet or a high fat diet of hen's egg yolk. The HPTLC analysis of neutral lipids showed that the DGG of infected snails fed the yolk diet contained significantly greater amounts of free sterols and cholesteryl esters but not triacylglycerols than that of the infected snails fed the lettuce diet.

Eidam et al. (185) used HPTLC to determine the concentration of lipids in *Biomphalaria glabrata* snails fed the leafy portion of Romaine lettuce versus the midrib portion. HPTLC was also used to analyze the concentrations of lipids in the two diets. The concentrations of lipids were significantly higher in snails fed the leafy diets; likewise, the concentrations of lipids were higher in the leafy portion than in the midrib portion. Muller et al. (186) used HPTLC to examine the effects of adult *Schistosoma mansoni* infection on the neutral lipid profile of experimentally infected laboratory mice. They found that the triacylglycerol and cholesteryl ester levels in the liver and ileum of the mice decreased significantly as the infection progressed. Hossain et al. (187) used HPTLC to study the structural analyses of glycolipids from *Borrelia burgdorferi*, the causative agent of Lyme disease. Lipids made up about 25–30% of the dry cell weight. HPTLC allowed for the separation of lipids into 11 components. Staining of the components revealed two glycolipids and two phospholipids. The glycolipids composed about 50% the total lipids and had only galactose and monosaccharide constituents.

Pintea et al. (188) reported that sea brickthorn (*Hippophae rhamnoides* of the family Elaeagnaceae) fruits contain abundant lipids in the fleshy mesocarp but that data on their polar lipids are not available. They noted that polar lipids play important structural and physiological roles in cell membranes and may be useful as emulsifiers and nutrients in cosmetic applications. Polar lipid information was obtained from *H. rhamnoides* fruit by the use of HPTLC and other analytical techniques.

Intercellular lipids in the stratum corneum are responsible for the barrier function of mammalian skin. The main components of stratum corneum lipids are ceramides, cholesterol, and free fatty acids. Wertheim and Ponec (189) developed a method to determine human stratum corneum lipid profiles by tape stripping in combination with HPTLC. Vietzyke et al. (190) used HPTLC to investigate human stratum corneum ceramides. They noted that the stratum corneum requires ceramides, cholesterol esters, and fatty acids to provide a cutaneous permeability barrier. They combined HPTLC and other analytical techniques for detailed ceramide analysis.

IX. CONCLUDING REMARKS

This chapter has examined the more important aspects of qualitative and quantitative TLC of lipids, particularly as related to the various classes of neutral lipids, phosphoglycerides, glycolipids, and gangliosides. Most attention has been paid to the separation and identification of lipids at the class level. Although some work on the analysis of the molecular species of lipids is available (46), TLC is not a primary method for such analyses. Molecular species analysis has not been considered herein.

The chapter has examined advantages of TLC, definitions, structure, occurrence, function, sample preparation, sorbents, mobile phases, usual modes of development, and detection procedures for lipids. Although a discussion of 2-D lipid analysis has been provided, mention was not made of the newer technique of "multiphase TLC," in which components are separated in two different directions according to different parameters, e.g., conventional silica gel in one direction and reversed phase in the other direction. Ritchie and Jee (191) used this technique for the analysis of triacylglycerols.

Quantification of lipids mainly by in situ densitometry has been described, and a detailed description has been provided of this procedure from the work of Morris et al. (87) on the quantification of cholesterol in hen's egg yolk. In situ quantification techniques continue to become more automated and will be used more frequently in the future for lipid analyses in clinical, industrial, and research labs.

Poole (192) provided some insight into how TLC will be practiced in the future. Although his review is not specific to lipid TLC, many of his remarks are appropriate to this chapter. He emphasizes the complementary features of thin-layer and column chromatographic separations. Some reasons for selecting TLC for a particular lipid analysis are that it uses a disposable stationary phase and provides simultaneous parallel separations and observations of all the sample components in the chromatogram. Poole noted that there are future prospects for improved separation performance in TLC using zone refocusing force-flow and electro-osmotic flow methods; also, it may be possible to increase zone capacity by using two-dimensional development coupled with column chromatography. Advances in coupling TLC with spectroscopic methods for structural

elucidation of lipids were also considered by Poole. For a prediction of how TLC will be practiced in the future, see Poole (192).

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Lipophilic Vitamins**Alina Pyka***Silesian Academy of Medicine, Sosnowiec, Poland***I. INTRODUCTION**

Vitamins are defined as biologically active organic compounds, controlling agents that are essential for an organism's normal health and growth, not synthesized within the organism, available in the diet in small amounts, and carried in the circulatory system in low concentrations to act on target organs or tissues. Vitamins are classified according to their solubility in water and in fats. Lipophilic vitamins are vitamins A, D, E, and K. Chromatography is useful in the identification and determination of vitamins in pharmaceutical preparations, the identification and determination of vitamins and related substances in natural materials and foodstuffs, and the chemical and biochemical determination of vitamins and their metabolites in fats and tissues. The isolation of the vitamins, their metabolites, and related substances from natural material is the most difficult task (1–4). Vitamins that are soluble in fat (lipophilic vitamins) are the object of wide investigations because of their biological properties. HPLC, TLC, and GC are the principal techniques used for the qualitative and quantitative investigations of lipophilic vitamins. Analysis of lipophilic vitamins by liquid chromatography (TLC and HPLC) is the subject of many scientific publications (1–18).

Generally TLC is useful for the investigation of a wide range of lipophilic vitamin applications, i.e., purification of samples, qualitative detection, quantitative determination, and the use of new visualizing agents and also for the separation of some optical isomers. The aim of this chapter is to present selected works that describe the analytical separation of lipophilic vitamins by means of TLC.

II. VITAMIN A**A. Introduction**

Physiological forms of vitamin A include retinol (vitamin A₁) and its esters, 3-dehydroretinol (vitamin A₂) and its esters, retinal (retinene, vitamin A aldehyde), 3-dehydroretinal (retene-2), retinoic acid, neovitamin A, and neo-b-vitamin A₁. Active

analogues and related compounds known as vitamins A are α -, β -, and γ -carotene; neo- β -carotene B, cryptoxanthine, myxoxanthine, torularhodin, aphanicin, and echinenone (19). Kitol, xanthophyll, and others are inactive analogues of vitamin A (19).

Vitamin A supports the formation of the cells of the skin and is essential to the process of vision. It is involved in the viability of the reproductive system by acting as a hormone and regulating the expression of specific genes. Good sources of vitamin A are fish liver oil from cod, salmon, halibut, and shark; chicken; eggs; milk; cheese; butter; and liver (see Table 1). Vitamin A occurs as retinyl esters in foods of animal origin.

Table 1 General Characteristics of Vitamin A₁ (Retinol)

Molecular weight	286.4
Requirements	1.7–2.7 mg
Chirality	–
Achirality	+
Melting point	63–64°C
Boiling point	120–125°C
λ	325 nm
Occurrence	Apricots, peaches, broccoli, carrots, endive, spinach, fish oil, egg milk, mushrooms

Vitamin A₁ is susceptible to oxidation and degradation. Therefore, control of the vitamin A level in foodstuffs is recommended. Foods of plant origin do not contain vitamin A but are rich sources of provitamin A. About 50 of the 500 known carotenoids can be converted to vitamin A. β -Carotene is the most important of all the carotenoids that occur in nature. β -Carotene (provitamin A), precursor of vitamin A, is found in plants. Good sources are yellow-orange fruits and vegetables and green leafy vegetables (carrots, apricots, collard greens, sweet potatoes, spinach, mangos, mustard greens, turnips, and sweet red peppers) (see Table 1). Both vitamin A and carotenoids with and without provitamin A activity appear to show anticancer effects. Vitamin A plays an essential role in protecting the body from infection. The earliest symptom of vitamin A deficiency is night blindness (20, 21). β -Carotene is a very effective antioxidant and is suspected to reduce the risk of cancers known to be initiated by the production of free radicals (22, 23). β -Carotene reduces the risk of lung cancer in smokers. Therefore, the separation and determination of these compounds are important. Structures of vitamin A and related compounds as well as β -carotene are shown in Fig. 1.

B. Basic Investigations of Vitamin A and Its Derivatives and β -Carotene

On silica gel thin-layer chromatography (TLC) plates eluted with a mixture of cyclohexane–ethanol (97:3), vitamin A₁ alcohol and *retro*-vitamin A₁ alcohol (R_f values

0.10 and 0.09, respectively) are separated from the pair vitamin A₁ acetate and *retro*-vitamin A₁ acetate (R_f values 0.48 and 0.45, respectively), vitamin A₁ palmitate (R_f value 0.78), and anhydrovitamin A₁ (R_f 0.87) (24). Similar investigations were presented in 1964 by Varma et al. (25). Geometric isomers of retinol are separated on silica gel plates using hexane-ether (50:50) as mobile phase. *Syn* and

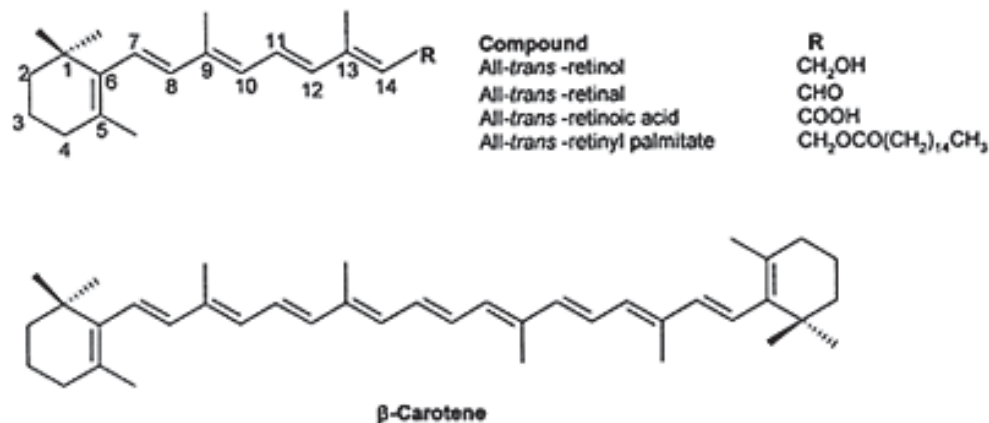


Figure 1 Structure of vitamin A, related compounds, and β -carotene.

anti forms of retinal oximes are separated from each other by using cyclohexane–toluene–ethyl acetate (50:30:20) as mobile phase (1, 2). On magnesium hydroxide plates eluted with benzene, retinol (R_f 0.29) is separated from retinal (R_f 0.61) and retinyl acetate (R_f 0.75). On magnesium oxide layers, and eluted with petroleum ether (boiling point 90–110°C)-benzene (50:50) as the solvent, the carotenes are separated; ϵ -carotene, α -carotene, β -carotene, δ -carotene, and γ -carotene (R_f 0.70, 0.66, 0.49, 0.20, and 0.11, respectively) (4). In partition TLC on kieselguhr G, plates impregnated with paraffin oil (8% paraffin oil in petroleum ether) and eluted with acetone–methanol–water (50:47:3), cryptoxanthin, echinenone, torularhodin methyl ester, and β -carotene (R_f 0.91, 0.69, 0.57, and 0.22, respectively) are separated (4).

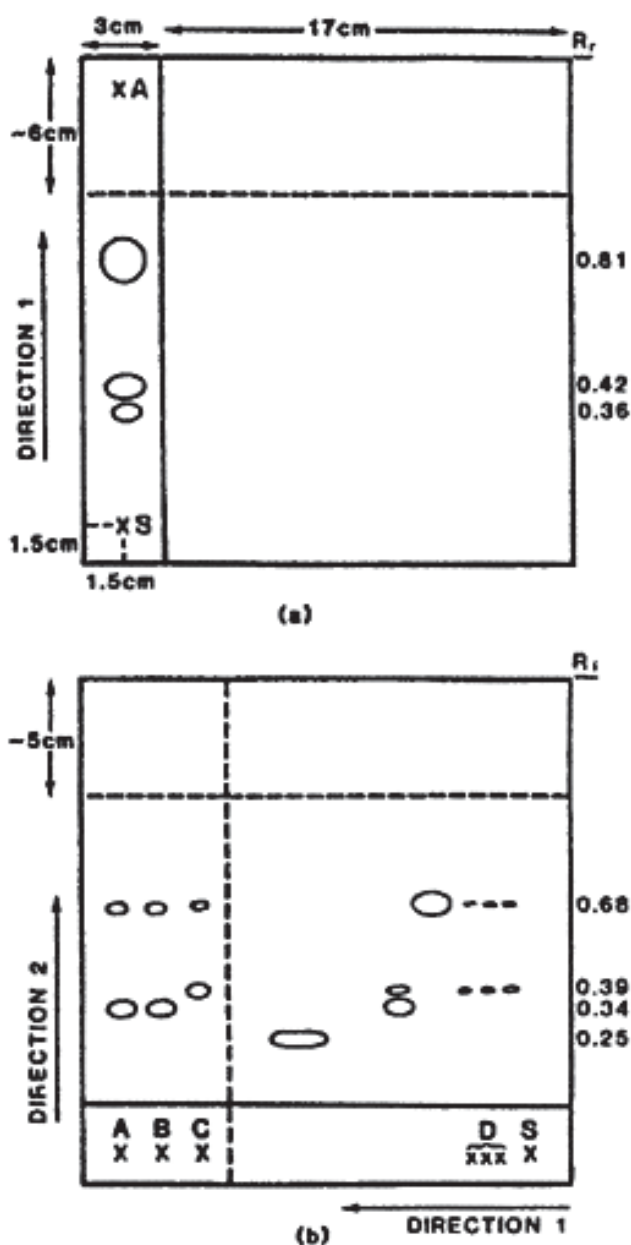
Two separate methods for all-*trans*- and 13-*cis*-retinoic acids, one for gel samples and one for cream samples, were described by De Paolis (26). A methanol extract of the gel formulation may be analyzed directly on high-performance thin-layer chromatography (HPTLC) silica gel plates eluted with diethyl ether–cyclohexane–acetone–glacial acetic acid (40:60:2:1). This method gives fast and complete resolution of the two isomers with a detection limit of about 20 ng for each of them. Methanol extracts of the cream samples contain interfering excipients, which require precleaning prior to chromatography. This was accomplished conveniently on a reversed-phase C₁₈ and normal-phase silica gel two-dimensional TLC plate. The C₁₈ reversed-phase separated the cream excipients from the isomers of retinoic acid using ethanol-distilled water (80:20) as mobile phase. The normal phase on silica gel resolved both isomers, all-*trans*- (R_f 0.34) and 13-*cis*-retinoic acid (R_f 0.39). This two-dimensional separation of all-*trans*- and 13-*cis*-retinoic acid is shown in Fig. 2. McKenzie et al. (27) purified the labeled retinoic acid by thin-layer radiochromatography and HPLC. TLC was performed on silica gel or cellulose plates using several mobile phases. A major impurity, present in ¹⁴C- and ³H-labeled retinoic

acid, was also isolated, characterized, and tentatively identified as an epoxide of retinoic acid.

Groenendijk et al. (28) separated several geometric isomers of retinol, retinal, retinal oxime, and retinyl ester using silica gel plates. All-*trans*-, 9-*cis*-, 11-*cis*-, and 13-*cis*-retinol (R_f 0.21, 0.23, 0.28, 0.28, respectively), all-*trans*-, 9-*cis*-, 11-*cis*-, and 13-*cis*-retinal (R_f 0.46, 0.50, 0.53, 0.55, respectively), the *syn* form of all-*trans*-, 9-*cis*-, 11-*cis*-, and 13-*cis*-retinal oxime (R_f 0.45, 0.40, 0.47, 0.39, respectively), the *anti* form of all-*trans*-, 9-*cis*-, 11-*cis*-, and 13-*cis*-retinal oxime (R_f 0.21, 0.23, 0.27, 0.33, respectively), and all-*trans*- and 11-*cis*-retinyl esters (R_f for both esters = 0.70) were chromatographed with cyclohexane–toluene–ethyl acetate (5:3:2). Under these conditions, the retinyl ester isomers and 11-*cis*- and 13-*cis*-retinol cannot be separated. But 11-*cis*- and 13-*cis*-retinol (R_f 0.28 and 0.23, respectively) were separated with hexane–diethyl ether (1:1) as mobile phase (28). Dobrucki (29) converted retinol isomers into 2,4-dinitrophenylhydrazones for the best chromatographic separation. 2,4-Dinitrophenylhydrazones of all-*trans*-, 9-*cis*-, and 13-*cis*-retinals (R_f 0.32, 0.39 and 0.16, respectively) were separated on silica gel G using petroleum benzene–chloroform–ethyl acetate (30:3:1). The retinoids complexed to cyclodextrin were also separated by TLC on silica gel (30). Ultraviolet-visible electronic absorption, spectrometry, thermogravimetric analysis, and thin-layer chromatography were used to detect the formation of retinoid- β -cyclodextrin complexes. Tsukida et al. (31) separated *syn* and *anti* isomers of all-*trans*-, 9-*cis*-, and 11-*cis*-retinaloxime on preparative TLC silica gel plates using cyclohexane–benzene–ethyl acetate (5:3:2), 3% diethyl ether in benzene, and 5% diethyl ether in benzene.

Retinol acetate in ethyl ether was determined by TLC with densitometric detection (32). TLC was performed on Silufol plates using ethyl ether–hexane (1:1) as mobile phase. Analyte concentration was determined by peak area. The relative error of the method was $\pm 3.15\%$. Parizkova and Blatna (33) used preparative TLC to separate retinyl acetate oxidation products. Fourteen oxidation products of retinyl acetate were separated on silica gel HR with a mixture of hexane–diethyl ether (95:5 to 10:90, depending on the polarities of the substances to be separated) as mobile phase.

Sliwiok et al. (34) used TLC and HPLC to compare the hydrophobicity of vitamin A derivatives. TLC was performed on RP-2 F₂₅₄ and kieselguhr F₂₅₄ (impregnated with 10% paraffin oil in cyclohexane) with methanol–water (95:5). Chromatographic data and log *P* values calculated from fragmental constants for the vitamin A derivatives are listed in Table 2. The separations of the vitamin A derivatives on the paraffin oil-impregnated plates were better than those on the



designated as follows: S=sample; A and B=tretinoin standard stock solution at the beginning of analysis and just before development in direction 2, respectively; C and D=13-*cis*-RA standard test solutions. The broken lines represent solvent fronts. The R_f values in (a) are 0.81 for polar excipients, 0.42 for tretinoin and 13-*cis*-RA, and 0.36 for butylated hydroxytoluene (BHT). In (b), the R_f values are 0.25 for polar excipients, 0.34 for tretinoin, 0.39 for 13-*cis*-RA, and 0.68 for BHT. (From Ref. 26.)

RP-2 plates. The relationships obtained by the authors confirm the following sequence of increasing hydrophobicity: *all-trans* retinoic acid < *all-trans* retinal < vitamin A acetate < vitamin A palmitate.

Retinol, retinal, β -carotene, retinyl acetate, and α -carotene were separated on magnesium hydroxide with carbon disulfide (R_f 0.03, 0.15, 0.29, 0.39, and 0.43, respectively) (35). The separation and simultaneous determinations of β -carotene, cantaxanthin, lutein, violaxanthin, and

Table 2 Chromatographic Data and log P Values for Vitamin A Derivatives

Substance	R_M		log P
	I ^a	II ^b	
All- <i>trans</i> retinoic acid	-0.60	-1.59	4.85
All- <i>trans</i> retinal	-0.51	-0.84	6.48
Vitamin A acetate	-0.37	-0.22	7.92
Vitamin A palmitate	0.09	1.18	15.30

^aI=RP-2 plates.

^bII=Paraffin oil—impregnated plates

Source: Ref. 34.

neoxanthin were obtained using TLC on Chromarods, flame ionization detection (FID), and a two-stage development technique. For example, β -carotene, the internal standard, and the non-saponifiable neutral lipid fraction were separated with a mobile phase of light petroleum-chloroform-acetone (89.5:10:0.5); the xanthophylls did not move from the injection point (36). Satisfactory separations of carotenoids, including β -carotene,

were obtained on silica gel glass plates using a mixture of *tert*-alcohol (t-butyl or t-pentyl alcohol) and petroleum ether (boiling point 40–60°C) as mobile phase (37).

C. Separation and Determination of Vitamin A in Biological Samples

Skurikhin et al. (38) used microcolumn liquid chromatography and thin-layer chromatography coupled with spectrophotometry to perform serial analyses for the determination of vitamin A in milk. Results obtained by HPLC agreed with those obtained by TLC-spectrophotometry. An older paper (39) presented a simple, rapid, and reproducible method for the resolution of anhydrovitamins A₁ and A₂ as well as vitamin A₁ and A₂ palmitate, acetate, aldehyde, alcohol, and acid and epoxy derivatives of vitamin A on silica gel using a mobile phase of 6% acetone in light petroleum (bp 40–60°C). These experimental conditions were successfully applied to the separation of vitamins A₁ and A₂ compounds in fish liver oils and rat liver extracts.

Physiologically active vitamin A compounds (retinol, retinal, and retinoic acid) were determined in homogenates of nuclei, mitochondria, and microsomes of cultured HeLa cells. Vitamin A compounds were identified by determining their relative mobilities in thin-layer chromatograms on silica gel G, with a mixture of acetone and petroleum ether (18:82) as mobile phase. After development and elution, the vitamin A compounds were prepared for spectrophotometric determinations or placed in vials and assayed for radioactivity (40). Retinyl palmitate (vitamin A palmitate) was determined in homogenates and subcellular fractions of rat liver by TLC and HPLC (41). TLC was performed on silica gel using petroleum ether–isopropyl ether–acetic acid–water (180:20:2:5) as mobile phase. After development of the chromatograms, the plates were examined under UV light to detect the fluorescent retinyl palmitate. The validity of the TLC results was confirmed by HPLC and spectrophotometric techniques.

Kawanabe (42) described a simple technique for identifying A vitamins in preparations containing crude drugs listed in the Pharmacopoeia of Japan by using thin-layer stick chromatography (TLSC). TLSC is an advanced version of TLC in a cylindrical form. Vitamin A palmitate (retinyl palmitate) and vitamin A acetate (retinyl acetate) were separated (*R_f* 0.84 and 0.51, respectively) on a mixture of silica gel (Wako FM-BO, Wako, Japan) and microcrystalline cellulose (Abricel SF) using benzene as mobile phase.

D. Separation and Determination of β -Carotene as Precursor of Vitamin A in Biological Samples

Recent publications concern the qualitative and quantitative determination of β -carotene by TLC. β -Carotene, pheophytin a, chlorophyll a and b, lutein, violaxanthin, and neoxanthin extracted from barley leaves were separated on HPTLC CN-coated plates using a mobile phase of chloroform–hexane–methanol (5:14:1) (*R_f* 0.83, 0.51, 0.41, 0.31, 0.19, 0.18, 0.13, respectively) (43). Typical chromatograms of chloroform extracts of barley leaves were also given.

Photoisomerization of individual all-*trans*- α - and β -carotene solutions produced several iso-mers each. Nyambaka and Ryley (44) described a TLC method to isolate and collect major isomers of β -carotene (13-*cis*-, all-*trans*-, 9-*cis*-, and 7-*cis*-). Two-

dimensional TLC was done on calcium hydroxide plates with 1.2% acetone in petroleum ether as mobile phase. After extraction of the zones by TLC, the isomers were identified by their behavior in UV-Vis absorbance spectra. This method was applied to dark green leafy vegetables (Italian spinach, spring cabbage, and cowpea leaves). Identical conditions were used to develop and separate α - and β -carotene isomers by two-dimensional TLC. Expected isomers from the iodine-catalyzed reaction were neo isomers U and B for β -carotene and neo isomers U, W, and B for α -carotene. These isomers were detected by TLC in fresh and processed vegetables (spinach, cucumber, pickle, sweet potato, carrot) (45). β -Carotene was identified and separated from six other chloroplast pigments (46). Quantification of β -carotene and other pigments in spinach leaves was performed by scanning densitometry on the C₁₈ layer at their wavelengths of maximum absorption (Fig. 3). β -Carotene and lutein were also identified and quantified in extracts from snail samples (Pennsylvania and Colorado strains of *Helisoma trivolvis* and *Biomphalaria glabrata*) (46).

The carotenoid composition, including β -carotene, of *Rosa canina* fruit was determined by TLC with densitometric analyses and also by HPLC. The peaks of the extracts obtained from the TLC densitograms were identified as β -carotene, lycopene, rubixanthin, β -cryptoxanthin, and zeaxanthin mixed with lutein (R_f 0.96, 0.90, 0.62, 0.53, and 0.32, respectively) on silica gel with 15% v/v acetone in petroleum ether. The distribution of these compounds was reproducible by TLC as well as by HPLC (47).

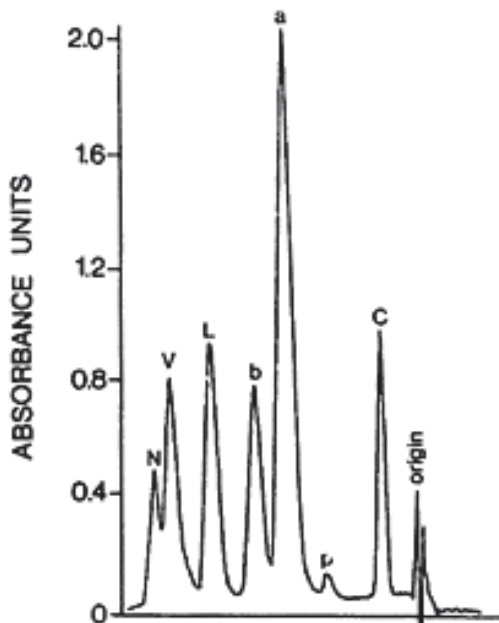


Figure 3 Reflectance densitogram at $\lambda=429$ nm of a spinach leaf extract separated on a C-18 plate developed with petroleum ether-acetonitrile-methanol (2:4:4). N=neoxanthin, V=violaxanthin, L= lutein,

b=chlorophyll b, a=chlorophyll a,
p=pheophytins, C= β -carotene. (From
Ref. 46.)

E. Detection of Vitamin A and Its Derivatives

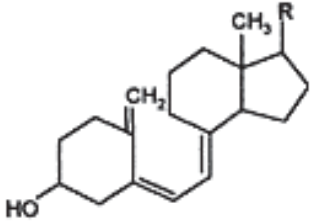
Small amounts of intensely colored pigments can be detected in ultraviolet (UV) light and also in visible light; the limit of detection is 0.01 μg of the carotenoids. Retinal (0.02–0.03 μg) can be detected after spraying with rhodamine (orange-red color of the spot of retinal). Many vitamin A compounds fluoresce yellow-green in light at 365 nm (limit of detection 0.05 μg). Vitamin A compounds can be detected with antimony(III) chloride (Carr—Price reagent) (24, 48–50) and antimony(V) chlorides (blue spots) (32, 42), with concentrated sulfuric acid (blue spots) (26, 48), with molybdophosphoric acid (green-blue spots), and with potassium dichromate in sulfuric acid (limits of detection 0.1–0.3 μg) (1, 2, 4). The limit of detection of retinol isomers converted to 2,4-dinitrophenylhydrazones of retinals is 1 μg (29). Wardas and Pyka (51) tested 11 visualizing reagents in 13 visualizing systems for the detection of E vitamins in adsorption and partition TLC and suggested the use of bromophenol blue (3 μg) for the detection of vitamin A after adsorption TLC.

III. VITAMIN D

A. Introduction

Physiological forms of vitamin D include vitamin D₂ (calciferol, ergocalciferol), vitamin D₃ (cholecalciferol), and phosphate esters of D₂ and D₃—25-hydroxycholecalciferol, 1,25-dihydroxycholecalciferol, and 5,25-dihydroxycholecalciferol. Vitamins D₂ and D₃ are 9,10-secosteroids, which differ structurally in the degree of saturation of an isoprenoid side chain. The structures and physicochemical properties of vitamins D₂ and D₃ are given in Table 3.

Table 3 Physicochemical Data of Vitamins D₂ and D₃

Data		
	D ₂ ergocalciferol ^a	D ₃ cholecalciferol ^b
MW	396.66	384.65
MP	118–119°C	85–87°C

$[\alpha]^{20}$ (acetone)	+82.6°	+53.3°
$\lambda_{\max}$ (nm)	265	264.5
Requirements	0.01 mg	0.01 mg
Chirality	+	
Achirality	+	
Occurrence	Mushrooms, fish oil	Vegetable oil, fish liver oil

$^a R = \begin{array}{c} \text{CH}_3 \\ \\ -\text{CH}-\text{CH}=\text{CH}-\text{CH}-\text{CH} \\ \quad \\ \text{CH}_3 \text{ CH}_3 \end{array}$	
$^b R = \begin{array}{c} \text{CH}_3 \\ \\ -\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH} \\ \\ \text{CH}_3 \end{array}$	

It is apparent from the literature (52) that the biological activity of vitamin D₃ is greater than that of vitamin D₂. Vitamin D₂ is of vegetable origin, whereas D₃ is formed in the skin of humans and animals. From a chemical standpoint, ergocalciferol (vitamin D₂) is a relatively stable vitamin. Active analogs and related compounds known as D vitamins include 22-dihydroergosterol (vitamin D₄), 2-dehydrostigmasterol (vitamin D₆), and 7-dehydrositosterol (vitamin D₅) (19). Lumisterol, tachysterol, ergosterol, and 7-dehydrocholesterol are inactive analogs and related compounds of vitamin D. Vitamins D₂ and D₃ are photochemically derived from their precursors ergosterol (provitamin D) and 7-dehydrocholesterol, respectively. Irradiation of sterols leads to various photolysis products, including tachysterol, lumisterol, and provitamin D (53). Vitamins D₂ and D₃ are precursors of hormones that are involved in the regulation of calcium and phosphate metabolism and are therefore important for growth and maintenance of bone (54). The D vitamins do not have significant biological activity. Rather they must be metabolized within the body to the hormonally active forms. Vitamin D₃, which has little biological activity, is converted into biologically active metabolites by oxidation. A first oxidation step occurs in the liver, converting vitamin D₃ into 25-hydroxyvitamin D₃ [25(OH)D₃] (Fig. 4). The second oxidation reaction takes place in the kidney and converts 25(OH)D₃ into either 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] (Fig. 4), the hormonal metabolite of the vitamin D₃ endocrine system, or 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃]. Hydroxycholecalciferols are hormonally active functional metabolites of vitamin D₃, and their detection and determination have significance in clinical investigations. As hormones, these compounds play a key role in the maintenance of serum calcium and phosphate by stimulating their intestinal absorption, bone resorption, possible reabsorption in the kidney, and other significant biological activities (54–61). For these reasons, investigative methods were developed to study hydroxycalciferols in body fluids.

Thin-layer chromatography has several applications in the vitamin D area. Some of these are of historical value only because they have been superseded by HPLC. TLC as an analytical technique can be considered for the following purposes. In investigations of vitamins D₂ and D₃, TLC is useful in basic investigations as well as in detecting

vitamins D₂ and D₃ and their metabolites in various samples of natural origin. TLC and mainly HPTLC as analytical techniques are used for various purposes, including differentiation of vitamin D analogs; separation of vitamin D from lipids, including sterols and fat-soluble vitamins, which may interfere with its quantification in foods, oils, and drug formulations; determination of the purity of radiolabeled vitamin D derivatives; and analysis of metabolites of vitamin D as part of radioligand assays of these metabolites in human plasma or serum.

B. Basic Investigations of Vitamins D₂ and D₃ and Their Analogs

On silica gel plates using chloroform as mobile phase, irradiation mixtures of tachysterol, vitamin D, and ergosterol (R_f 0.64, 0.44, and 0.25, respectively) were separated by Norman et al. (62).

Kocjan and Sliwiok (63) determined the hydrophobicity of vitamins D₂ and D₃ by RPTLC, thin-layer adsorption chromatography, and infrared spectrometry. Partition TLC separations of vitamins D₂ and D₃ were done on TLC plates precoated with kieselguhr F₂₅₄ impregnated with 10% paraffin oil in benzene and developed to 10 cm with binary mixtures of methanol–water and acetonitrile–water. The R_f values of the best separations of vitamins are listed in Table 4. Adsorption TLC was done on glass plates precoated with activated silica gel 60 F₂₅₄, with a mobile phase of benzene–methanol (9:1). Measurement of the surface of chromatographic spots obtained by adsorption TLC gave hydrophobicity coefficients. The results indicate that the hydrophobicity of vitamin D₃ is greater than that of vitamin D₂. This conclusion was proved by the lower R_f values for vitamin D₃ obtained by partition chromatography, the higher values of the respective hydrophobicity coefficients, and the greater relative decreases in the ratio of free to bonded OH groups (obtained by spectrometric measurement).

The oxidative degradation of ergocalciferol has been known for over 50 years. Stewart et al. (64) investigated the products of the degradation of crystalline ergocalciferol. These studies showed that numerous acidic and neutral oxidation products were formed, resulting in complete destruction of the triene functionality. Separation of the neutral products by preparative TLC (silica gel with fluorescent indicator, four mobile phases) led to material identified as the Windaus ketone.

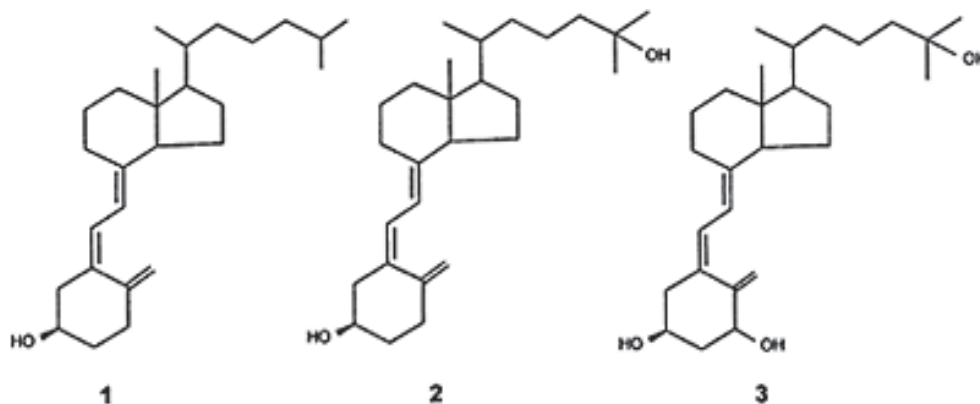


Figure 4 Structure of principal metabolites of (1) vitamin D₃; (2) 25(H)D₃; and (3) 1,25-(OH)₂D₃.

For the separation and determination of photothermally induced isomers and ergocalciferol in the product formed by UV irradiation of ergosterol, the extracted sample is spotted on silica gel G TLC plates developed with benzene-acetone (93:7) as the mobile phase (65).

C. Separation of Vitamin D from Biological Materials

In order to identify vitamin D₂ in shiitake mushrooms (*Lentium edodes*), vitamin D₂ was isolated by thin-layer chromatography (using Wako-gel B5FM silica gel TLC with benzene-acetone 95:5 as mobile phase) and high-performance liquid chromatography and identified by thermospray-interfaced mass spectrometry (66). This procedure prevents the decomposition of the vitamin by heat, which is a common problem in the gas chromatography-mass spectrometry of vitamin D₂.

After extraction of the lipid fraction from milk powder, the vitamin D was separated by adsorption TLC with cyclohexane-diethyl ether (1:1) as mobile phase and was measured by densitometry under UV (67). Grace and Bernhard (68) conducted similar investigations on whole milk. They separated the vitamin D from the methyl esters by preparative TLC, using hexane-isopropanol (85:15) as mobile phase. Final analysis was achieved by RP-HPLC with methanol-water (97:3) as mobile phase. Ergocalciferol and cholecalciferol were separated from retinyl acetate or palmitate and tocopheryl acetate using TLC on silica gel HF₂₅₄ (69). These vitamins were

Table 4 R_f Values with RP-TLC and Chromatographic Spot Areas (s) and Hydrophobicity Coefficients (h_f) Obtained by Adsorption TLC Measurement of Vitamins D₂ and D₃

Vitamin	R_f obtained by RP-TLC in			Adsorption TLC	
	Methanol-water	Acetonitrile-water		Spot area s (mm ²)	Hydrophobicity coefficient h_f
	(9.5:0.5)	(9.5:0.5)	(9:1)		
D ₂	0.56	0.50	0.38	141.0	6.09
D ₃	0.48	0.41	0.29	99.5	9.05

Source: Ref. 63.

then determined by spectrophotometry. This method was applied for the determination of ergocalciferol in the preparations Jecoderm (Galenika, Belgrade, Yugoslavia) ointment

and Vidaylin-M (Galenika, Belgrade, Yugoslavia) syrup and of cholecalciferol in Oligovit (Galenika, Belgrade, Yugoslavia) coated tables.

Das (53) presented results obtained from analysis of an extract of cod liver oil; parts per billion levels of vitamin D₃ were measured by using densitometric analysis in the sample extract. The samples, e.g., fish liver oils or feed components, were saponified by the addition of an internal standard before extraction with light petroleum and cleanup of the extract by TLC. Next, cholecalciferol and ergocalciferol were determined by HPLC on LiChrospher C18 with methanol–water–propan-2-ol–hexane (200:4:4:1) as mobile phase and detection at 265 nm. The method was efficient and reliable (70). An efficient separation of lipophilic vitamins, including vitamins D₂ and D₃, was also reported on silica gel (9, 71).

D. Separation of Vitamin D Metabolites and Application to Biological Materials

Justova and Starka (72) described TLC separations of vitamin D hydroxymetabolites. They tested three stationary phases (three commercial silica gel sorbents) and one relatively polar mobile phase (chloroform-ethanol–ater, 183:16:1). R_f values of vitamin D₃ and its functional hydroxyme-tabolites are listed in Table 5. The optimal separation was achieved on Kieselgel 60 F₂₅₄ foils produced by Merck. In biological samples, the vitamin D₃ hydroxymetabolite contents are at nanogram levels, which is below the limit of sensitivity of the applied visualization methods (UV light and the anisaldehyde reagent). Therefore, the hydroxycholecalciferols were localized with their corresponding metabolites used as markers. The vitamin D₃ metabolites were then scraped off and extracted with ethanol, and the eluates were examined for radioactivity. The effect of the complete chromatographic procedure on the accuracy and precision of the determination is given in Table 6. Listed values in Table 6 indicate that the methods of TLC separation and determination of vitamin D₃ and its hydroxymetabolites are accurate and precise.

Esparza et al. (73) applied the same conditions to investigate vitamin D₃ hydroxylated metabolites in *Solanum malacoxylon*. Justova et al. (74) used identical conditions to identify and determine 1,25-dihydroxyvitamin D₃. It was found that TLC can substitute for HPLC; that is, quantitative separation of femtomole amounts of compounds in biological material can be done in biochemical laboratories that do not have specially designed apparatus. These conditions were used to determine [³H] 1,25-dihydroxycholecalciferol in plasma (75). Plasma samples were saturated with (NH₄)₂SO₄, and the mixture was extracted with methanol and toluene before HPTLC of the extracts on silica gel 60, with chloroform-ethanol-water (183:16:1) as mobile phase. Vitamin D metabolites and calcitriol were separated; the silica gel corresponding to the R_f value of calcitriol was scraped off and dispersed in water for competitive protein binding assay (CPBA).

Table 5 R_f Values of Vitamin D₃ and Its Functional Hydroxymetabolites on Silica Gel Layers With Chloroform–Ethanol–Water (183:16:1) as Mobile Phase

Compound	Kieselgel 60 F ₂₅₄ ^a	Silufol UV 254 ^b	Silica gel 6061 ^c
D ₃	0.69	0.56	0.65
1—OH—D ₃	0.35	0.39	0.40
25—OH—D ₃	0.56	0.43	0.57
1,25—(OH) ₂ —D ₃	0.20	0.15	0.33
24,25—(OH) ₂ —D ₃	0.41	0.26	0.51
25,26—(OH) ₂ —D ₃	0.30	0.17	0.35

^a Merck, Germany.

^b Kavalier, Sazava, Czech Republic.

^c Eastman Kodak, Rochester, NY.

Source: Ref. 72.

Table 6 Recovery of Labeled Vitamins D₃ and 25-Hydroxycholecalciferol After Chromatography on Thin Layers of Kieselgel 60 F₂₅₄

Compound	Amount applied (ng)	Amount found ² (ng)	Coefficient of variation (%)
D ₃	4.68	4.44±0.33	5.85
25(OH)D ₃	0.34	0.33±0.01	3.03

^a Mean±S.D. (n=5).

Source: Ref. 72.

Intestinal cytosol from chickens was used as binding protein, and ³H-labeled calcitriol was used as radioligand. HPTLC separation does not affect the detection limit (10 fmol for 1 mL plasma samples) and precision of CPBA. Recoveries were 98.9% for extraction and HPTLC and 73% for the precipitation procedure.

High-performance thin-layer chromatography (HPTLC) to separate the hydroxylated metabolites of vitamin D₃ was used for the first time by Thierry-Palmer and Gray (76). They developed a solvent system for separating mono-, di-, and trihydroxylated metabolites of vitamin D₃ by HPTLC and compared their results with those from the separation of these compounds by conventional TLC. The efficiency of separation by conventional TLC is similar to that by HPTLC. The choice of mobile phase depended on which metabolite was determined. However, for routine analysis, HPTLC may be better than HPLC in the speed of analysis because of its ability to separate many samples at one time (77). The choice between TLC and HPTLC depends on the material to be purified (76).

Unconjugated vitamin D and its metabolites were investigated by Saden-Krehula and Tajic (78) in the pollen of *Pinus nigra* Ar. and *Pinus sylvestris* L. by TLC. During TLC, standards were used to check elution and loss of analytes during removal of lipids. However, vitamins D₂ and D₃ were not separated. TLC was also used to purify extracts, including vitamin D₃ and its derivatives, of *Solanum malacoxylon* (79). Cheng et al. (80) observed unknown spots on plates of extracted urine samples. GC-MS analysis indicated that the unknown compounds were ergocalciferol metabolites from a vitamin D supplement.

Calcitriol, the hormonally active form of vitamin D, regulates transcription of target genes through activation of its intracellular receptor. Barsony et al. (81) characterized the purity and structure of three BODIPY-labeled calcitriol derivatives using preparative TLC, HPLC, and ¹H-NMR spectroscopy.

E. Detection of Vitamin D and Its Derivatives

Spots of vitamin D derivatives can be inspected in short-wavelength UV light (limit of detection 0.025–0.5 µg). The mono- and dihydroxycholecalciferols can be visualized under UV light at 254 nm or by spraying the foil with a solution of anisaldehyde in sulfuric acid (2%, w/v) mixed with glacial acetic acid (1:10, v/v) at 40°C (72). Vitamins D₂ and D₃ can be detected with antimony(III) chloride (Carr—Price reagent) (48–50) and antimony(V) chloride (gray-blue and orange-red spots, respectively; limit of detection 0.025–0.3 µg), with concentrated sulfuric acid (brown and green spots, respectively, of vitamins D₂ and D₃, limit of detection 30 µg), with tungstophosphoric acid (gray-brown spots; limit of detection 0.2 µg) (71), with molybdophosphoric acid (gray-blue spots; limit of detection 0.3 µg), and with trichloroacetic (48) and trifluoroacetic (3) acids (limit of detection 0.1–0.2 µg). Vitamins D₂ and D₃ separated by adsorption TLC were visualized with 0.005% aqueous new fuchsine solution (63). A densitogram of vitamin D₃ after a spray of antimony(III) chloride (Carr-Price Reagent) is given by Jork et al. (48). Vitamin D₃ was also detected with sodium hydroxide (yellow and orange spots in visible and UV light, respectively; limit of detection 1–8 µg), with iron(III) chloride (brown-red spot in visible light; limit of detection 8 µg), with iodoplatinate (black and brown spots in visible and UV light, respectively; limit of detection 1–8 µg) (82, 83), and with phosphomolybdate (pinkish brown spot in visible light; limit of detection 8 µg) (82). Ergocalciferol was located by UV radiation (the blue spot) (65). Wardas and Pyka (51) tested 11 visualizing reagents in 13 visualizing systems for detection of vitamins D₂ and D₃ using adsorption and partition TLC. They suggested the use of bromocresol green and bromothymol blue as well as helasol green for the detection of vitamins D₂ and D₃ after adsorption TLC (limit of detection 5 µg) and partition TLC (limit of detection 50 µg), respectively.

IV. VITAMIN E

A. Introduction

Vitamin E has been an enigma in nutrition research for over 60 years. In 1937, Emerson et al. (84) described various vitamin E homologs with different capacities to prevent vitamin E deficiency. General characteristics of vitamin E are given in Table 7. The known physiological forms of vitamins E are *d*- α -tocopherol and tocopheronolactone and their phosphate esters. Active analogs and related compounds known as vitamins E are *dl*- α -tocopherol, *l*- α -tocopherol, esters (succinate, acetate, phosphate), and β -, ζ_1 , and ζ_2 -tocopherols. δ -, ϵ -, and η -Tocopherols are inactive analogs and related compounds of vitamin E (19).

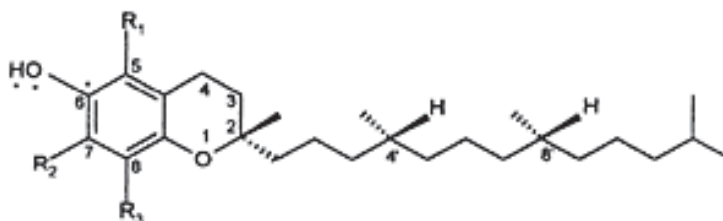
In nature, vitamin E occurs in eight different forms (α -, β -, γ -, and δ -tocopherols and α -, β -, γ -, and δ -tocotrienols) with varying biological activities. Tocopherols have been intensively studied owing to their medical, biological, and physicochemical significance (85–87). The biological properties of α -tocopherol are of particular importance (88, 89). Of these eight compounds, α -tocopherol has the highest biological activity (90). Tocopherol possesses three asymmetric carbon atoms, and there are eight possible stereoisomeric tocopherols. Natural α -tocopherol occurs as the enantiomer about the configuration *2R,4'R,8'R*. Semisynthetic α -tocopherol is a mixture of the diastereoisomers about configurations *2R/S,4'R*, and *8'R*. Although γ -tocopherol is a more effective free radical scavenger than α -tocopherol in vitro (91), the reverse is true in vivo (92). The biological activity of vitamin E has generally been associated with its well-defined antioxidant property, specifically against lipid peroxidation in biological membranes (93–99). The antioxidative effect of the different tocopherols may not be identical. It has been shown in antioxidation tests with foodstuffs that the antioxidative activity of the tocopherols increases in the order γ -, δ -, β -, and α -tocopherol (100, 101). Vitamin E occurs mainly in wheat germ, vegetable oil, and vegetables (102). α -Tocopherol and γ -tocopherol are the most common of the eight naturally occurring vitamin E homologs in the human diet. Tocopherol is nearly insoluble in water but soluble in ethanol, ether, chloroform, acetone, and vegetable oils. The problem of separating α -, β -, γ -, and δ -tocopherols has been the subject of numerous reviews (5, 85, 103). Table 8 lists general physicochemical data for α -, β -, γ -, and δ -tocopherols.

B. Basic Investigations of Vitamin E and Its Analogs

α -, β -, γ -, and δ -Tocopherols were separated (R_f values 0.26, 0.48, 0.50, and 0.65, respectively) on kieselguhr G plates impregnated with a 10% solution of paraffin oil in hexane and a mobile

Table 7 General Characteristics of Vitamin E

Molecular weight	430.71
Requirements	30 mg
Chirality	+
Achirality	—
Melting point	2.5–3.5°C
λ	284 nm
Occurrence	Apples, olives, lettuce, spinach, corn, soybean (oil), green peppers, cauliflower, coconuts, palm oil, oats, brown rice, eggs, milk

Table 8 Structures, Molar Weights (M), Partition Coefficient by Rekker ($\log P$), Relative Affinity of the Tocopherols Investigated, and Net Electron Charge (Σ NEC) on—C—O—H Groups

Compound	R ₁	R ₂	R ₃	M (g/mol)	$\log P$	Σ NEC	Relative affinity (%)
DL- α -Tocopherol	—CH ₃	—CH ₃	—CH ₃	430.71	12.37	0.0039	100
DL- β -Tocopherol	—CH ₃	—H	—CH ₃	416.68	11.86	0.0223	38.1±9.3
DL- γ -Tocopherol	—H	—CH ₃	—CH ₃	416.68	11.86	0.0327	8.9±0.6
DL- δ -Tocopherol	—H	—H	—CH ₃	402.65	11.36	0.0432	1.6±0.3

Source: Ref. 105.

phase of methanol–water (9.5:0.5) by Sliwiok and Kocjan (87). They correlated their results with those of quantum-mechanical calculations and with respective steric effects. It was established that the investigated tocopherols can be arranged with respect to their hydrophobic properties in the order α -tocopherol> β -tocopherol> γ -tocopherol> δ -tocopherol. But enantiomers of DL- α -tocopherol were separated on Chiralplates (Machery-Nagel, Germany) with 2-propanol–water–methanol (17:2:1) as mobile phase (104). Under these conditions two bands were generated with R_f values of 0.72 and 0.62. Tocotrienols were separated on silica gel G plates using a mobile phase of methanol–benzene (1:99); the R_f values for ζ -, ϵ -, and η -tocopherol and δ -T-3 are 0.55, 0.41, 0.39, and 0.29, respectively (4).

α -, β -, γ -, and δ -Tocopherols were separated by reversed-phase high-performance thin-layer chromatography (RP-18-HPTLC), normal-phase high-performance liquid chromatography (HPLC), reversed-phase high-performance liquid chromatography (RP-18-HPLC), and gas chromatography (GC). R_f values of the α -, β -, γ -, and δ -tocopherols investigated with RP-18-HPTLC are shown in Table 9. The chromatographic conditions used allowed for the separation of the four tocopherols in various biological samples. The selected topological indices based on the connec-

Table 9 R_f Values of α -, β -, γ -, and δ -Tocopherols Investigated by RP-18-HPTLC

Compound	R_f^a		
	S1	S2	S3
α -Tocopherol	0.49	0.31	0.18
β -Tocopherol	0.53	0.37	0.20
γ -Tocopherol	0.57	0.41	0.22
δ -Tocopherol	0.63	0.47	0.24

^aMean values, $n=5$. S1, S2, S3 contained ethanol and water in the volume proportions 10:0, 9.5:0.5, and 9:1, respectively.

Source: Ref. 105.

tivity—adjacency matrix (M^v , ${}^1\chi^v$), on the distance matrix (W , ${}^0B$, MTI), and on information theory (Q_{AC} , I_{AC}) were calculated for these tocopherols. The observed chromatographic separations of investigated tocopherols were compared. The comparison indicated that RP-18-HPTLC, HPLC, and GC are the best techniques for the separation of these tocopherols. The topological index ${}^0B$ was the most significant. A definite dependence between the numerical values of the topological index ${}^0B$ and the chromatographic separation of the investigated tocopherols was obtained (105). α -, β -, γ -, and δ -tocopherols were also separated by reversed-phase thin-layer chromatography on C_{18} plates using seven different mobile phases (methanol, ethanol, n -propanol, and mixtures containing ethanol and water and n -propanol and water in the volume proportions 9.5:0.5, and 9:1, v/v). The R_M values of the compounds were correlated with the numerical values of the topological indices, the sum of the net electron charge (Σ NEC) on the tocopherols'—C—O—H groups, the moment dipoles (μ_{mph}), and the permittivities (ϵ_{mph}) of the mobile phases. The most accurate prediction of the R_M values of the tocopherols in all the mobile phases investigated was achieved by the use of two parametric equations employing the dipole moments of the mobile phases and one topological index from among the topological indices ${}^2\chi^v$, ${}^0B$, C or the sum of the net electron charge (Σ NEC) (106).

Ruggeri et al. (107) determined α -, γ -, and δ -tocopherol, α -tocotrienol, and tocol. The TLC system employed silica gel GF plates, hexane-isopropyl ether (17:3) as mobile phase, and a scanning densitometer operating at 350 nm; the HPLC system used a Varian MCH 10 C_{18} Micropak column (30 cm $\times$ 4 mm), methanol–water (95:5) as mobile phase

(2 mL/min), and detection at 296 nm. The two systems were considered comparable in sensitivity, reproducibility, recovery (~91%), and ease of application (107).

C. Separation of Vitamin E from Biological Materials

Olejnik et al. (108) determined α - and γ -tocopherols and plastochromanol-8 in linseed oil. TLC was used for purification of the unsaponifiable fraction of linseed oil. TLC was done on silica gel G with chloroform as the first mobile phase (Fig. 5) and hexane–diethyl ether (19:1) as the second phase (Fig. 6) (108).

TLC was used to estimate the deodorized distillate of soybean oil as a source for tocopherols that can be used instead of the synthetic antioxidants as food preservatives (109). TLC for ana-

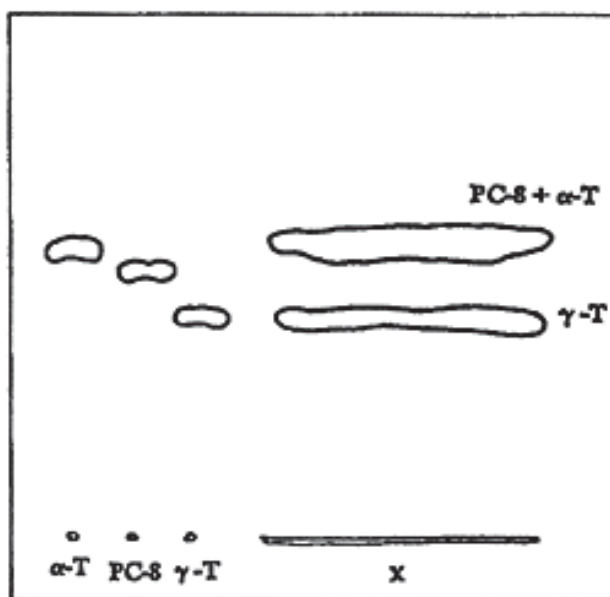


Figure 5 Preparative separation of plastochochromanol-8 (PC-8) and α -tocopherol (α -T) on TLC (silica gel G, CHCl_3); α -T, PC-8, γ -T standards; x sample (the unsaponifiable fraction of linseed oil). (From Ref. 108.)

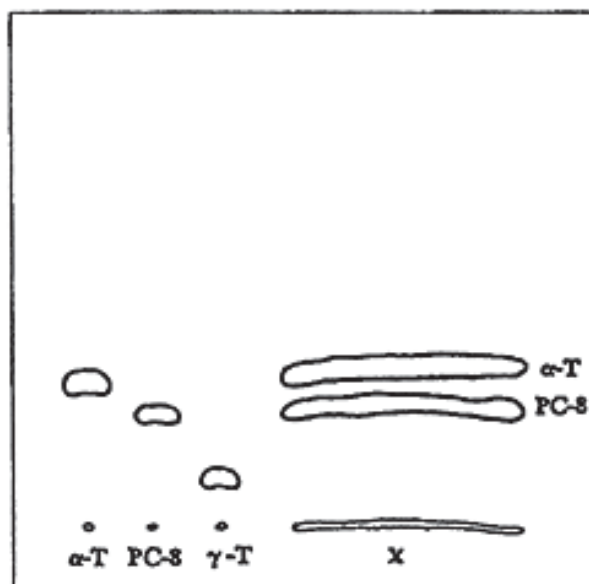


Figure 6 Rechromatography of plastocholesterol-8 (PC-8) and α -tocopherol (α -T) on TLC (silica gel G, hexane–diethyl ether, 19:1); α -T, PC-8, γ -T standards; x sample (the unsaponifiable fraction of linseed oil). (From Ref. 108.)

lytical and preparative purposes was carried out on silica gel 60 G F₂₅₄ (Merck) with a mobile phase of *n*-hexane–benzene–diethyl ether (40:40:20). Analytical TLC indicated the presence in soybean oil of three isomers, the α -, β -, and γ -tocopherols. Dimeric oxidation products from the α -tocopherol were checked by TLC using the solvent system *n*-hexane–benzene (1:1). These oxidation products showed R_f values of 0.7, 0.79, and 0.85 compared with 0.63 for α -tocopherol and gave dark red, light red, and pale red spots, respectively, under UV light. Results agreed with those obtained by TLC and were higher than those of the Emmerie and Engel method (109).

The separation of α -tocopherol, α -tocotrienol, (β + γ)-tocopherol, (β + γ)-tocotrienol, δ -tocopherol, and 5-tocotrienol from the sterols in the unsaponifiable part of palm oil was achieved by TLC on silica gel 60, using benzene–ethyl acetate (96:4) as mobile phase (110). Mixtures of β - and γ -tocopherol and of β - and γ -tocotrienol could also be separated by GLC (110). TLC was also used for the separation and determination of tocopherol isomers in peanut oil (111), in wheat germ, cottonseed, and soybeans (112), and in other foods (113, 114). The derivatives (hydroxy fatty acids), minor lipid compounds, e.g., tocopherol, and aromatic compounds, e.g., menthol, can be purified by TLC and separated and determined by HPLC (115). Askinazi et al. (116) reported a modified TLC method to measure tocopherol isomers. TLC was done on a Silufol UV₂₅₄ plate. The mobile phase was hexane–ethyl ether (49:1), and after 15–20 min it was changed to chloroform. R_f values for α -, γ -, and δ -tocopherols were 0.63, 0.44, and 0.29, respectively.

The α -, γ -, and δ -isomers of tocopherol were determined by spectrophotometry in sunflower seed oil, cottonseed oil, soybean oil, and margarine and compared with those determined by gas chromatography. Both techniques demonstrated consistent results. The TLC technique may be useful in the analysis of the isomeric composition of tocopherols in fats and vegetable oils. Koswig and Moersel (117) described a simple method for the determination of tocopherols in vegetable oils. TLC on silica gel 60 separated the tocopherols from a sample. Tocopherols were then eluted from the separated spots with ethanol and determined by UV spectrophotometry.

Manz et al. (118) described a simple and universal method for the selective and quantitative determination of α -tocopherol in the presence of other tocopherols in food. After alkaline saponification of the sample, the unsaponifiable matter was purified with silicic and sulfuric acids and finally analyzed by thin-layer chromatography. TLC was done on silica gel plates using chloroform-isooctane (50:50) as mobile phase. After elution the α -tocopherol was colorimetrically determined. The recovery of α -tocopherol was about 90%.

Bapçum (119) investigated fats from the seed of cotton plants grown in different regions for separating α -, β -, γ -, and δ -tocopherols by TLC. TLC was done on silica gel H₂₅₄ with light petroleum (boiling range 50–70°C)-isopropyl ether–acetone–ethyl ether–glacial acetic acid (160:30:10:2:1) as mobile phase. The quantities of tocopherols were determined by photometric methods. Rectilinear calibration graphs were obtained for 2–8 $\mu\text{g/mL}$ of α - or γ -tocopherol in the final solution. No β - or δ -tocopherol was detected (119).

Leray et al. (120) described a reliable procedure for the joint analysis of tocopherols, cholesterol, and phospholipids in the same small samples of human platelets and human cultured endothelial cells. Phospholipids, cholesterol, and tocopherols in total lipid chloroform extracts were separated by TLC on Whatman LK5 silica gel plates impregnated with boric acid. The solvent system was chloroform-ethanol-water-triethylamine (35:30:7:35) containing 0.10 g/L of ascorbic acid and 0.15 g/L of butylated hydroxytoluene, and visualization was by UV light after a primuline spray. R_f values of cholesterol and tocopherols were 0.86–0.89. α -, γ -, and δ -tocopherols, tocopherol acetate, and cholesterol were purified after TLC by column chromatography on silica gel G60 and determined by HPLC. Recoveries were >98% for cholesterol and phospholipids and >98% for tocopherol when ascorbic acid and butylated hydroxytoluene were included in the TLC solvent system. The method can be used to determine tocopherol, cholesterol, and phospholipid fatty acids in the same microsample of human platelets or cultured human endothelial cells (120).

Lipids of different classes present in the lungs of rats were separated by TLC. Fatty acids and plasmalogens were determined as methyl esters and dimethylacetals, respectively, by GC. Cholesterol and vitamin E were determined enzymatically and by HPLC, respectively. Three types of subfractions were distinguished. Vitamin E was present in the less dense subfraction (121).

Many papers have described the use of adsorption thin-layer chromatography for purification of biological samples or of extracts of biological samples (122, 123). The purified extracts can be determined by other methods such as HPLC, GC, and spectrophotometry. Tandem TLC-spectrophotometry was used to determine vitamin E in

animal tissues (123). HPLC and TLC were also used to estimate the effects of α -tocopherol on the oxidative transformation of arachidonic acid in human platelets (124).

TLC was used for the estimation of the antioxidant activity of vitamin E and of various biological samples containing α -tocopherol (125–127). Preparative TLC was used for the purification of the extracts of the biological samples. These investigations indicate that α -tocopherol is an active antioxidant (127). Antioxidants containing the α -tocopherol can be qualitatively evaluated in extracts by TLC separations (128). α -Tocopherol (R_f 0.51) was also separated from five other antioxidants on silica gel plates with cyclohexane–dioxane–acetic acid (80:15:5) as mobile phase (126). On RP-18 plates, using methanol–water–acetic acid (82:16:2) as mobile phase, α -tocopherol has an R_f of 0 and is separated from 10 other antioxidants (126).

Mono-, di-, and trimethylated tocopherols and tocotrienols were separated, identified, and determined by TLC-MS by the use of mass-analyzed ion kinetic energy spectra. The monomethyl compounds were separated by TLC on silica gel G with light petroleum–ethyl ether (41:9) as mobile phase. The occurrence of δ -tocopherol and γ -tocotrienol in cyanobacteria was reported (129).

Hachuła and Buhl (130) described analytical methods to determine α -tocopherol in Vitaminum E capsules (Polfa, Poznan, Poland) and soybean oil. Determination of α -tocopherol (after extraction of samples) was performed by TLC on silica gel plates with benzene–ethanol (99:1) as mobile phase.

D. Detection of Vitamin E and Its Derivatives

Vitamin E compounds can be detected (about 20 μ g) as dark spots by UV light. They appear violet, and detection is more sensitive (0.02 μ g) on layers that contain 0.02% Na-fluorescein. Moreover, these are visible in daylight as red spots (limit of detection 2 μ g). Spraying with fluorescein or dichlorofluorescein reagent produces the same effect. Nonspecific visualization procedures for tocopherols and tocotrienols are based on spraying with sulfuric acid, molybdophosphoric acid (48, 119, 131), antimony(V) chloride, dipyridyliron reagent (48, 105, 116, 132), nitric acid, copper(II) sulfate–phosphoric acid (1–4), or bathophenanthroline (112, 123). α -Tocopherol was also visualized by a coupled redox-complexation reaction with iron(III), phenanthroline, and bromophenol blue (130). Eleven visualizing reagents in 13 visualizing systems for the detection of E vitamins were tested using adsorption and partition TLC by Wardas and Pyka (51). They indicated that aniline blue, alkaline blue, and bromothymol blue (1 μ g) were effective for detection of E vitamins after adsorption TLC. When β -carotene was used as a TLC spray reagent, tocopherols (10 μ g) appeared as yellow spots against a white background (133). Densitograms of E vitamins after spraying with dipyridyliron reagent were given by Jork et al. (48).

V. VITAMIN K

A. Introduction

The physiological forms of vitamin K are vitamin K₁ (phyloquinone, phytonadione) and vitamin K₂ (farnoquinone). Active analogs and related compounds known as K vitamins are menadiol diphosphate, menadione (vitamin K₃), menadione bisulfite, phthiocol, synkayvite, menadiol (vitamin K₄), menaquinone-*n* (MK-*n*), ubiquinone (Q-*n*), and plastoquinone (PQ-*n*) (19). Structures of vitamins K₁, K₂, and K₃ are given in Fig. 7. Reduced vitamin K is an inactive analog. Dicoumarol, sulfonamides, antibiotics, α -tocopherol quinone, dihydroxystearic acid glycide, salicylates, iodinin, and warfarin are antagonists of vitamin K. Vertebrates and some bacteria (intestinal bacteria in humans) are exogenous sources of vitamin K. Endogenous sources of vitamin K are plants, bacteria, and all other organisms that require it. Phylloquinone (K₁) and menaquinone-4 (MK-4)

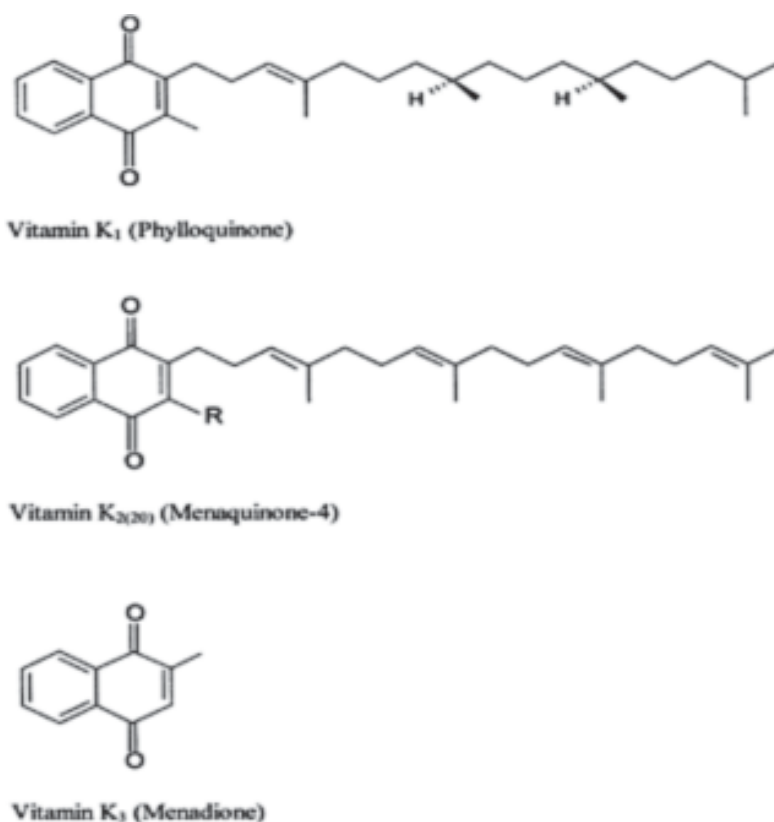


Figure 7 Structures of vitamins K₁, K₂, and K₃.

are natural K vitamins and are often used as medicine to prevent intracranial hemorrhage in the newborn (134–139). General characteristics of vitamin K₃ are given in Table 10.

Vitamin K contributes to the formation and regulation of numerous proteins in the body, but most significantly to prothrombin, a protein essential for blood clotting. Vitamin K is also necessary for converting prothrombin to thrombin, which is also required for blood clotting. Vitamin K is a key factor in the creation of many important nutrients and proteins necessary for essential body functions (139).

Ubiquinones and menaquinones are constituents of bacterial plasma membranes, where they play an important role in respiratory electron transport (140). Structural differences in these respiratory quinones have been used as a basis for bacterial classification (141). Menaquinones of bacteria are lipophilic components of the cytoplasmic membrane that undergo reversible oxidation and reduction to form a quinone or hydroquinone, respectively.

B. Basic Investigations of K Vitamins and Their Analogs

Selected K vitamins and related quinones were separated on silica gel G impregnated with silver nitrate and with diisopropyl ether as mobile phase. The R_f values for vitamin K (*trans*-K-4), MK-3, MK-4, MK-5, MK-6, MK-7, and menadione are 0.72, 0.58, 0.49, 0.41, 0.29, 0.21, and 0.45, respectively (4). Argentation TLC was also used to separate vitamins K₁, K₂, and K₃ (142). Ubiquinones Q-30, Q-35, Q-40, Q-45, Q-50 and vitamins K_{2(30)}}, K_{2(35)}}, K_{2(45)}}, and K₁ were also separated on silica gel G impregnated with 5% paraffin oil in petroleum ether using acetone–water (95:5) mobile phase (143).

C. Separation of Vitamin K from Biological Materials

Hachula (144) developed a sensitive spectrophotometric method for the determination of vitamin K₄ in drugs (Styptobion, Merck) after chromatographic separation on silica gel with benzene–acetone (9:1) as mobile phase. Beer's law was obeyed for 0.3–1 µg/mL vitamin K₄ ($\epsilon=196000$). No detection limit was given. Pharmaceutical preparations, including tablets, coated tablets, and injection solution, containing vitamin K₁ (phyloquinone, I), vitamin K₃ (menaphthone, II), or vitamin K₄ (acetomenaphthone, III) were analyzed by TLC-spectrophotometry (145). TLC was done on silica gel H F₂₅₄ (Merck) with the following mobile phases: benzene–ethyl acetate (97:3) for K₁, cyclohexane–chloroform–methanol–acetic acid (2:15:3:1) for K₂, and benzene–acetone (9:1) for K₃. The spots, located with 254 nm radiation, were scraped off, extracted with ethanol (for I), water (for II), or methanol (for III), and the vitamins were determined at 251, 234, or 225 nm, respectively, by using suitable calibration graphs (145).

HPLC was used to measure endogenous K vitamins in human and animal feces, and these vitamins in human feces were also identified by mass spectrometry by Sakano et al. (146). K vitamins extracted from human and animal (monkey, dog, guinea pig, rat, mouse, rabbit, chicken) feces were purified by thin-layer chromatography using plates precoated with silica gel 60 F₂₅₄ and petroleum ether–diethyl ether (85:15) as mobile phase. In this system the R_f values of standard K vitamins ranged from 0.53 (MK-4) to 0.61 (MK-13). Vitamins K were also measured by RP-HPLC with fluorometric detection. Usui et al. (147) measured vitamin K in human liver by gradient elution high-performance liquid chromatography after preparative thin-layer chromatog-

Table 10 General Characteristics of Vitamin K₃

Molecular weight	172
Requirements	0.15 mg
Chirality	—
Achirality	+
Melting point	105–107°C
Occurrence	Nettles, oranges (peel), tomatoes, spinach, cabbage, brussels sprouts, hemp seed

raphy, using platinum black catalyst reduction and fluorometric detection. Adsorption preparative thin-layer chromatography was done on silica gel 60 F₂₅₄ with the mobile phase petroleum ether–diethyl ether (85:15). After preparative TLC using benzene or methanol–benzene (1:2 or 1:4) as mobile phase, menaphthone (vitamin K₃) was also determined by GC (148).

A case of hemorrhage of unknown origin was observed in cattle; their liver samples were submitted to the diagnostic laboratory for assay of vitamin K by Madden and Stahr (149). After evaluating normal-phase and reversed-phase thin-layer chromatography plates with different solvents, reversed-phase TLC plates and a mobile phase of methylene chloride–methanol (7:3) were selected for the determination of vitamin K. The *R_f* value of vitamin K was 0.75. Levels as low as 0.2 µg were detected. Gas chromatography and densitometry can be used to quantify vitamin K in bovine liver. Mass spectroscopy can be used to confirm vitamin K present in the extracts. The method involves cleanup of tissue homogenate extracts on a Sep-Pak silica cartridge followed by separation of the vitamins by TLC on silica gel 60 F₂₅₄ as given by Hirauchi et al. (150). Separated vitamins were extracted from the silica and determined by HPLC. The eluate was subjected to coulometric reduction. The method was used in the analysis of liver, spleen, kidney, heart, and muscle. Recoveries were 73.5–91.8%, and detection limits were in the picograms per gram or picograms per liter range.

Mazulin and Kaloshina (151) described a very simple, highly sensitive method for the quantitative determination of vitamin K in milfoil plants. A portion of extracted, filtered, coagulated, cooled, and filtered sample was used for spectrophotometric determination at 265 nm. Linear calibration was followed in the range of 2–42 µg/mL. The presence of vitamin K was confirmed by using TLC on Silufol UV254 plates (Kavalier, Czech Republic) with two mobile phases—cyclohexane–diethyl ether (4:1) and hexane–diethyl ether–acetic acid (9:1:0.1)—with phosphomolybdic acid as the detection agent. This method can be used by analytical laboratories for crude drug analysis.

Menaquinone-7 was isolated from *Pseudomonas* N.C.I.B. 10590 and identified by reversed-phase thin-layer chromatography and gas chromatography mass spectral analysis (152). Ubiquinones extracted from 24 strains of *Legionella pneumophila* and from 44 strains of other *Legionella* species were also analyzed by reversed-phase TLC on octadecylsilane-bonded reversed-phase KC₁₈F TLC plates (Whatman) using acetone–water (19:1) as mobile phase. Ubiquinone profiles as determined by this method were reproducible, both qualitatively and semiquantitatively, and provided information to aid

in the identification of species of *Legionella* (140). Ubiquinone was also analyzed in the rat tapeworm *Hymenolepis diminuta* and in yeast, using TLC for isolation and HPLC for determination (153, 154). Menaquinone-6 and a methyl-substituted menaquinone-6 were the major isoprenoid quinones found in membrane preparations of *Campylobacter jejuni* and *Campylobacter fetus*. By reversed-phase HPLC and TLC the faster-eluting menaquinone-6 co-chromatographed with a menaquinone-6 standard. The identity of menaquinone-6 was confirmed by UV spectrophotometry, mass spectrometry, and nuclear magnetic resonance (NMR). The slower-eluting methyl-substituted menaquinone-6 cochromatographed with a menaquinone-7 standard by reversed-phase TLC on C-18 RPTLC plates (Analtech, Newark, DE, USA) with a solvent system of methanol–acetone (1:1) but eluted between menaquinone-6 and menaquinone-7 standards by HPLC (155).

Menaphthone (vitamin K₃), extracted from food products (butter, margarine, yogurt, beef, pork, chicken, cheese, eggs, milk), was purified on a Sep-Pak silica cartridge and/or a Sep-Pak silica cartridge followed by TLC, then measured by HPLC on an ODS-UH column with 45 dioxane saturated with argon and containing 0.2% NaClO₄. The detection limit for vitamin K₃ was 50 pg/g or pg/mL in foods (156).

Sakamoto et al. (134) determined phylloquinone (K₁) and menaquinone-4 (MK-4) in plasma and liver. They used adsorption TLC on silica gel (Merck) with 85% petroleum ether–15% ethyl ether for purification. Final separation, however, was done by HPLC. This method is useful for vitamin K studies on rats, which require micro- and multisampling methods.

D. Detection of Vitamin K

All lipoquinones at levels of 0.5 μ g or more are visible as dark spots on layers containing inorganic fluorescent material when illuminated with UV light, after adding Na-fluorescein or rhodamine B or 6G to the adsorbent or spraying the chromatographed layer with fluorescein or dichlorofluorescein reagents. These compounds can be detected in daylight and, with high sensitivity, in UV light. Vitamin K compounds can be detected with iodine vapor (brown spots), with concentrated sulfuric acid followed by heating (violet spots, limit of detection 3 μ g), with molybdophosphoric acid (gray-blue spots, limit of detection 0.5 μ g) (1–4), and with potassium hexaiodoplatinate reagent (83). Long- and shortwave UV light were used to detect vitamin K; under shortwave UV light, vitamin K showed an intense purple color (149). Vitamin K₁ was also detected with antimony(III) chloride (pink spots in visible light, limit of detection 8 μ g), with iron(III) chloride (blue spot in visible light, limit of detection 8 μ g), with sulfuric acid (green-brown spot in visible light; limit of detection 8 μ g), with iodoplatinate (yellow and brown spots in visible and UV light, respectively; limit of detection 1–8 μ g), and with phosphomolybdate (black spot in visible light; limit of detection 8 μ g) (82).

VI. SEPARATION OF MIXTURES OF LIPOPHILIC VITAMINS AND OF LIPOPHILIC VITAMINS FROM OTHER VITAMINS AND OTHER COMPOUNDS

Lipophilic vitamins A, D₂, and E (α -tocopherol) (R_f 0.34, 0.44, and 0.62, respectively) were separated on silica gel plates with a mobile phase of benzene–chloroform–acetone (88.5:8.8:2.7) (157). Information about detection and the hR_f values of lipophilic vitamins in mixtures (β -carotene, vitamin A alcohol, vitamin A acetate, vitamin A palmitate, vitamin D₂, α -tocopherol, α -tocopherol acetate, vitamin K₁, and vitamin K₃), using the various layers and solvents were given by Bolliger and König (4).

Srivastava and Parakash (71) used scolecite (200 mesh) as an adsorbent (after activation at 110°C) for TLC separation of thiamine (I), ascorbic acid (II), ergocalciferol (III), cholecalciferol (IV), and biotin (V). The R_f values with benzene-methanol-acetone-acetic acid (14:4:1:1) as mobile phase were 0.32, 0.27, 0.90, 0.80, and 0 for I–V, respectively. In chloroform the R_f values for ergocalciferol (D₂) and cholecalciferol (D₃) were 0.92 and 0.42, respectively.

Chromatographic systems have been developed for the reversed-phase TLC separation of lipophilic vitamins on RP-18 as stationary phase (158). A mixture of lipophilic vitamins (A acetate, E, E acetate, and D₃) was separated using acetonitrile–benzene–chloroform (10:10:1) as mobile phase (Table 11).

Applied chromatographic conditions do not permit the separation of vitamin E and vitamin E acetate. Derivative spectrometry was used to determine vitamin A acetate in mixtures of lipo-

Table 11 R_f Values of Lipophilic Vitamins
Separated on Various Supports

Vitamin	Support			
	RP-18 ^a	Starch ^b	Cellulose ⁵	Talc ^b
A acetate	0.86	0.82	0.85	0.86
A palmitate	—	0.25	0.33	0.27
K ₁	—	0.40	0.53	0.45
E	0.80	0.63	0.72	0.67
E acetate	0.80	0.52	0.63	0.56
D ₂	—	0.79	0.82	0.83
D ₃	0.62	0.79	0.82	0.83

^aMobile phase: acetonitrile–benzene–chloroform (10:10:1).

^bMobile phase: acetone–concentrated acetic acid (3:2); stationary phase impregnated with paraffin oil.

Source: Refs. 158, 160.

philic or water-soluble vitamins. A satisfactory separation for α -, β -, γ -, and δ -tocopherol and vitamin A acetate was obtained on silica gel G using cyclohexane–*n*-hexane–isopropyl ether– ammonium hydroxide (40:40:20:2) as mobile phase (159). Perisic-Janjic et al. (160) described a method for the quantitative analysis of lipophilic vitamins by thin-layer chromatography on starch, cellulose, and talc impregnated with paraffin oil. Vitamins A acetate, A palmitate, K₁, DL- α -tocopherol, DL- α -tocopherol acetate, D₂, and D₃ were separated with acetone–concentrated acetic acid (3:2) while vitamins K₃, K₄, and K₅ migrated with the front (Table 11). Differences between the R_f values were satisfactory (except for vitamins D₂ and D₃) and allowed for accurate identification.

On silica gel plates using diphacinone, pindone, valone, warfarin, and bromadiolone, vitamins K₁ and D₃ were separated with three mobile phases. No phase used alone could separate all seven compounds. However, vitamins K₁ and D₃ were separated with a mixture of dichloromethane– methanol–acetic acid (45:4:1) (R_f 0.75 and 0.59, respectively) and with a mixture of chloroform– methanol (97:3) (R_f 0.92 and 0.68, respectively) (82).

Thielemann (161, 162) separated vitamins A, D₂, and E from vitamins B₁, B₂, B₆, and C; nicotinamide; and panthenol, which occur in the multivitamins Summavit® (Jenapharm) and Turigeran® (Jenapharm). Lipophilic vitamins were separated on silica gel with benzene–petroleum ether–acetic acid (35:65:1). R_f values for vitamins A, D₂, and E were 0.71, 0.18, and 0.07, respectively. This method can be used in pharmaceutical investigations. Bączyk et al. (163) described a method for the determination of vitamins D₂ and K₁ in the presence of rutin added as stabilizer and assessed the rate of breakdown of these vitamins when exposed to ultraviolet light. TLC was done on silica gel H. The best developing agent was chloroform. The R_f values were 0.60 for vitamin K₁, 0.34 for vitamin D₂, and 0 for rutin. Quantitative determination of vitamins D₂ and K₁ was done by spectrophotometric analysis. Riboflavin, ascorbic acid, and nicotinamide in pharmaceutical preparations were separated by TLC on silica gel H F₂₅₄ with chloroform–95% ethanol–acetic acid–water (54:27:9:4) as mobile phase by Ni et al. (164). Vitamin A, vitamin D, and α -tocopherol were extracted from the sample with light petroleum, and the extract was subjected to TLC on silica gel H F₂₅₄ with light petroleum–ethyl ether (4:1) as mobile phase. Detection was by scanning at 440 nm (700 nm reference wavelength) for riboflavin and at 250, 260, and 300 nm (400 nm reference wavelength) for ascorbic acid, nicotinamide, and vitamin A, respectively. Recovery was 99.9%, and the corresponding coefficient of variation was 1.9% for vitamin A.

A method was described for the simultaneous determination of retinol and α -tocopherol in plasma by Chavan and Khatri (165). The sample was mixed with methanol, then extracted with heptane containing α -tocopheryl acetate (internal standard). After vortex mixing, a portion of the extract was applied to a silica gel F₂₅₄ HPTLC plate, which was developed with chloroform– cyclohexane (11:9) and evaluated densitometrically with a Camag TLC Scanner II or by diffuse reflectance absorbance at 290 nm. Calibration graphs were rectilinear for 0.2–1.4 and 3–21 μ g/mL of retinol and α -tocopherol, respectively. The corresponding detection limits were 0.16 and 1.2 μ g/mL.

Avocado (*Persea americana*) is a fruit of unusually high oil content and is relatively rich in chlorophyll. TLC detected the presence of avocado seed oil in various avocado oils. Avocado oils, extracts, and mixtures were subjected to cold ethanol precipitation. The precipitate was discarded and the ethanol was evaporated. Samples (10 mg) were

applied as a single spot on a TLC plate and eluted with petroleum ether (60–80°C)–ethyl ether (1:1). Standards of β -sitosterol, α -tocopherol, squalene, β -carotene, and β -amyrin were used to characterize unsaponifiable components and separated by TLC. The plates were sprayed with 50% H_2SO_4 , and the color was developed at 115°C for 10 min (166).

Most of the prenyl lipids, such as chlorophylls, carotenoids, and prenylquinones, as well as tocopherols and vitamin K_1 , which occur in plant lipid extracts, can be separated by TLC using silica gel plates or special mixtures of silica gel with other adsorbents (142, 167). But the compounds with one double bond per isoprene and others with a partially or fully unsaturated isoprenoid chain can be separated efficiently by argentation TLC. The separations of prenylquinones and prenols using adsorption TLC and argentation TLC are demonstrated in Fig. 8. The R_f values

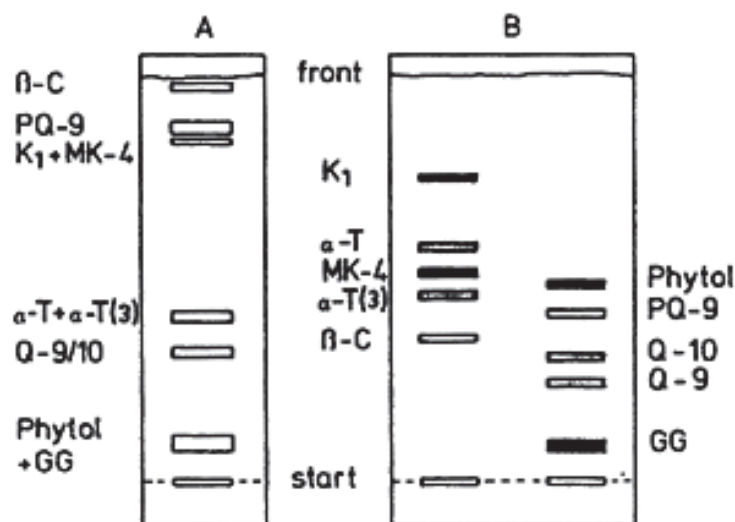


Figure 8 Thin-layer chromatography of selected lipophilic vitamins.

Adsorption chromatography on (A) silica gel plates with light petroleum–diethyl ether (9:1) as mobile phase, and (B) on silica gel plates impregnated with AgNO_3 , with light petroleum–chloroform–acetone (50:10:17) as mobile phase. K_1 , phylloquinone, vitamin K_1 ; MK-4, menaquinone-4; PQ-9, plastoquinone-9; Q-9 and Q-10, ubiquinone-9 and -10; $\alpha\text{-T}$, α -tocopherol; $\alpha\text{-T(3)}$, α -tocotrienol; $\beta\text{-C}$, β -carotene; GG, geranyl-geraniol. (From Ref. 142.)

Table 12 R_f Values of Selected Fatty Vitamins and Provitamins Separated by Argentation TLC^a

	$R_f \times 100$				
	S1	S2	S3	S4	S5
β -Carotene (provitamin A)	7	10	64	35	—
α -Tocopherol	60	70	68	56	54
α -Tocotrienol (ξ -tocopherol)	48	58	55	45	—
β -Tocopherol	58	69	64	52	—
β -Tocotrienol (ϵ -tocopherol)	46	55	49	40	—
Vitamin K ₁ (phylloquinone)	67	71	76	73	70
Vitamin K ₂₍₂₀₎ (menaquinone-4)	50	63	62	50	49
Desmethylvitamin K ₁	63	70	73	63	—
Vitamin K ₃ (menadione)	48	57	56	47	46
Plastoquinone-9	18	31	50	41	—
Ubiquinone-6	25	42	48	40	—
Ubiquinone-9	5	21	35	24	—
Ubiquinone-10	9	28	38	30	—
α -Tocoquinone	—	—	—	59	50
Vitamin A alcohol	—	—	—	62	51
Vitamin A palmitate	—	—	—	82	77
Vitamin D ₂	—	—	—	32	19
Vitamin D ₃	—	—	—	39	25

^aSolvents: S1=hexane–ethyl acetate–diisopropyl ether (2:1:2); S2=hexane–ethyl acetate–diisopropyl ether (2:2:1); S3=light petroleum (bp 50–70°C)–chloroform–acetone (50:10:24); S4=light petroleum (bp 50–70°C)–chloroform–acetone (50:10:17); S5=hexane–ethyl acetate–diisopropyl ether (2:1:1).

Source: Ref. 142.

of selected fatty vitamins and their provitamins separated by argentation TLC using different mobile phases are listed in Table 12.

The fat-soluble vitamins D- α -tocopherol and K₁ (phyllochinone) as well as β -carotene were determined in spinach by reflectance photometry after chromatography of the nonsaponified raw extracts on HPTLC silica gel plates using benzene or petroleum ether–benzene (6:1) as mobile phase. Densitograms of K₁ and E vitamins as well as β -carotene were also given (168).

Retinol, α -tocopherol, and cholecalciferol were also determined in foods, vegetables, and fruits by HPLC, spectrophotometry, and TLC scanning. Results were satisfactory and

similar with all the applied techniques (169). TLC was also used for purification and determination of lapachol and vitamin K in pau d'arco (170).

VII. GENERAL CONCLUSIONS

This chapter has discussed the use of TLC in the investigation of lipophilic vitamins. The use of thin-layer chromatography as a separation method allows for the qualitative and quantitative investigation of lipophilic vitamins. The use of TLC techniques together with densitometry gives precise and sensitive quantification of vitamins A, D, E, and K compounds on TLC plates. Thin-layer chromatography is an important investigative technique for the analysis of lipophilic vitamins.

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24

Natural Pigments

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I. INTRODUCTION

Thin-layer chromatography (TLC) remains a popular method for the analysis of natural pigments. The readily available equipment is easy to use, and operating costs are low. The progress of separation can be followed throughout the separation, the time required for completion of the process is usually short, and the results are immediately visible. Together with the robust nature of most of the systems that have been developed, these facts ensure that TLC will continue to enjoy popularity where rapid qualitative analysis required. Good separations of pigments obtained from these TLC systems are important to demonstrate chromatography to students of chemistry and biology, but the systems are also suitable for field work in biology and agriculture. It should be noted that the quantities of pigments present in most natural sources are such that TLC is often the method of choice for preparative separation. Provided that suitable precautions are taken, good quantitative analysis can often be obtained.

II. TLC IN GENERAL

A. Stationary Phase

A wide variety of TLC plates are commercially available, and many of these have found applications in the analysis of pigments. In this chapter, individual sorbents are discussed under specific pigment groups. It is also possible and cheaper to prepare TLC plates in the laboratory. Such laboratory plates can, with practice, provide excellent results and thus allow access to stationary phases or phase mixtures that are not otherwise available. The descriptions given below should allow preparation of a 0.25 mm layer with a surface area of about 2 m×20 cm.

1. Preparation of Layers

a. Silica Layers. Silica gel G (30 g) is mixed with water (60 mL), and the slurry is transferred to the TLC plates with a commercial spreader. The plates are allowed to dry for 30 min followed by activation at 120°C for 1–2 h.

b. MgO Layers. MgO (10 g) and kieselguhr G (10 g) are passed through a 60 mesh sieve followed by mixing with 80 mL of distilled water. The slurry is transferred to the plates with a commercial spreader. The plates are allowed to dry for 12 h.

c. Cellulose Layers. Cellulose powder (15 g) is mixed with distilled water (100 mL) and homogenized in a blender for 30 s. The slurry is transferred to the plates with a commercial spreader unit. The plates are allowed to dry for 6 h.

d. Sucrose Layers. Sucrose (65 g) is passed through a 60 mesh sieve followed by mixing with 100 mL of petroleum ether (60–80°C). The slurry is transferred to the plates with commercial spreading equipment. The plates are allowed to dry for 30 min.

B. Solvent

The choice of solvent is described under the respective pigment groups. Mobile-phase optimization is described elsewhere in this Handbook, but applications of optimized systems for the separation of pigments are given in this chapter.

C. Development

Development is usually performed in rectangular glass tanks lined with filter paper. A convenient volume of solvent for a 21×21×6 cm tank is 100 mL. After an equilibration period of 20 min, the plate is placed in the tank and allowed to stand until the desired developing distance is obtained.

Circular TLC is performed with the apparatus shown in Fig. 1. The system permits improved separation of the polar pigments.

Overpressured TLC (OPTLC) is described elsewhere in this Handbook. In general, the method ensures a more rapid and selective separation of mixtures. Within the realm of pigment analysis, the system has been used to separate a variety of pigments (1–3).

D. Detection

Although most pigments are immediately obvious on the plate, the use of longwave ultraviolet light may improve detection in some cases. Spray reagents have been used extensively in the flavonoid and quinonoid groups, and a silver nitrate spray has been used to distinguish certain carotenoid endgroups.

The temptation to believe that a compound that is pure with respect to other pigments is pure with respect to all contamination should be resisted. Spray reagents are valuable in identifying both the presence and identity of colorless contaminants. The use of a spray containing a good general oxidant followed by charring will give an overall picture of colorless contaminants. Where specific noncolored compounds are suspected, suitable reagents for disclosing their presence should be used.

E. Quantification

Quantification may be carried out by recovering the separated zones from the plates and measuring them by spectrophotometry in solution. This has been successfully shown for flavonoids (4), anthocyanins (5), photosynthetic pigments (6), and porphyrins (7, 8). Alternatively, densitometry may be used with visible, ultraviolet, and fluorescence detection. The results obtained from optimum densitometric measurements have recently been compared with those obtained by use of a video camera equipped with a charge-coupled device (CCD) (8a). These results are regarded as equivalent for the detection of flavonoids and other phenolics at wavelengths of 254 and 366 nm. All densitometric measurements given in this chapter were performed with a Desaga Quick Scan R & D (Helena Laboratories) apparatus.

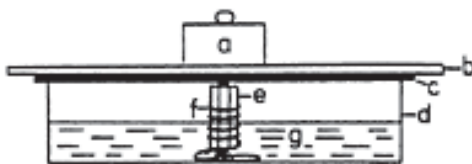


Figure 1 Simple apparatus for circular TLC. a, Weight; b, glass plate; c, chromatographic plate; d, Petri dish; e, glass cylinder filled with glass wool; f, inert metal wire as holder; g, developing solvent.

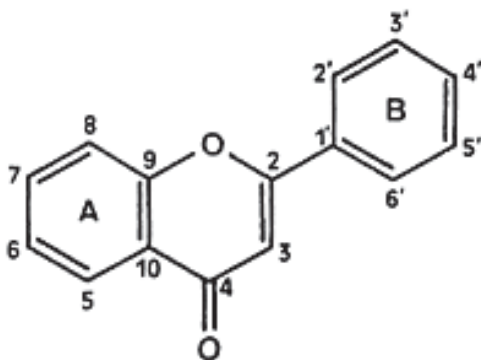


Figure 2 The 2-phenylbenzo- γ -pyrone skeleton is the parent nucleus of flavones and flavonols, the most numerous flavonoid classes. Note that the flavonols have an oxygen function in the 3-position. See Tables 2 and 3 for some typical flavones and flavonols.

III. FLAVONOIDS

A. General

1. Structures

Flavonoids occur in a variety of structural forms. They are phenolic compounds with a basic $C_6-C_3-C_6$ skeleton. The two phenyl rings may be linked by an open three-carbon chain (chalcones) or by a three-carbon chain formed into a five-membered ring (aurones) or the more usual six-membered heterocyclic ring (flavones and flavonols) (Figs. 2 and 3). Conveniently, the 5000 different flavonoids are divided into about 12 classes according to the oxidation level of the central C_3 unit. Most flavonoids occur in vivo as glycosides, which may have an aliphatic or aromatic acyl substituent on the sugar moieties. Other flavonoids are conjugated with sulfate groups. Different flavonoid classes may be linked through a common acyl moiety, and some flavonoids occur as dimers, trimers, and polymers.

2. Distribution

The flavonoid pigments are one of the most numerous and most widespread groups of natural products (9, 10, 10a). They are universally present in vascular plants. The flavonoid class anthocyanins, which is treated separately in Section IV, is the source of most orange to blue colors in petals, fruits, leaves, and roots. Other flavonoids contribute to yellow flower color either by cooccurring with carotenoids or by replacing them in about 15% of all plant species. Many colorless flavonoids contribute to flower color by acting either as copigments to anthocyanins or as the source of pastel colors. The biological properties of flavonoids have been reviewed (10b).

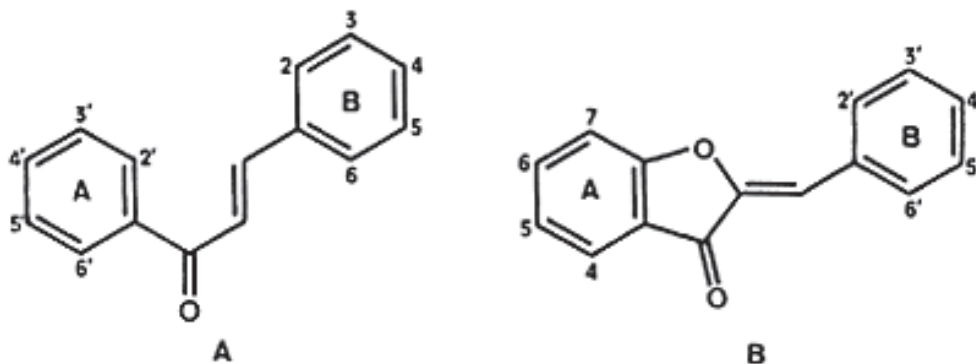


Figure 3 Parent nuclei of the yellow chalcones (A) and the orange aurones (B).

B. TLC

Thin-layer chromatography is a technique that is applicable to all classes of flavonoids and is especially useful for rapid analysis of partly purified mixtures derived from paper or column chromatography. Various TLC systems for the separation of flavonoid aglycones and glycosides have been described in the literature (Table 1) (11–13).

Aglycones of flavonols and flavones are easily separated on silica layers with traditional solvents (14, 15). Diol-bonded silica gel layers were successfully used for various types of flavonoids and coumarins (15a). Polyamide layers are often used for separation of medium polar to apolar flavonoids (16), and a solvent system containing methanol–formic acid–water (58:10:16) is ideal for the separation of several aglycones on reversed-phase layers (17). The examination of the retention behavior of some flavonoids in 2-D TLC systems performed in normal- and reversed-phase systems on plates with chemically bonded cyanopropyl stationary phase (17a) is also very interesting. For the separation of flavonoids on the preparative scale, centrifugal TLC on silica gel was used for the final purification of flavanones with toluene–ethyl acetate–acetic acid (80:19:1) as mobile phase (18) and for the isolation of flavonol glycosides by using benzene–butane–2-one–methanol (17:2:1, 15:3:2, and 13:4:3 mixtures) as mobile phase (19).

A chemometric approach in which the R_f values of 47 flavonoids in seven TLC systems were studied using principal component and cluster analyses made it possible to choose the minimum number of chromatographic systems needed to perform the best separation (20). Another method (the PRISM A model) based on Snyder's solvent selectivity triangle to aid mobile-phase optimization has been described (21). This model is reported to give good separation of flavonol glycosides from *Betula* spp. (1). When tested in our laboratory, no improvements were obtained in comparison with established systems (22) such as ethyl acetate–formic acid–acetic acid–water (100:11:11:27) on silica support, which can be used for separation of a wide range of flavonoids. In recent years methods based on information theory and numerical taxonomy have been used with success for selection of the most suitable mobile phase in TLC separations of flavonoids in various extracts (22a).

The separation of complex flavonoid mixtures demands in many cases the use of several chromatographic techniques. Thus, a comparison of the HPTLC and high-performance liquid

Table 1 TLC Systems for Selected Flavonoid Classes

Flavonoid class ^a	Layer	Solvent	Detection ^b
Aurone	Cellulose	<i>n</i> -BuOH–HOAc–H ₂ O (4:1:5)	Daylight
Biflavonoids	Silica	C ₆ H ₅ CH ₃ –HCO ₂ Et–HCO ₂ H (5:4:1)	UV
Chalcones	Cellulose	<i>n</i> -BuOH–HOAc–H ₂ O (4:1:5)	Daylight
Dihydrochalcones	Cellulose	H ₂ O	UV

Flavanones	Cellulose	HOAc (5%)	UV
Flavones/flavonols Aglycones	Silica	C ₆ H ₅ CH ₃ –HCO ₂ Et–HCO ₂ H (5:4:1)	UV
	Polyamide	C ₆ H ₅ CH ₃ –C ₂ H ₅ COCH ₃ –MeOH (60:25:15)	UV
	RP-18	MeOH–HCO ₂ H–H ₂ O (58:10:16)	UV
	Cellulose	HOAc–conc HCl–H ₂ O (30:3:10)	UV
Glycosides	Silica	CH ₃ CO ₂ C ₂ H ₅ –HCO ₂ H–HOAc–H ₂ O (100:11:11:27)	UV
	Cellulose	<i>n</i> -BuOH–HOAc–H ₂ O (4:1:5)	UV
	Cellulose	HOAc (15%)	UV
Isoflavones	Silica	CHCl ₃ –MeOH(89:11)	UV

^aSee Section IV for anthocyanins.

^bSpray reagent may improve detection.

chromatographic (HPLC) behavior of 26 flavonoids and a method for establishing HPLC gradient elution conditions by using TLC data (23) is useful.

Being colorless to yellow, the flavonoids may be difficult to detect in visible light. By using a TLC support treated with fluorescent indicator, it is possible to detect most flavonoids as quenching bands or spots. The measurement of the fluorescence emitted by flavones and flavonols in situ on TLC plates can be improved by dipping the developed plates in appropriate solutions (23a). Alternatively, a number of spray reagents may be used to enhance spot detection (13). The strength of, for instance, 1% of the diphenylboric acid–ethanolamine complex in methanol (Naturstoffreagenz A, NP) as a spray reagent lies in its ability to distinguish between different substitution patterns. Inspected under longwave UV, flavones and flavonols with 4'-hydroxylation produce green spots, whereas similar flavonoids with 3',4'- and 3',4',5'-hydroxylation are revealed as yellow and orange spots, respectively. The sensitivity may be improved by further spraying with a 5% ethanolic (or methanolic) solution of polyethyleneglycol 4000 (PEG 4000) (24). The lower limit of detection is now assumed to be 0.5 ng. The influence of the derivatization technique, plate drying, reagent concentration, and selected detector parameters on the densitometric determination after treatment with NA and PEG 4000 was investigated using the two main flavonoid glucuronides from *Malva silvestris* leaves (25). Diphenyltin dichloride has proved to be a useful chromogenic reagent for the detection of flavones and flavonols by forming fluorescent complexes of different colors (26).

C. Practical Experiments

1. Extraction

Fresh plant tissue is macerated in a blender with hot methanol for a few minutes. An alternative procedure uses a solvent mixture of methanol and water. The water content is

usually 15% in the first extraction step and 50% in the final extraction. The two extracts are then combined before chromatographic treatment.

2. Hydrolysis

The extract (or purified compound) in methanol is mixed with an equal volume of 2 M HCl and refluxed on a water bath for 60 min. The mixture is cooled, and the liberated aglycones are extracted with ethyl acetate or diethyl ether and analyzed by TLC. The C-glycosides and some *O*-glucuronides are not hydrolyzed under these conditions, and their presence may be checked for by their relatively high TLC mobility on cellulose using H₂O as the mobile phase.

3. Separation of Flavone and Flavonol Aglycones

The TLC systems presented for flavone and flavonol aglycones (Table 2) reveal different separation mechanisms. Thus, a combination of various systems gives valuable information during the analysis of these pigments.

a. Silica Gel Layers. Solvent system 1, benzene–ethyl acetate–formic acid (40:10:5), and system 2, toluene–ethyl formate–formic acid (50:40:10), give acceptable separation of many flavonoid aglycones on silica gel. The plates are developed over 8.5 cm (15–20 min), and the aglycones are sprayed with the NP/PEG 4000 reagent before inspection under longwave UV. Typical colors and R_f values are given in Table 2.

Systems 1 and 2 reveal similar selectivity and resolution with respect to flavone and flavonol aglycones; however, these compounds are generally more slow-moving (more retained) in system 1 (Table 2). Although addition of a hydroxyl group at position 5 in the A-ring decreases retention, addition of hydroxyl groups to other positions in flavones results in a marked increase in retention. For instance, 5-hydroxyflavone, flavone, and 7-hydroxyflavone have R_f values of 0.74, 0.60, and 0.48, respectively, in system 2. The intramolecular hydrogen bond between the 5-hydroxyl group and the keto group at the 4-position is responsible for the reduced polarity of 5-hydroxyflavone. The same effect is observed in flavonols; the R_f values of robinetin and myricetin are 0.22 and 0.32, respectively, in system 2 (Table 2). The flavonols possess a hydroxyl group at the 3-position, which also may interact with the keto group by forming a hydrogen bond. As a consequence, the flavones are in general more slow-moving than the corresponding flavonols in systems 1 and 2.

Table 2 R_f Values and Colors of Flavonoid Aglycones on Silica Gel, Polyamide, and Reversed-Phase Layers^a

Pigment, spot color ^a	Substituent position ^b		System ^c			
	OH	OCH ₃	1	2	3	4
Flavones						
Flavone, b			0.50	0.60	0.86	0.29
5-Hydroxyflavone, br	5		0.63	0.74	0.88	0.22
6-Hydroxyflavone, gr	6		0.31	0.50	0.65	0.39
7-Hydroxyflavone, b	7		0.29	0.48	0.51	0.41
7,8-Dihydroxyflavone, gr-br	7,8		0.22	0.43	0.32	0.57
Chrysin, br-g	5,7		0.48	0.57	0.52	0.33
Apigenin, g	5,7,4'		0.29	0.46	0.18	0.48
Acacetin, gr-g	5,7	4'	0.42	0.54	0.45	0.30
Apigenin-7', 4'-dimethyl ether, gr-g	5	7,4'	0.56	0.66	0.86	0.14
Luteolin, y	5,7,3',4'		0.22	0.38	0.06	0.53
Diosmetin, gr	5,7,3'	4'	0.23	0.43	0.41	0.46
Tangeretin, gr		5,6,7,8,4'	0.25	0.48	0.89	0.26
Flavonols						
Galangin, g	3,5,7		0.51	0.59	0.41	0.38
Kaempferol, y-g	3,5,7,4'		0.32	0.48	0.14	0.57
Kaempferid, b-g	3,5,7	4'	0.48	0.59	0.38	0.37
Kaempferol-7, 4'-dimethyl ether, b-g	3,5	7,4'	0.63	0.73	0.74	0.18
Kaempferol-3, 4'-dimethyl ether, ol	5,7	3,4'	0.43	0.55	0.61	0.35
Kaempferol- 3, 7, 4'-trimethyl ether, g	5	3,7,4'	0.61	0.69	0.87	0.19
Quercetin, br-o	3,5,7,3',4'		0.23	0.41	0.06	0.67
Tamarixetin, g	3,5,7,3'	4'	0.31	0.48	0.19	0.53
Rhamnetin, o	3,5,3',4'	7	0.29	0.48	0.24	0.45
Quercetin-3,7-dimethyl ether, o	5,3',4'	3,7	0.27	0.46	0.47	0.46
Morin, g	3,5,7,2',4'		0.17	0.29	0.02	0.73

Fisetin, o	3,7,3',4'	0.21	0.36	0.08	0.70
Robinetin, o	3,7,3',4',5'	0.08	0.22	0.01	0.82
Myricetin, o-br	3,5,7,3',4',5'	0.13	0.32	0.02	0.76
Quercetagenin, r-br	3,5,6,7,3',4'	0.00	0.00	0.01	0.95

^aPigment colors are observed under UV, 366 nm after spraying plates with Naturstoffreagenz A followed by polyethyleneglycol (PEG-4000). b=blue, br=brown, g=green, gr=gray, o=orange, ol=olive, r=red, y=yellow.

^bSee Fig. 2 for structures.

^cSystems: 1 and 2, silica gel (60 F₂₅₄, 0.25 mm), benzene–ethyl acetate–formic acid (40:10:5) and toluene–ethyl formate–formic acid (50:40:10); 3, polyamide (DC-Alufolien F₂₅₄, 0.15 mm), toluene–butan-2-one–methanol (60:25:15); 4, reversed-phase (C-18 F₂₅₄, 0.25 mm) methanol–formic acid–water (58:10:16).

Replacing a hydroxyl with a methoxyl function usually results in higher R_f values. However, a methoxyl group at the 3-position cancels the effect of the existing hydrogen bond in the parent hydroxyl compound, and increased retention is observed.

b. Polyamide Layers. Samples (3 μ L) are applied on a polyamide sheet and developed over 8.5 cm (25 min) with toluene–2-butanone–methanol (60:25:15) as mobile phase. NP/PEG 4000 is used as detection reagent, and the colors are similar to those produced in the preceding system. R_f values are given under system 3 in Table 2.

Introducing hydroxyl functions onto the B-ring produces the same increase in retention as was observed for flavonoid aglycones chromatographed on silica systems. However, the effects of hydroxyl substituents at positions 5 and 3 are somewhat different. No significant change in retention is observed when a hydroxyl function is introduced in the 5-position. This may indicate that the contribution of intramolecular hydrogen bonding is less effective in the case of polyamide than in the case of silica layers. Polyamide is known to form extensive hydrogen bonds between the amide carbonyl function and the hydroxyl substituents of phenolic compounds. This effect can be observed for flavonols, which are more retained than the corresponding flavones. Instead of taking part in internal hydrogen bonding, the 3-hydroxyl group interacts with the polyamide layer. Methylation of any hydroxyl group prevents the formation of hydrogen bonding between the flavonoid and the stationary phase, and a marked decrease in retention is observed.

c. Octadecyl-Bonded Silica (RP-18) Layers. Samples (3 μ L) are applied on RP-18 support and developed over 8.5 cm (35 min) with methanol–formic acid–water (58:10:16) as the mobile phase. The pigments are visualized with NP/PEG-4000 reagent, and the plate is inspected under longwave UV. The colors observed are comparable to those seen with the preceding systems. Measured R_f values are given in Table 2 under system 4.

The retention of flavones and flavonols is partly determined by their polarity, as evidenced by the decreased retention with increasing hydroxylation on the pigments (Table 2). In contrast to this trend, a 5-hydroxyl group takes part in intramolecular hydrogen bonding to the carbonyl group, and increased retention is observed; the R_f values of 7-hydroxy- and 5,7-dihydroxyflavone are 0.41 and 0.33, respectively, in system 4 (Table 2). Whereas addition of a new methoxyl function on the pigment has little effect

on its mobility on RP-18 layers, the replacement of a hydroxyl with a methoxyl function gives markedly increased retention (Table 2).

4. Separation of Flavonoid Glycosides

Samples (3 μ L) of flavones and flavonols are applied on a silica plate and developed over 8.5 cm (35 min) with ethyl acetate–formic acid–acetic acid–water (100:11:11:27) as the mobile phase. After development, the plate is dried and inspected under longwave UV before and after it has been sprayed with the NP/PEG 4000 reagent. Typical R_f values and colors for some flavonoids are given in Table 3.

The flavonoids that are separated on silica (Table 3) give well-defined bands with R_f values ranging from 0.20 to 0.72. The monoglycosides are less retained than the diglycosides. The retention with respect to the type of monosaccharide substituent increases as follows: arabinofuranoside>rhamnoside>arabinopyranoside>glucoside>galactoside.

The separation of flavonoid glucosides in *Betula* spp. with fluorescence quenching as the detection mode is shown in Fig. 4.

Methanolic extracts of *Coreopsis* spp., *Dahlia* spp., and *Helichrysum bracteatum* containing chalcones and aurones were tested on silica gel plates with ethyl acetate–formic acid–water (60:12:16) as the mobile phase. The plates were developed over 8.5 cm (about 40 min), dried, and sprayed with NP/PEG 4000. The clear yellow zones turned to violet and red, and the red-toorange fluorescent colors seen under longwave UV light confirmed the presence of chalcone and aurone pigments. Ammonia vapor intensifies the colors after spraying.

IV. ANTHOCYANINS

A. General

1. Structure

Anthocyanins are water-soluble glycosides of anthocyanidins and are part of the phenolic group known collectively as flavonoids (see Sec. III). The anthocyanidins (aglycones) are polyhydroxy and polymethoxy derivatives of the 2-phenylbenzopyrylium cation (Fig. 5). Eighteen different anthocyanidins (aglycones) have been reported, but only six of them (Table 4) are widespread. With the exception of the rare deoxyanthocyanins, the 3-hydroxyl is always replaced by a sugar; however, glycosylation at the 7-, 3'-, 5'-, and, especially, the 5-hydroxyl groups is encountered

Table 3 R_f Values of Selected Flavones and Flavonols on Silica Gel 60 F₂₅₄ (0.25 mm, Merck)

Pigment ^a		Source ^b	Substituent position ^c		<i>R_f</i>
			OH	OCH ₃	
Flavones					
Apigenin-8- <i>C</i> -glu	Vitexin	a	5,7,4'		0.56
Apigenin-6- <i>C</i> -glu-7- <i>O</i> -glu	Saponarin	b	5,7,4'		0.20
Apigenin-7- <i>O</i> -glu		c	5,7,4'		0.57
Apigenin-7- <i>O</i> -apiosylglu	Apiin	d	5,7,4'		0.39
Luteolin-7- <i>O</i> -glu		c	5,7,3',4'		0.54
Diosmetin-7- <i>O</i> -rhaglu	Diosmin	e	5,7,3'	4'	0.31
Flavonols					
Kaempferol-3- <i>O</i> -rha		f	3,5,7,4'		0.72
Kaempferol-3- <i>O</i> -glu	Astragalin	g	3,5,7,4'		0.65
Kaempferol-3- <i>O</i> -gal		f	3,5,7,4'		0.59
Quercetin-3- <i>O</i> -ara(f)		h	3,5,7,3',4'		0.72
Quercetin-3- <i>O</i> -rha	Quercetin	h	3,5,7,3',4'		0.69
Quercetin-3- <i>O</i> -ara(p)		h	3,5,7,3',4'		0.61
Quercetin-3- <i>O</i> -glu	Isoquercitrin	i	3,5,7,3',4'		0.53
Quercetin-3- <i>O</i> -gal	Hyperoside	h	3,5,7,3',4'		0.51
Quercetin-3- <i>O</i> -rut	Rutin	j	3,5,7,3',4'		0.30
Isorhamnetin-3- <i>O</i> -glu		k	3,5,7,4'	3'	0.58
Isorhamnetin-3- <i>O</i> -rut	Narcissin	k		3,5,7,4',3'	0.36
Tamarixetin-7- <i>O</i> -rut		l	3,5,7,3'	4'	0.34
Myricetin-3- <i>O</i> -rha		m	3,5,7,3',4',5'		0.58
Myricetin-3- <i>O</i> -glu		n	3,5,7,3',4',5'		0.46
Myricetin-3- <i>O</i> -gal		o	3,5,7,3',4',5'		0.45

Developing solvent: Ethyl acetate-formic acid-acetic acid-water (100:11:11:27).

^aglu=glucoside, rut=rutinoside, gal=galactoside, rha=rhamnoside, ara(f)=arabinofuranoside, and ara(p)=arabinopyranoside.

^bSuitable pigment sources: a, *Cratageus monogyna*; b, *Saponaria officinalis*; c, *Anthemis nobilis*; d, *Petroselinum* spp.; e, *Diosma crenulata*; f, *Menyanthes trifoliata*; g, *Astragalus* spp.; h, *Betula* spp.; i, *Fanisetum arvense*; j, *Ruta graveolens*; k, *Calendula officinalis*; l, *Tamarix* spp.; m, *Myrica*

rubra; n, *Primula sinensis*; o, *Camellia sinensis*.

^cSubstituent position refers to the corresponding aglycone and the numbering pattern in Fig. 2.

quite frequently. Acylation of sugars with aromatic and aliphatic acids is also of widespread occurrence. To make the analysis even more complicated, each anthocyanin may occur in different structural forms depending on factors such as pH, copigmentation, and metal chelation.

2. Distribution

In addition to the more restricted occurrence of the red carotenoids and the red to purple betalains and anthraquinones, anthocyanins are largely responsible for the scarlet through purple to blue colors of flowers, fruits, roots, and leaves of higher plants, fruit juices, red wines, etc. (27). They accumulate in the vacuoles of epidermal or subepidermal cells, but they may also be confined to the leaf mesophyll. The number of identified anthocyanins has increased dramatically in recent years to a total of 600 (author's records).

B. TLC

Paper chromatography, TLC, HPLC, column chromatography (ion-exchange resins, poly amide powder, gel material), and countercurrent chromatography have been the most commonly used

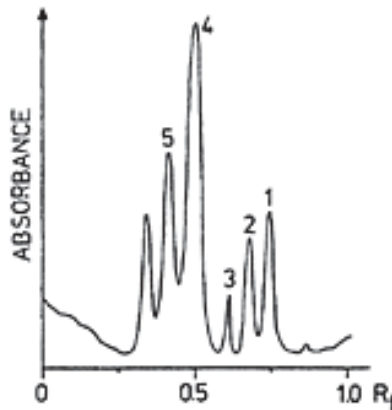


Figure 4 Separation of flavonol glycosides from *Betula* spp. Solvent system: ethyl acetate–formic acid–acetic acid–water (100:11:11:27). Stationary phase: silica gel 60 F₂₅₄ (0.25 mm, Merck). Developing distance: 8.5 cm. Detection: absorbance at 254 nm (fluorescence quenching in reflection mode). Peak

identities: (1) quercetin-3-*O*-ara(f), (2) quercetin-3-*O*-rha, (3) quercetin-3-*O*-ara(p), (4) quercetin-3-*O*-gal, (5) myricetin-3-*O*-gal (tentative).

methods for the separation and purification of anthocyanins (28, 28a). Among the TLC systems, cellulose exhibits the best properties for the separation of both anthocyanins and anthocyanidins. A solvent system composed of concentrated hydrochloric acid (25%), formic acid (24%), and water (51%) (FHW) is used for the simultaneous separation of anthocyanidins and their mono-, di-, and triglycosides (29), which is particularly valuable in structure elucidation of anthocyanins by hydrolytic techniques. Complete separation of the six common anthocyanidins is best performed using two-dimensional TLC (30). An HPTLC densitometric method for quantitative analysis of the anthocyanins of mallow flowers on cellulose plates has proved to be more sensitive than an HPLC-DAD system (detection at 530 nm) (30a). Anthocyanins from grapes have been separated by means of repeated chromatography on reversed-phase material (Macherey-Nagel, SIL-RP-18) using first 20%, and then 40% methanol (in 0.25% cone, hydrochloric acid) as the mobile phase (31). Other TLC systems for separation of anthocyanins have been described in detail (32).

For assessment of structure of the anthocyanin, it is useful to develop the cellulose plate first in an aqueous solvent such as FHW and then in an alcoholic solvent such as 1-butanol–acetic acid–water (4:1:5, upper phase) (BAW). With otherwise similar structures the R_f values of the anthocyanins in BAW normally increase with the addition of acyl moieties and decrease with increasing number of sugar units, whereas the addition of aromatic acyl moieties results in a decrease in R_f values in FHW and a corresponding increase with addition of aliphatic acyl groups and as the number of sugar units increases. The chromatographic behavior of anthocyanins sub-

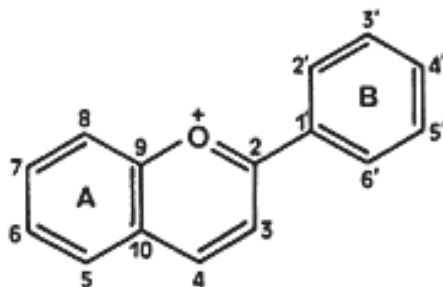


Figure 5 The 2-phenylbenzopyrylium ion, the skeleton of anthocyanidins. See Table 4 for typical anthocyanidins and anthocyanins.

Table 4 R_f Values of Anthocyanidins and Anthocyanins on Cellulose (0.1 mm, Merck)

Pigment ^a	S ^b	Substituent position ^c		Solvent system		
		OH	OCH ₃	1	2	3
Aglycones						
Delphinidin	a	3,5,7,3',4',5'		—	0.11	0.03
Petunidin	a	3,5,7,4',5'	3'	—	0.20	0.05
Cyanidin	a	3,5,7,3',4'		—	0.22	0.06
Malvidin	a	3,5,7,4'	3',5'	—	0.27	0.07
Peonidin	a	3,5,7,4'	3'	—	0.31	0.08
Pelargonidin	a	3,5,7,4'		—	0.35	0.11
Monoglycosides						
Dp-3-glu	b			0.08	0.38	0.13
Pt-3-glu	c			0.13	0.49	0.23
Cy-3-glu	b			0.17	0.51	0.25
Mv-3-glu	c			0.22	0.64	0.34
Pn-3-glu	c			0.25	0.64	0.38
Pg-3-glu	d			0.32	0.65	0.40
Diglycosides (biosides)						
Dp-3-rut	b			0.24	0.69	0.36
Cy-3-rut	b			0.35	0.69	0.49
Pn-3-rut	e			0.47	0.76	0.63
Cy-3-sam	f			0.47	—	0.64
Cy-3-sop	f			0.62	0.81	0.75
Diglycosides						
Cy-3,5-diglu	g			0.38	0.70	0.52
Pn-3,5-diglu	g			0.49	0.81	0.67
Triglycosides						
Cy-3-glurut	f			0.80	0.86	0.88

^aDP=delphinidin, Pt=petunidin, Cy=cyanidin, Mv=malvidin, Pn=peonidin, Pg=pelargonidin, glu=glucoside, rut=rutinoside, sam=sambubioside, sop=sophoroside, glurut=(2^G-glucosyl)rutinoside.

^bPigment source: a, hydrolysis product; b, *Ribes nigrum* berry; c, *Vitis vinifera* fruit; d, *Fragaria* spp. berry; e, *Prunus* spp. fruit; f, *Rubus idaeus* berry; g, *Fuchsia* spp. flowers.

^cSubstituent position refers to the numbering in Fig. 5.

^dA mixture of cone, hydrochloric acid, formic acid, and water is used as follows: System 1 (19:19:62), system 2 (7:51:42), and system 3 (25:24:51).

jected to cellulose TLC and paper chromatography is comparable, and R_f data for a large number of anthocyanins have been provided by paper chromatography (10).

Anthocyanins are visible at the concentration levels encountered on chromatograms. Examination under UV light is worthwhile, however, because the 3,5-diglycosides of pelargonidin, peonidin, and malvidin are distinguished by their fluorescence from the corresponding 3-glycosides. After the dried chromatograms have been sprayed with aluminum chloride (3% in methanol), anthocyanins with their free adjacent hydroxyls on the B-ring of the aglycone (delphinidin, cyanidin, and petunidin derivatives) turn blue.

C. Practical Experiments

1. Extraction

Anthocyanin extraction should be performed using methanol or ethanol mixed with a weak acid such as acetic acid (5%) or trifluoroacetic acid (3%). The low pH of the extract facilitates the

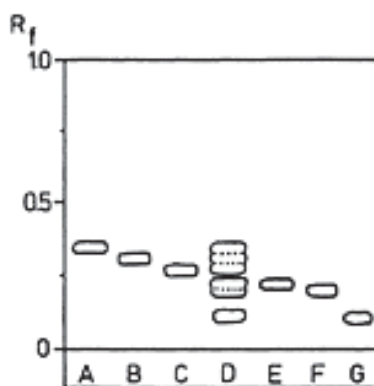


Figure 6 TLC separation on cellulose (0.1 mm, Merck) of the six common anthocyanidins using concentrated hydrochloric acid–formic acid–water (7:51:42) as mobile phase. Band identities: (A) pelargonidin, (B) peonidin, (C) malvidin, (D) mixture of pigments A–C and E–G, (E) cyanidin, (F) petunidin, (G) delphinidin.

formation of the favorable flavylium ion. When anthocyanins acylated with aliphatic acids are isolated by using solvents containing a mineral acid (such as HCl), they are rapidly degraded to the corresponding unacylated glycoside. Acetone has been used for extraction in recent years, although in some cases its use may lead to artifacts. Anthocyanins in solution are generally prone to degradation, and the pigments should be stored as cold as possible in the dark, preferably in the dried state.

2. Anthocyanidin Formation by Hydrolysis

The extract (or isolated anthocyanin in alcoholic solvent) is mixed with 4 M hydrochloric acid (1:1) and refluxed for 30 min at 100°C. After cooling, the liberated anthocyanidins are extracted into 1-pentanol, transferred to a Petri dish placed on a thermoplate (40°C), and dried under a nitrogen stream.

3. Separation of Anthocyanidins on Cellulose

Separation of the six common anthocyanidins with solvent system 2 (Table 4) is illustrated in Fig. 6. It is evident that complete separation of anthocyanidins with closely related R_f values is difficult, and two-dimensional TLC may thus be preferable (Fig. 7).

The sample (dissolved, e.g., in methanol containing 1% concentrated hydrochloric acid) is spotted on a corner of a 10×10 cm plate and developed with acetic acid-conc. hydrochloric acid-water (30:3:10). After complete drying, the plate is turned 90° and developed with metha-

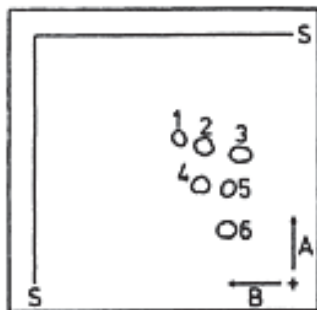


Figure 7 Two-dimensional separation on cellulose (0.1 mm, Merck) of the six common anthocyanidins. Mobile phase A: acetic acid-conc. hydrochloric acid–water (30:3:10). Mobile phase B: methanol– conc hydrochloric acid–water (190:1:10). Developing distances: 8 cm in each direction. Spot identities: (1) pelargonidin, (2) peonidin, (3)

malvidin, (4) cyanidin, (5) petunidin,
(6) delphinidin. S=solvent front.

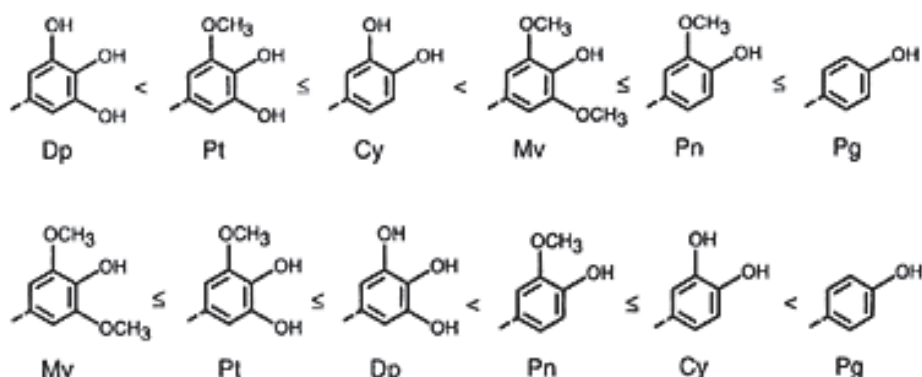


Figure 8 Typical TLC retention order on cellulose of anthocyanidins with respect to their B-ring substituents. See Table 4 for explanation of abbreviations. Top row: Aqueous solvents as mobile phase. This retention order is also observed for anthocyanin 3-*O*-monoglycosides. Bottom row: Alcoholic solvent as mobile phase.

nol–conc. hydrochloric acid–water (190:1:10). The chromatogram (Fig. 7) reveals complete separation of the six common anthocyanidins.

The observed R_f values are related to structural differences in the B-ring. After development in the first direction, the anthocyanidins are located in three groups according to the number of hydroxyl groups on their B-rings (Fig. 8, top). The solvent used in the second direction resolves the pigments largely according to the number of oxygen-containing substituents on the B-rings (Fig. 8, bottom).

4. Separation of Anthocyanins on Cellulose

Separation of typical anthocyanins is carried out on cellulose layers in three solvent systems that all contain concentrated hydrochloric acid, formic acid, and water in different proportions: system 1, 19:19:62; system 2, 7:51:42; and system 3, 25:24:51. The mobile phase is run 18 cm (about 120 min). The R_f values of the individual compounds are given in Table 4.

The anthocyanidins are completely separated from the anthocyanins in systems 2 and 3. System 2 gives the best division between the anthocyanidins; however, none of the six common anthocyanidins is completely separated (Fig. 6). Because the A-ring substituents of the common anthocyanidins are identical, the observed R_f values seem to be related to

the number of hydroxyl groups on the B-rings (Fig. 8, top). The introduction of sugar units gives higher R_f values; however, the same separation pattern with respect to the B-ring substituents is observed.

Densitometric profiles of the separation of anthocyanins isolated from black currant (*Ribes nigrum*) and raspberry (*Rubus idaeus*) are given in Figs. 9 and 10, respectively.

V. CAROTENOIDS

A. General

1. Structure

The carotenoids are yellow to red tetraterpenoids in which eight isoprene units are arranged in a symmetrical linear pattern and after biosynthetic dehydrogenation provide a polyene chromophore that absorbs light in the visible part of the spectrum. Skeletal variation is largely confined to cyclization of the endgroup, whereas oxygenation yields alcohols, ketones, *etc.* (see Fig. 11). The carotenoid hydrocarbons are sometimes distinguished from the oxygenated carotenoids by using the class names carotene for the former and xanthophyll for the latter.

2. Distribution

Carotenoids are essential components of all photosynthetic tissue, in which they are largely located in the chloroplasts. They are also responsible, either alone or together with other pigments, for

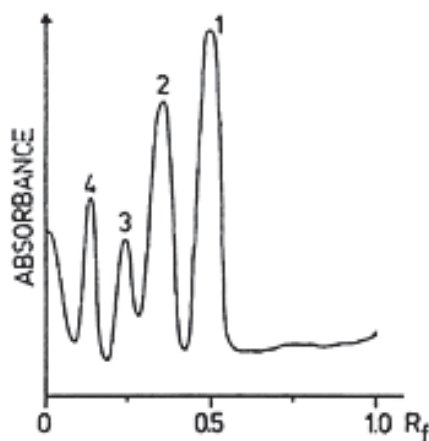


Figure 9 TLC separation on cellulose (0.1 mm, Merck) of anthocyanins from black currant (*Ribes nigrum*) with densitometric detection at 525 nm using concentrated hydrochloric acid—

formic acid– water (25:24:51) as mobile phase. Peak identities: (1) cyanidin-3-rutinoside, (2) delphinidin-3-rutinoside, (3) cyanidin-3-glucoside, (4) delphinidin-3-glucoside.

the colors of many fruits and flowers. Their occurrence in fungi and nonphotosynthetic bacteria is more sporadic, but their contribution to animal coloration in both vertebrates and invertebrates is important.

Although the relationship between carotenoids and chlorophylls in photoharvesting explains their presence in plants, a multitude of other functions is emerging for these compounds in all types of organisms. It has been suggested that these, including the photofunctions, are all adaptations from their origin as cell membrane reinforcers in archaeobacteria (32a). Such an explanation helps explain why these compounds are now being discovered in specific tissues in a wider range of organisms.

Although frequently analyzed as free C_{40} compounds, carotenoids often occur as esters or in the form of more or less tight lipid and protein complexes. Reviews describing the distribution of carotenoids in general (33) or in special groups of organisms i.e., plants (34, 35), microorganisms (34), and animals (36), not only provide useful background material for new investigations but also represent handy guides to reference compounds. A book by Britton et al. (37) describing

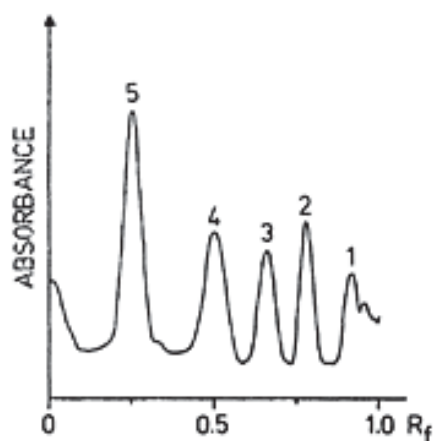


Figure 10 TLC separation on cellulose (0.1 mm, Merck) of anthocyanins from raspberry (*Rubus idaeus*) with densitometric detection at 525 nm using cone, hydrochloric acid–formic acid–water (25:24:51) as mobile phase. Peak identities: (1) cyanidin-3-(2^G-glucosyl)-rutinoside, (2) cyanidin-3-sophoroside, (3) cyanidin-3-

sambubioside, (4) cyanidin-3-rutinoside, (5) cyanidin-3-glucoside.

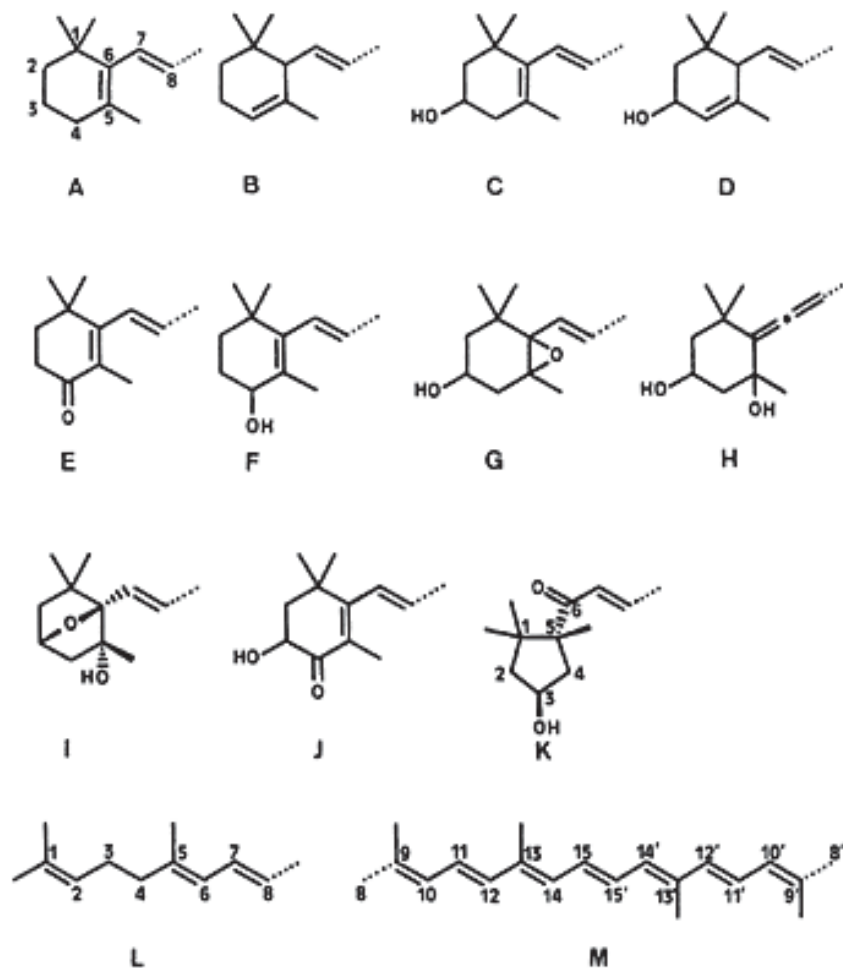


Figure 11 Structures of some carotenoids. Refer to Table 5 for compound identities.

both the isolation and analysis of carotenoids is an invaluable guide for all those working in the field.

B. TLC

Numerous systems exist for the normal-phase (38, 39) or reversed-phase TLC separation of carotenoid pigments (6, 40). Continuing progress is still being made on systems for the separation and isolation of groups of compounds of more limited distribution. Algal carotenoids, in particular, are of increasing interest, and useful studies dealing with the isolation of peridinin and diadinoxanthin and of carotenoids in the family Prasinophyceae have appeared (40a, 40b). A specialized study of the chromatography of compounds

containing the 5-hydroxy-3,6-epoxy endgroup (Fig. 11, group I) was carried out by Deli (40c).

In general, silica gel layers given efficient separation of the most common pigments with solvent systems containing a polar modifier in petroleum ether. Acetone or a tertiary alcohol is recommended as the polar component (41). These systems can also be used in the investigation of animal pigments. MgO layers should be employed for the separation of isomeric carotenoids (42), although alumina layers have also been used for this purpose (43).

The power of TLC in the isolation of pure carotenoids is emphasized by the claim that TLC and mass spectrometry in combination allow the identification of carotenoids from complex sources without the need for the presence of primary standards (43a).

C. Practical Experiments

1. Isolation

a. Isolation from Plant Tissue. A fresh sample is macerated and extracted in a blender with methanol–acetone (2:1), a procedure that has the advantage of dehydrating the plant material. The mass is filtered and reextracted with portions of acetone until a colorless filtrate is obtained. When large amounts of carotenes are present, it may be advantageous to include one or more extractions with petroleum ether or hexane, or with mixtures of these with acetone, after most of the water in the tissue has been removed in the first extraction. In a similar vein, when highly polar carotenoids, e.g., carotenoid glycosides, are present, it may be necessary to extract with pure methanol to remove the bulk of these compounds. The combined filtrate is concentrated under reduced pressure at not more than 40°C. The pigments are extracted with diethyl ether and washed with 10% aqueous sodium chloride. Finally the extract is washed with water and concentrated. Any remaining traces of water are azeotropically distilled with benzene-methanol under reduced pressure. The isolated pigment mixture is dissolved in diethyl ether and should then be stored under a nitrogen atmosphere at low temperature (−30°C).

b. Isolation from Animal Tissue. Animal tissue is commonly extracted with acetone, and a further cleanup procedure is followed as for plant pigments. Carotenoproteins, widely found in animals, may be isolated as such by specialized procedures (36).

c. Saponification. The xanthophylls are usually esterified with long-chain fatty acids, especially in fruits, flower petals, and animal tissue, and a saponification step is required to liberate the free pigments. The extract is taken to dryness and mixed with diethyl ether and 10% methanolic sodium hydroxide (1:1). The mixture is allowed to stand for 6 h. Saponified pigments are extracted with diethyl ether and washed to neutrality with portions of water containing 10% NaCl. The extract is concentrated, and the last traces of water are removed azeotropically under reduced pressure. Finally the pigments are dissolved in acetone and stored at low temperature. Precipitated compounds (e.g., sterols and proteins) are removed by centrifugation or by filtering through a wad of glass wool.

Saponification of extracts from animal sources is usually omitted to prevent formation of artifacts. Many animal pigments contain the 3-hydroxy-4-keto combination (see group J in Fig. 11), which is readily converted to the corresponding diketo artifact when treated

with base. Astaxanthin, one of the most common pigments in animals, is easily oxidized to astacene in the presence of base.

2. Separation

a. Silica Gel Layers. The extract is applied in 3 μL samples as bands (1 cm) and developed over 8.5 cm (15 min) with different portions of a polar solvent in light petroleum (40–60°C). β -Carotene moves close to the solvent front in all systems and is used as a standard with a value of 100. R_{β} values are given in Table 5. The colors are mainly orange to yellow. Spraying with silver nitrate may be used to distinguish compounds varying in structure only with respect to α and β endgroups, e.g., the pairs α -carotene– β -carotene and lutein–zeaxanthin (44). Epoxides may be identified by exposing the plate to hydrochloric acid fumes, at which time the epoxides are decomposed and the affected zones rapidly turn blue or green, it is clear that the acetone-based system separates the pigments into groups mainly according to the oxygen-bearing functional groups. The monools are located with canthaxanthin in the upper part of the plate. The diols, represented by lutein and zeaxanthin, are inseparable from the recently discovered cucurbitaxanthin-thin B even though the latter contains an additional epoxy group. Taraxanthin and antheraxanthin are slightly more polar than lutein and zeaxanthin owing to the presence of an additional 5,6-epoxy function. A keto group seems to affect the retention in the same manner as an epoxy substituent, and capsanthin is thus located in the group with the two former pigments at R_{β} about 0.40. Violaxanthin and capsanthin are grouped in the lower part of the plate and are completely separated from neoxanthin.

Carotenoids are commonly separated on silica gel with acetone as the polar component of the developing solvent. Such a system readily separates the most commonly found carotenoids

Table 5 R_{β} Values (β -Carotene=100) of Pigments on Silica Gel 60 (0.25 mm, Merck)^a

Pigment	Structure ^b	S ^c	Acetone (%)			<i>t</i> -Butanol (%)			<i>t</i> -Pentanol (%)		
			40	20	10	40	20	10	40	20	10
β -Carotene	A-M-A	a	100	100	100	100	100	100	100	100	100
Lycopene	L-M-L	b	100	97	87	100	100	100	100	100	100
β -Cryptoxanthin	A-M-C	a	72	34	11	90	78	51	91	76	47
Canthaxanthin	E-M-E	c	71	36	—	82	69	43	82	66	33
Gazaniaxanthin	C-M-L ^d	d	70	33	—	90	79	51	92	80	49
Cucurbitaxanthin A	I-M-C	i	66	11	—	80	59	26	82	56	18
Cucurbitaxanthin B	I-M-G	i	44	7	—	75	49	18	74	41	10
Lutein	C-M-D	e	44	7	—	80	56	23	82	53	15
Zeaxanthin	C-M-C	g	44	7	—	80	55	22	80	50	13
Taraxanthin	D-M-G	g	41	—	—	73	43	16	72	37	7

Antheraxanthin	C-M-G	h	40	—	—	70	40	12	69	31	—
Capsanthin	C-M-K	f	39	—	—	69	38	11	67	28	—
Violaxanthin	G-M-G	e	33	—	—	62	30	—	58	19	—
Capsorubin	K-M-K	f	30	—	—	60	24	—	56	14	—
Neoxanthin	H-M-G	e	18	—	—	48	13	—	42	8	—

^aDeveloping solvents as percent by volume polar component in petroleum ether (40–60°C).

^bFor carotenoid structures indicated by letters, refer to Fig. 11.

^cCarotenoid source: a, *Sorbus aucuparia* berry; b, *Solanum lycopersicum* fruit; c, commercial; d, *Gazania rigens* flower; e, *Petroselinum crispum*; f, *Capsicum annum* fruit; g, *Taraxacum officinale* flower; h, *Lilium × hollandicum* flower; i, *Cucurbita maxima* fruit.

^dGazaniaxanthin contains a 5'-cis bond in the L end group.

associated with photosynthetic activity. In addition, this system is of general use in carotenoid screening, although it has limitations with respect to groups of compounds at each end of the polarity scale. It is valuable for preparative TLC when high purity is less important.

Replacing acetone with tertiary alcohols has a marked effect on the separation. The resolution of compounds of closely related polarity is improved, and a more efficient spread of the polar pigments is observed. The distinct change in selectivity toward canthaxanthin is obvious.

A system that was 20% tertiary alcohol in petroleum ether separated all the investigated pigments except β -carotene and lycopene. A typical separation is shown in Fig. 12, where an extract from parsley was used as the carotenoid source, and the efficient spread of the polar pigments is evident. The separations are even better on plastic-backed silica gel (0.2 mm, Merck), where an extremely narrow bandwidth can be produced. Combined with circular TLC, the system provides valuable information about samples with complicated pigment patterns in the polar area.

b. MgO-Kieselguhr Layers. The extracts (1 μ L) were carefully applied as bands (1 cm) and developed over 10 cm (60 min). Two solvent systems were tested: acetone–petroleum ether (40–60°C) (15:85), system 1 and (40:60), system 2. Approximate R_f values are given in Table 6 based on the average of four separations.

Magnesium oxide layers exhibit great selectivity toward carotenoids that differ in the nature, number, and/or positions of the double bonds. The layer is very efficient in separating a wide variety of structural and geometrical isomers. An illustrative example of carotene separations is given with solvent system 1 as the mobile phase. Carotenes with cyclic endgroups are less sorbed than acyclic compounds, and a decrease in conjugated double bonds shows the same tendency. Consequently, lycopene, which possesses 11 conjugated and two isolated double bonds, is strongly sorbed compared with α - and β -carotene. The difference in retention between α - and β -carotene is based solely on the position of the double bond in the nonidentical ring systems.

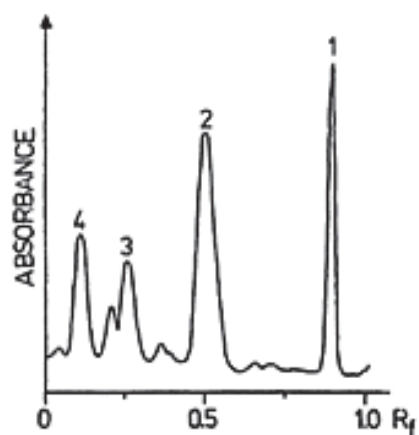


Figure 12 Separation of carotenoids from *Petroselinum crispum*. Solvent system: light petroleum (40–60°C)–*t*-butanol (80:20). Stationary phase: silica gel 60 (0.25 nm, Merck). Developing distance: 8.5 cm. Detection: absorbance at 445 nm. Peak identities: (1) β -carotene, (2) lutein, (3) violaxanthin, (4) neoxanthin.

Oxygenated carotenoids require a more polar solvent system, and the acetone content should be adjusted to 30–45% to effect acceptable separation. A combination of oxygenated sites and double-bond positioning determines the retention, and the highly oxygenated violaxanthin is less sorbed than expected, mainly because of the short chromophore. Pairs of compounds that differ

Table 6 R_f Values of Carotenes and Xanthophylls on MgO-Kieselguhr G (1:1) Layers (0.25 mm, Koch-Light/Merck)

Pigment	Structure ^a	S ^b	Solvent system ^c	
			1	2
Carotenes				
α -Carotene	A-M-B	a	0.86	—
β -Carotene	A-M-A	a	0.82	—
δ -Carotene	B-M-L	a	0.65	—
γ -Carotene	A-M-L	a	0.33	—
Lycopene	L-M-L	a	0.04	—

Xanthophylls				
Taraxanthin	D-M-G	c	—	0.77
Violaxanthin	G-M-G	b	—	0.70
β -Cryptoxanthin	A-M-C	e	—	0.63
Lutein	C-M-D	b	—	0.42
Neoxanthin	H-M-G	b	—	0.33
Antheraxanthin	C-M-G	d	—	0.27
Zeaxanthin	C-M-G	a	—	0.12

^aFor carotenoid structures, refer to Fig. 11.

^bSources for pigments are as follows: a, *Hippophae rhamnoides* fruit; b, *Petroselinum crispum*; c, *Taraxacum officinale* flower; d, *Lilium × hollandicum* flower; e, *Sorbus aucuparia* berry.

^cSystem 1: Light petroleum (40–60°C)–acetone (85:15). System 2: Light petroleum (40–60°C)–acetone (60:40).

only within the ring system, such as lutein–zeaxanthin and taraxanthin–antheraxanthin, are clearly separated.

Separation of carotenoids on magnesia layers may serve as a useful supplement to the more commonly used silica gel systems. Used alone, however, magnesia layers can easily lead to confusion, especially in the case of the xanthophylls. Relatively sharp bands are obtained, but the R_f values may vary and the tabulated values should therefore be used only as a rough guideline. It is advisable to prepare standards from established sources with a more consistent system before testing the properties of the magnesia layers.

c. *Octadecyl-Bonded Silica (RP-18) Layers*. The extracts are applied as bands (2 cm) and developed with solvent systems containing the following compositions of petroleum ether–acetonitrile–methanol: system 1 (10:40:50), system 2 (20:40:40), and system 3 (25:25:50). The plates are developed over 8.5 cm (14–17 min). A chromatogram (system 3) of parsley pigments is given in Fig. 13.

Reversed-phase separation of carotenoids on octadecyl-bonded silica provides a mild and sensitive method for the TLC analysis of chloroplast pigments. The separation can be explained on the basis of partition between the mobile phase and the hydrophobic surface of the modified silica gel layer, but the separation mechanism is not fully understood. Less polar compounds such as the carotenes are strongly held according to their lipophilic nature. The retention of the xanthophylls is determined mainly by the nature and the number of the oxygenated substituents. Representative R_f values found for some carotenoids with this system are shown in Table 7. Solvent systems 2 and 3 are most suitable for general separations, but system 1 gives better separation of the more polar compounds.

The RP-TLC system seems to be generally applicable in carotenoid analysis when used either separately or in combination with other thin-layer systems. The strength of the system lies in its ability to resolve pigments from apparently pure fractions obtained from other sorbents. High loading capacity makes the system useful for preparation separation, especially of polar compounds that are difficult to recover from the sorbents in normal-phase separations.

VI. CHLOROPHYLL AND CHLOROPHYLL DERIVATIVES

A. General

1. Structure

The chlorophylls are macrocyclic tetrapyrrole pigments that in their native form contain a chelated magnesium ion. The general structures for the chlorophylls and the derivatives discussed in this section are given in Fig. 14.

2. Distribution

Chlorophylls occur ubiquitously in the plant kingdom, where they function as photoreceptors in photosynthesis. Although they are responsible for the green colors in higher plants, their presence can be masked by copigmentation with compounds belonging to other pigment classes. They are also widespread in algae and certain bacteria (45).

B. TLC

Chlorophyll and chlorophyll derivatives are highly sensitive compounds. Exposure to light, heat, acids, bases, and certain solvents and sorbents is reported to result in structural alteration (46). Facts about this sensitivity are becoming clearer, and the dangers that are implied for quantitative work are emphasized in Ref. 46a. An extensive review by Sesták (47), which includes over 220 reports for the period 1967–1982, gives valuable information on several systems commonly used for the TLC analysis of the chlorophylls.

Organic sorbents including sucrose and cellulose, regarded as “mild” sorbents, give acceptable separation of chloroplast pigments (48, 49). Separation with diethyl ether–acetone–isooctane (20:20:60) as the mobile phase is included as an example.

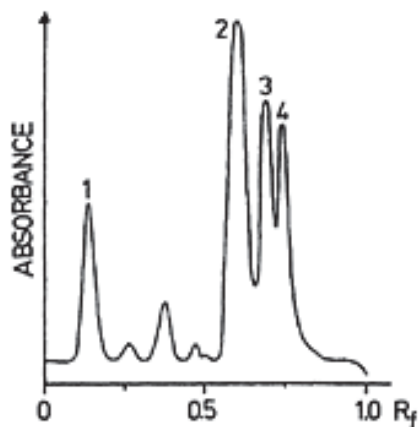


Figure 13 Separation of carotenoids from *Petroselinum crispum*. Solvent system: light petroleum (40–60°C)–

acetonitrile–methanol (25:25:50).
 Stationary phase: RP-18 F₂₅₄ (0.25 mm, Merck). Developing distance: 8.5 cm. Detection; absorbance at 445 nm.
 Peak identities: (1) β -carotene, (2) lutein, (3) violaxanthin, (4) neoxanthin.

C. Practical Experiments

1. Extraction

a. General Method. A sample of 50 g of material is macerated with 100 mL of acetone. After filtration, the procedure is repeated until the extract is almost colorless. Finally, the less polar pigments are extracted with portions of petroleum ether. The extracts are combined, then

Table 7 R_f Values for Carotenoids on RP-18 F₂₅₄ Layers (0.25 mm, Merck) Using Light Petroleum (40–60°C)–Acetonitrile–Methanol as Developing Solvent

Pigment	Structure ^a	S ^b	Solvent system ^c		
			1	2	3
β -Carotene	A-M-A	a	0.10	0.13	0.16
Lycopene	L-M-L	b	0.17	0.23	0.25
β -Cryptoxanthin	A-M-C	a	0.22	0.31	0.37
Gazaniaxanthin	C-M-L ^d	c	0.26	0.38	0.43
Canthaxanthin	E-M-E	d	0.32	0.51	0.55
Lutein	C-M-D	e	0.37	0.55	0.59
Isozeaxanthin	F-M-F	f	0.38	0.57	0.62
Antheraxanthin	G-M-C	g	0.44	0.60	0.63
Taraxanthin	G-M-D	h	0.47	0.62	0.64
Capsanthin	C-M-K	i	0.48	0.64	0.66
Violaxanthin	G-M-G	e	0.55	0.68	0.68
Neoxanthin	H-M-G	e	0.63	0.72	0.74

^aFor carotenoid structures, refer to Fig. 11.

^bCarotenoid source: a *Sorbus aucunaria* berry; b *Solanum lycopersicum* fruit; c *Gazania regina*

flower; d, commercial (Fluka); e, *Petroselinum crispum*; f, lithium aluminum hydride reduction of d; g, *Lilium* × *hollandicum* flower, h, *Taraxacum officinale* flower; i, *Capsicum annuum* fruit.

^dGazaniaxanthin contains a 5'-cis bond in the L end group.

Figure 14 Structures of chlorophyll and chlorophyll derivatives. Refer to Table 8 for compound identities.

b. Purification as a Chlorophyll-Dioxane Complex. A finely cut or minced sample is successively extracted with portions of 2-propanol. Dioxane is added to the combined extracts (1:7), and water is then added drop wise until turbidity occurs. The resulting mixture is stored in a refrigerator, and a chlorophyll-dioxane complex forms within 2 h. This complex is collected by centrifugation. Repetition of this procedure is recommended to obtain extrapure pigments, i.e., free from carotenoids and lipids. The complex is dissolved in diethyl ether or acetone prior to TLC analysis. Alternatively, it can be dried in a vacuum apparatus at room temperature. The dried complex is best stored in an inert atmosphere at low temperature (-30°C).

2. Standard Markers

Parsley (*Petroselinum crispum*) is a convenient source of a wide range of natural chloroplast pigments including chlorophyll a (Chl a), chlorophyll b (Chl b), and their derivatives. The amounts of some of these derivatives is small in fresh extracts, and greater amounts may be obtained by chemical treatment of extracts containing the parent compounds.

Pheophytin a (Pheo a) and pheophytin b (Pheo b) are obtained by treating a stirred ether extract with 1 M HCl (2:1) for 2 min. Similarly, pheophorbides a and b are prepared from the ethereal pigment extract by mixing with 30% (9.5 M) HCl (1:2) and stirring for 5 min. The pigments are transferred to diethyl ether by adding water, and the resulting ethereal extract is washed repeatedly with distilled water to ensure neutrality. Heating a pyridine solution of the parent chlorophylls a and b (Chl a and Chl b) in a sealed tube at 60°C for 1 h readily produces chlorophyll a' (Chl a') and chlorophyll b' (Chl b').

3. Separation

a. Sucrose Layers. Ether extracts (1 μ L) are carefully applied as spots and developed over 18 cm (20 min) with light petroleum (40–60°C)–2-propanol (99:1) as mobile phase. A typical chromatographic pattern is indicated in Fig. 15, and a densitometric profile showing the separation of parsley pigments is given in Fig. 16.

The pigments appear as relatively large and irregular spots, but the separation is superior to those obtained with other systems commonly used in TLC analysis of the chlorophylls. The main pigments can be identified with considerable certainty on the basis of their characteristic colors as observed in daylight. Pheo a and Pheo b give gray and yellow-brown spots, respectively. Chl a and Chl a' are blue-green, whereas Chl b and Chl b' give a yellow-green coloration. The colors of pheophorbides a and b resemble those of Pheo a and Pheo b. Chlorophylls and their derivatives give an intense red fluorescence under longwave UV light.

The separation of chlorophyll pigments on sucrose layers is expected to be correlated with their ability to self-aggregate. Increased coordination facilitates self-aggregation and leads to greater retention. Thus the magnesium-free pheophytins are separated from the other pigments in the upper part of the plate, apart from Chl a', which overlaps with Pheo b in total extracts. In all cases pigments of type b are more retained than those of type a which is ascribed to the carbonyl substituent at C-3 in the type b compounds. The primed compounds Chl a' and Chl b', the 10*S* epimers of Chl a and Chl b, respectively, are well separated from their parent chlorophylls, because the *cis* arrangement of the bulky groups at C-7 and C-10 in the 10*S* isomers results in increased steric interaction and decreased coordination. The free carboxylic acid group found at C-7 in pheophorbides a and b results in these being more retained than all of the other compounds where the acid function is esterified as a phytol ester.

Sucrose layers give fast and cheap separation of chlorophylls and their derivatives with a minimum of pigment degradation and loss. The recovery in preparative separation is about 95% with sample loadings in the range of 50–100 μ g. The pigments are commonly recovered in diethyl ether after dissolution of the sucrose in water.

b. Cellulose Layers. Extracts (2 μ L) are applied as bands (1 cm) and developed over 8.5 cm (10 min) with petroleum ether (40–60°C)–acetone–2-propanol (90:10:0.45) as the

mobile phase. The R_f values obtained are only approximate, because tailing effects produce oblong zones. The separated pigments follow the pattern observed in the sucrose system, but distinct differences are apparent in the measured retention values. The most retained compounds, pheophorbides a and b, are better separated in this system than on sucrose in spite of their higher R_f values. Pheo b is also clearly separated from Chl a' on cellulose layers. Cellulose layers are easier to deal with than sucrose layers, which are loose and require great care in handling, and have the additional advantage of being commercially available. Cellulose systems give increased degradation, but recoveries in the range of 85–90% are generally acceptable. Although cellulose layers are useful in preparative separation, their loading capacity is slightly lower than that of sucrose layers.

c. Silica Layers. The extracts (2 μL) are applied as bands (1 cm) and developed over 8.5 cm (15 min) with diethyl ether–acetone–isooctane (20:20:60) as the mobile phase. Calculated R_f values are given in Table 8. The strength of this system is that it can be used to produce extremely narrow pigment bands. A major disadvantage of the system, however, is that the pigments are not spread as well over the surface area of the plate. Pigment degradation makes the system unsuitable for preparative separations.

VII. PORPHYRINS

A. General

1. Structure

Porphyrin pigments are yellow-orange fluorescent compounds in which four pyrrole units linked by methine bridges form a macrocyclic molecule (Fig. 17). Substitution of the peripheral positions of the pyrrole rings gives a large number of possible structures. Methyl, ethyl, vinyl, carboxy-

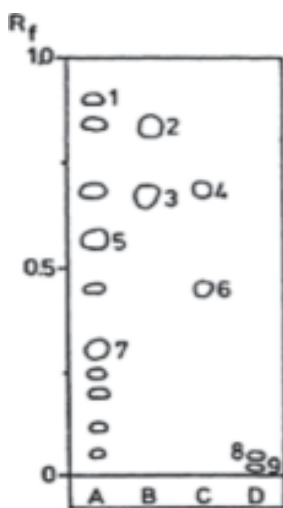


Figure 15 Separation of chlorophyll and chlorophyll derivatives. Solvent

system: light petroleum (40–60°C)–2-propanol (99:1). Stationary phase: sucrose (icing sugar, 0.4 mm, Tate & Lyle). Developing distance: 18 cm. Detection: visible light/UV 365 nm. Samples: (A) *Petroselinum crispum* extract, (B) pheophytin a/b, (C) chlorophyll a'/b', (D) pheophorbide a/b. Spot identities: (1) pyropheophytin, (2) pheophytin a, (3) pheophytin b, (4) chlorophyll a', (5) chlorophyll a, (6) chlorophyll b', (7) chlorophyll b, (8) pheophorbide a, (9) pheophorbide b.

methyl, and carboxyethyl substituents are most common. The number of carboxyl groups varies from one to eight (Table 9).

2. Distribution

The porphyrins are intermediates in the biosynthesis of heme and chlorophyll and are thus responsible for the most abundant colors in nature. Small amounts of porphyrins can be isolated

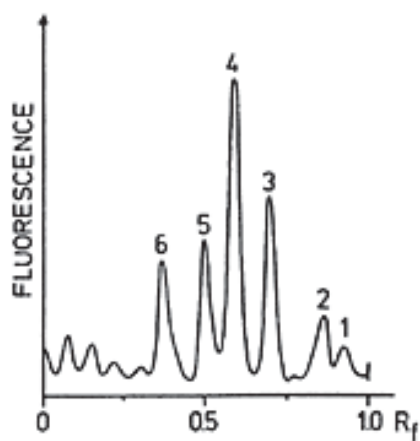


Figure 16 Separation of chlorophyll and chlorophyll derivatives from *Petroselinum crispum*. Solvent system: light petroleum (40–60°C)–2-propanol (99:1). Stationary phase: sucrose (0.4 mm, Tate & Lyle). Developing

distance: 18 cm. Detection: fluorescence (366 nm excitation in reflection mode). Peak identities: (1) pyropheophytin a, (2) pheophytin a, (3) pheopytin b and chlorophyll a', (4) chlorophyll a, (5) chlorophyll b', (6) chlorophyll b.

Table 8 R_f Values of Chlorophylls and Their Derivatives

Pigment	Structure				Solvent system		
	R	R ₁	Mg		1	2	3
Pyropheophytin a	CH ₃	Phy	—	2H at C-10	0.90	—	—
Pheophytin a	CH ₃	Phy	—		0.84	0.93	0.40
Pheophytin b	CHO	Phy	—		0.72	0.88	0.33
Chlorophyll a'	CH ₃	Phy	+	10(S)-Chl a	0.72	0.80	0.31
Chlorophyll a	CH ₃	Phy	+		0.56	0.76	0.27
Chlorophyll b'	CHO	Phy	+	10(S)-Chl b	0.44	0.60	0.25
Chlorophyll b	CHO	Phy	+		0.30	0.57	0.22
Pheophorbide a	CH ₃	H	—		0.05	0.36	—
Pheophorbide b	CHO	H	—		0.02	0.18	—

System 1: Sucrose (icing sugar) layer (0.4 mm, Tate & Lyle); light petroleum (40–60°C)–2-propanol (99:1). System 2: Cellulose layer (0.1 mm, Merck); light petroleum (40–60°C)–acetone (80:20). System 3: Silica gel 60 (0.25 mm, Merck); diethyl ether–acetone–isooctane (20:20:60). *Petroselinum crispum* was used as pigment source. The following abbreviations are used: Phy=phytyl; Chl=chlorophyll. Structure indication refers to Fig. 14.

from normal urine and feces, although certain metabolic disorders cause abnormally high concentrations of some of these pigments.

B. TLC

The porphyrins are best separated in their esterified forms on silica layers with benzene–ethyl acetate–methanol (85:13.5:1.5) as the mobile phase (7), but several other systems are reported in the literature (8, 50). A useful preparative system was reported by Cardinal et al. (51). TLC analysis of free porphyrins on normal- and reversed-phase silica layers was used for the diagnosis of different types of porphyria in human subjects (52, 52a).

C. Practical Experiments

1. Extraction

a. Extraction from Urine. A urine sample is acidified to pH 3–4 with acetic acid. Talcum powder is added (0.1 volume of urine), and the suspension is stirred for 60 min, allowed to settle, and filtered. The filtrate is tested for the absence of fluorescence, and the talcum powder is dried

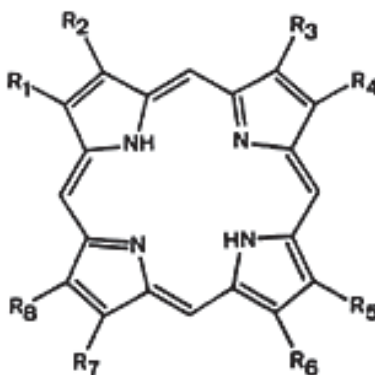


Figure 17 Structure of porphyrins.
Refer to Table 9 for compound identities.

Table 9 R_f Values of Porphyrin Methyl Esters on Silica Gel Layers (0.2 mm, Riedel-De Haen)

Pigment ^a	Substituent position ^b								R_f
	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	
Protoporphyrin IX	Me	V	Me	V	Me	Pr	Pr	Me	0.69
Coproporphyrin III	Me	Pr	Me	Pr	Me	Pr	Pr	Me	0.49
5-COOH	Me	Pr	Me	Pr	Ac	Pr	Pr	Me	0.42
6-COOH	Me	Pr	Ac	Pr	Ac	Pr	Pr	Me	0.33
7-COOH	Ac	Pr	Ac	Pr	Ac	Pr	Pr	Me	0.21
Uroporphyrin III	Ac	Pr	Ac	Pr	Ac	Pr	Pr	Ac	0.10

Solvent system: Benzene-ethyl acetate-methanol (85:13.5:1.5).

^aProto-, copro-, and uroporphyrin are commercial (Sigma). Penta-, hexa-, and hepta-porphyrin are isolated from human porphyrin urine.

^bSubstituent position refers to free pigments in Fig. 17, and the abbreviations are as follows: Me=CH₃, V=CH=CH₂, Ac=CH₂COOH, and Pr=CH₂CH₂COOH.

in a vacuum desiccator protected from light. Direct esterification of the adsorbed porphyrins is performed with a mixture of methanol and sulfuric acid (20:1). The talcum

powder is suspended in the reaction mixture and left for 12 h at room temperature or refluxed for 20 min on a water bath. After filtering, the filtrate is adjusted to pH 4 by adding concentrated aqueous sodium acetate. Chloroform is then added, and the mixture is again filtered. The precipitate is washed repeatedly with portions of chloroform, and the extracts are combined. The chloroform phase is then washed several times with portions of water. Finally, the chloroform phase is evaporated to dryness, and the residue is dissolved in chloroform. The extract is used directly for TLC analysis.

b. Extraction from Feces. A fecal sample (1 g) is defatted with acetone and centrifuged. The acetone is discarded and the residue dried. The residue is mixed with 20 mL of 5% sulfuric acid in methanol and refluxed for 20 min. The porphyrin esters are recovered following the extraction procedure as described above for urine.

2. Separation

The extracts (portions) are applied as streaks (1 cm) on aluminum-backed silica gel plates and developed over 8.5 cm (25 min) with benzene-ethyl acetate-methanol (85:13.5:1.5) as the mobile phase. The plate is dried and inspected under longwave UV light; porphyrin pigments give an intense red fluorescence. The R_f values given in Table 9 indicate that retention depends on the number of ester groups attached to the molecule. Protoporphyrin IX has only two ester groups and is less sorbed than the more carboxylated compounds. An increase in the polarity caused by the introduction of new methyl ester substituents leads to better retained compounds, as one would expect.

Preparative separations are best performed on silica gel layers coated on glass. Petroleum ether (40–60°C)–chloroform (1:1) is recommended as the mobile phase. The plate is developed in an NH_3 atmosphere produced by placing a beaker containing 10% ammonia in the developing tank. The order of elution follows the system described above. The recovery of the pigments varies in the range 65–85%.

VIII. QUINONES

A. General

1. Structure

The quinones are a rather heterogeneous collection of compounds with structures based on a unsaturated system of cyclic diketones. The biologically important plastoquinones, ubiquinones,

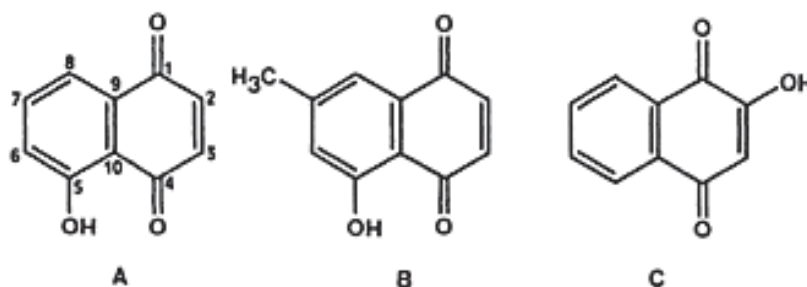


Figure 18 Common naphthaquinones.
(A) Juglone; (B) 7-methyljuglone; (C) lawsone.

and vitamin K are included in this group; however, as pigments the most widespread and most important quinones are the 1,4-naphthaquinones (Fig. 18) and the 9,10-anthraquinones (Fig. 19) (Table 10). Methyl, methoxyl, and hydroxyl groups are the most common substituents, and *O*- and *C*-glycosides (see Fig. 20) are frequently present in the anthraquinone group. Several structural modifications exist due to reduction, dimerization (Fig. 21), and addition of side chains.

2. Distribution

The quinones are widely distributed in nature, and altogether 1200 different quinones have been observed in bacteria, in all plant phyla except mosses, and in animal phyla like echinoderms (sea urchins) and arthropods (insects) (53, 54). They may occur in all parts of a plant; however, a large proportion are present in roots, heartwood, and bark.

The quinones range in color from yellow through red and purple to almost black. They make relatively little contribution to color in higher plants; their color is perhaps most conspicuous in some fungi, lichen, and insects (Coccidae).

B. TLC of Naphthaquinones

Several solvent systems have been reported for separation of naphthaquinone pigments on silica gel (55). A solvent containing 30% ethyl acetate in petroleum ether (60–80°C) gives acceptable separation of quinones from Plumbaginaceae. Many of the older systems include benzene or chloroform in the mobile phase, which impairs the separation on commercial plates. Polar naphthaquinones are better separated with hexane–acetone–acetic acid (75:25:1.5) (56). Using this latter solvent, a replacement of hexane with petroleum ether (40–60°C) may be beneficial.

Centrifugal TLC on silica gel using toluene–ethyl acetate (15:1) as mobile phase has been applied for the preparative isolation of potent molluscicidal naphthaquinones from the root bark of *Diospyros usambarensis* (57).

Quantitative determinations of naphthaquinones from *Arnebia densiflora* using TLC–densitometry and HPLC were compared and showed no significant differences in the results obtained with the two techniques (57a).

C. Practical Experiments

1. Isolation

A solution of 100 mL of diethyl ether containing 1% concentrated H_2SO_4 was mixed with 50 g of fresh *Drosera rotundifolia*. The mixture was macerated in a Waring blender and allowed to stand for 8 h. After filtration and evaporation, 50 mL of 2 M H_2SO_4 was added. The mixture was steam-distilled, and the yellow distillate was extracted with ether.

Powered leaf of *Lawsonia alba* (20 g) was mixed with 100 mL of 2 N Na_2CO_3 and stirred for 30 min. The mixture was filtered, and 2 M H_2SO_4 was added until neutrality. Precipitated pigments were extracted with ether.

The mesocarp from fresh fruits of *Juglans regia* was cut into small pieces and transferred to methanol. The methanolic extract was used directly. Bark and leaves can be extracted with methanol or ether.

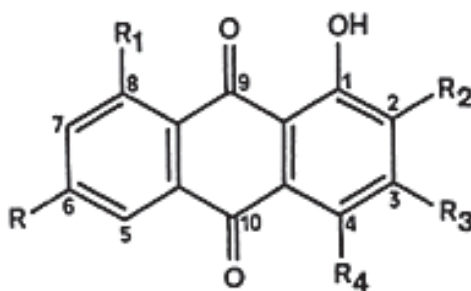


Figure 19 General structure of anthraquinone pigments. See Table 10 for common anthraquinones.

Table 10 Structures of Some Anthraquinones as Shown in Fig. 19

Pigment	Substituent				
	R	R ₁	R ₂	R ₃	R ₄
Chrysophanol	H	OH	H	CH ₃	H
Physcione	OCH ₃	OH	H	CH ₃	H
Emodin	OH	OH	H	CH ₃	H
Alizarin	H	H	OH	H	H
Purpurin	H	H	OH	H	OH
Rhein	H	OH	H	COOH	H
Aloe-emodin	H	OH	H	CH ₂ OH	H

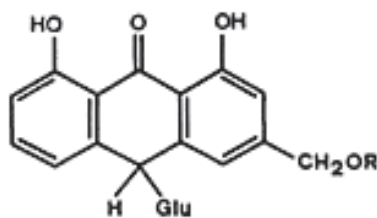


Figure 20 Structure of aloin (R=H) and aloinoside (R=rhamnosyl). Glu=glucose.

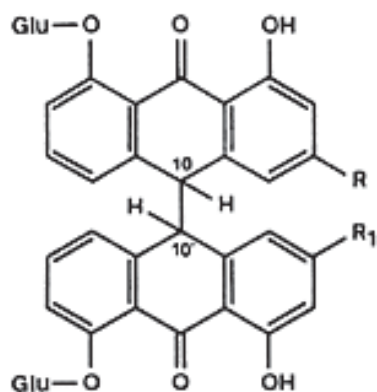


Figure 21 Structure of bianthrone from *Cassia senna*. Sennoside A: R=R₁=COOH(+)-form. Sennoside B: R=R₁=COOH, meso form. Sennoside C: R=COOH and R₁=CH₂OH(+)-form. Sennoside D: R=COOH and R₁=CH₂OH, meso form. Glu=glucose.

2. Separation

Extracts (3 μ L portions) were applied as bands (2 cm) on plastic-backed silica gel (0.2 mm) (Macherey-Nagel) and developed over 8.5 cm with petroleum ether (40–60°C)–acetone–acetic acid (75:25:1.5) as the mobile phase. Separated extracts showed several clearly located yellow and orange-yellow bands (Fig. 22). Spraying with 10% methanolic KOH intensified the pigments from *L. alba*, and the naphthaquinones in *J. regia* and *D. rotundifolia* changed to blue-violet. The main compounds were identified as lawsone (R_f 0.26), juglone (R_f 0.48), and 7-methyljuglone (R_f 0.50).

The location of the hydroxy substituent in compounds such as juglone and 7-methyljuglone (Fig. 18) has a great influence on the retention of these compounds. Intramolecular hydrogen bonding between the keto group and the hydroxyl substituent reduces the polarity and results in relatively high R_f values. In contrast, lawsone (Fig. 18) is strongly sorbed to the TLC layer.

D. TLC of Anthraquinones

Common anthraquinone glycosides are best separated on silica layers with ethyl acetate–methanol–water (100:13.5:10) as the mobile phase (58). More polar compounds, such as the sennoside pigments, are well separated on a silica gel system reported by Khafagy et al. (59).

Suitable solvent systems for the separation of aglycones on silica layers include petroleum ether–ethyl acetate–formic acid (75:25:1) (58) and petroleum ether–ethyl formate–formic acid (75:25:5). A mobile phase reported by Nyiredy et al. (60) offers no advantages compared with the former systems. Finally, a method developed by Ebel and Kaal (61) involves direct hydrolysis of the glycosides on the TLC plate; the solvent systems suggested may be advantageously replaced by the solvents described above.

A two-dimensional TLC separation on silica of a complex mixture of anthraquinone aglycones and glycosides from the fungus *Dermocybe sanguinea* was recently reported (61a). The eluent systems used were n-pentanol–pyridine–methanol (6:4:3) and toluene–ethyl acetate–ethanol–formic acid (10:8:1:2).

The prediction of retention data for quinones on the basis of their structure and the physical chemistry of the chromatographic system used also received attention, and results are reported to be consistent with the forecast behavior (61b, 61c).

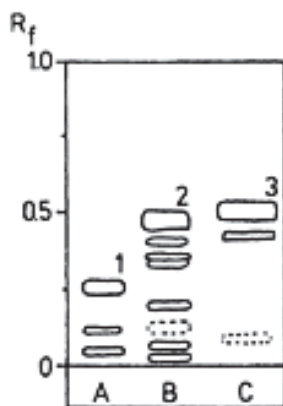


Figure 22 Separation of naphthaquinone pigments from (A) *Lawsonia alba*, (B) *Juglans regia*, and (C) *Drosera rotundifolia*. Solvent system: light petroleum (40–60°C)–acetone–acetic acid (75:25:1.5). Stationary phase: POLYGRAM silica gel N-HR/UV₂₅₄ (0.2 mm, Macherey-Nagel). Developing distance: 8.5 cm. Detection: visible light with KOH reagent. Band identities: (1) lawsone, (2) juglone, (3) 7methyljuglone.

E. Practical Experiments

1. Isolation

Small quantities of plant material are directly extracted with hot methanol, whereas Soxhlet extraction with the same solvent is used for larger quantities. Successive extractions with several solvents of increasing polarity are often performed when a complete analysis is required.

2. Hydrolysis of Glycosides

The glycosidic extract is evaporated to dryness and heated under reflux for 25 min with 7.5% hydrochloric acid. After cooling, the aglycones are extracted with portions of diethyl ether. Concentrated extracts are used directly for TLC.

3. Separation of Glycosides

Extracts from rhubarb root (*Rheum palmatum*), alder buckthorn bark (*Rhamnus frangula*), and aloe (*Aloe capensis*) are used as pigment sources. The extracts (3 μ L) are applied to silica gel as streaks (2 cm), and the plate is developed to a distance of 8.5 cm (35 min) with ethyl acetate–methanol–water (100:13.5:10) as the mobile phase. An illustrative example of Separated glycosides from *R. frangula* is given in Fig. 23. Yellow bands are observed in daylight, and the pigments produce orange-yellow colors under longwave UV. Spraying the plates with 5% ethanolic KOH gives red to purple bands in the visible and dull red bands in the longwave UV, except for the C-glycosides from aloe, which give an intense yellow-orange fluorescence.

R_f values for several anthraquinone glycosides are given in Table 11. The pigments are generally separated according to the number and nature of the sugar units. The monoglycosides are well separated from the diglycosides. Within each of these groups of glycosides, variation of the sugar moiety is reflected in further separation, although changes in the aglycone do not appear to result in similar separation. Rhein-8-O-glucoside does not follow the general pattern and is strongly sorbed due to the acid group. Rhein itself is located at R_f 0.28, and the system is thus useful for preparative separation of rhein from a hydrolyzed extract where the major compounds move with the solvent front.

4. Separation of Sennosides

Extracts (3 μ L) from leaves and fruits of senna (*Cassia angustifolia*) are applied as streaks (2 cm) on silica gel sheets and developed over 8.5 cm (100 min) with 2-propanol–ethyl acetate–water (36:36:28) as the mobile phase. The sennosides occur as pale yellow zones in daylight and give characteristic dull red zones in longwave UV. After spraying with KOH reagent, the colors of the sennoside pigments are intensified in daylight and a characteristic yellow-green fluorescence appears in UV light. Alternatively, the bianthrone can be detected indirectly by oxidation to the corresponding anthraquinones. The plate is first sprayed with concentrated nitric acid, then heated on a thermoplate for

10 min at 120°C to complete the reaction. Further spraying with 5% KOH gives the common colors of the free anthraquinones.

The separated pigments are indicated in Fig. 24. Bianthrone glycosides dominate in the extracts. Sennoside B and sennoside A appear at R_f 0.18 and 0.30, respectively. Sennoside D is located directly below rhein-8-glycoside at R_f 0.44, and small amounts of sennoside C are detected at R_f 0.52 in the leaf extract. An additional pigment occurs above the sennoside A band and reacts like the sennosides with the common spray reagents.

5. Separation of Aglycones

Hydrolyzed root extracts from rhubarb and madder (*Rubia tinctorum*) are applied as bands (2 cm) on silica gel layers and developed in three different solvent systems. All systems separated at least five yellow pigments from rhubarb and several pink-to-purple pigments from madder. Calculated R_f values are given in Table 12.

System 1 required development over 18 cm (100 min) with light petroleum (40–60°C)–ethyl acetate–formic acid (75:25:1) as the mobile phase. A representative separation of pigments from rhubarb is given in Fig. 25. It should be noted that this separation was achieved on a plate prepared

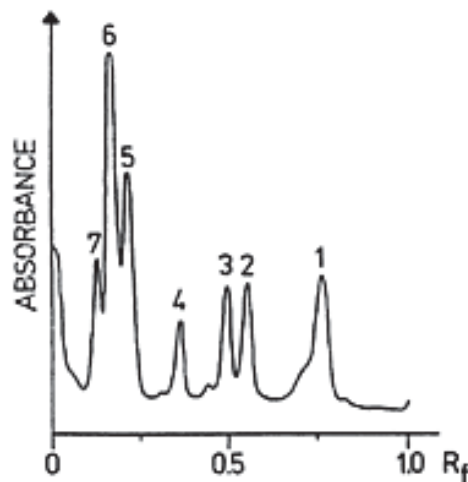


Figure 23 Separation of anthraquinone glycosides from *Rhamnus frangula*.

Solvent system: ethyl acetate–methanol–water (100:13.5:10).

Stationary phase: silica gel 60 F₂₅₄ (0.25 mm, Merck). Developing

distance: 8.5 cm. Detection:

absorbance at 420 nm. Peak identities:

(1) aglycones, (2) frangulin B, (3) frangulin A, (4) emodin

monoglucoside, (5) glucofrangulin B, (6) glucofrangulin A, (7) diglycosides of chrysophanol and physcione.

in the laboratory. Such a plate is better for the separation of the polar pigments than corresponding commercial plates, where separation of rhein and aloe-emodin depends on development distance.

System 2 involves development over 8.5 cm (20 min) with a mixture of 10.6% chloroform, 9.9% ethyl acetate, 9.0% dioxane, and 70.6% hexane; 0.5% acetic acid was added as a modifier. The system is an example of a mobile phase developed and optimized by the PRISMA model using the most common pigments from rhubarb as a test mixture. The system produces sharp bands, but the resolution of the components is not significantly better than in system 1. Only a

Table 11 R_f Values of Anthraquinone Glycosides on Silica Gel 60 F₂₅₄ (0.25 mm, Merck)

Pigment ^a	Source ^b	R_f
Emodin-6- <i>O</i> -api (frangulin B)	a	0.59
Emodin-6- <i>O</i> -rha (frangulin A)	a	0.50
Emodin-8- <i>O</i> -glu	b	0.41
Chrysophanol-8- <i>O</i> -glu	b	0.41
Physcione-8- <i>O</i> -glu	b	0.41
Aloe-emodin-8- <i>O</i> -glu	b	0.36
Aloe-emodinanthrone-10- <i>C</i> -glu (aloin A/B)	c	0.34
Emodin-6- <i>O</i> -api-8- <i>O</i> -glu (glucofrangulin B)	a	0.23
Alain-11- <i>O</i> -rha	c	0.19
Emodin-6- <i>O</i> -rha-8- <i>O</i> -glu (glucofrangulin A)	a	0.17
Emodin digly	b	0.15
Chrysophanol digly	b	0.10
Physcione digly	b	0.10
Rhein-8- <i>O</i> -glu	b	0.05

Solvent system: Ethyl acetate–methanol–water (100:13.5:10).

^aapi=apioside, rha=rhamnoside, glu=glucoside, and gly=glycoside.

^bExtracts of a, *Rhamnus frangula*; b, *Rheum palmatum*; and c, *Aloe capensis* were used as pigment sources.

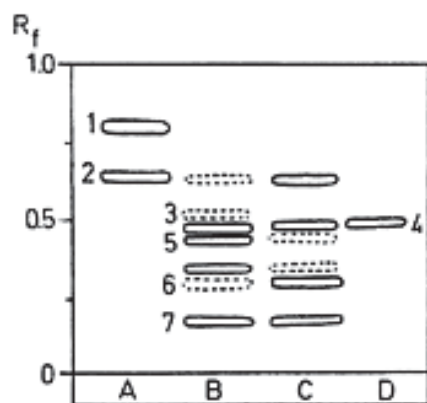


Figure 24 Separation of anthraquinone glycosides from *Cassia angustifolia* (B) fruits and (C) leaves. (A) and (D) are isolated from *Rheum palmatum*. Solvent system: 2-propanol–ethyl acetate–water (36:36:28). Stationary phase: POLYGRAM silica gel N-Hr/UV₂₅₄ (0.2 mm, MN). Developing distance: 8.5 cm. Detection: nitric acid–KOH reagent, visible light/UV₃₆₅. Band identities: (1) aglycones, (2) rhein, (3) sennoside C, (4) rhein-8-glucoside, (5) sennoside D, (6) sennoside B, (7) sennoside A.

limited area of the plate surface is utilized, and the system is not recommended for preparative separations.

System 3 chromatograms were developed twice (2×8.5 cm) with light petroleum (40–60°C)–ethyl formate–formic acid (75:25:5) as the mobile phase. Chrysophanol and physcione are completely separated in this system, and there are great differences in retention between rhein and aloe-emodin. Similar retention is observed on homemade plates, and the system is well suited for preparative separation.

Table 12 R_f Values of Anthraquinone Aglycones

Pigment	Source ^a	Solvent system ^b		
		1	2	3
Chrysophanol	a	0.65	0.49	0.66
Physcione	a	0.59	0.44	0.56
Emodin	a	0.36	0.18	0.30

Alizarin	b	0.25	0.25	0.32
Purpurin	b	0.20	0.17	0.34
Rhein	a	0.15	0.04	0.22
Aloe-emodin	a	0.13	0.12	0.15

^aPigments were obtained by hydrolysis of extracts of *Rheum palmatum* (a) and *Rubia tinctorum* (b).

^bSystem 1: Silica gel 60 F₂₅₄ (0.25 mm, Merck), light petroleum (40–60°C)–ethyl acetate–formic acid (75:25:1). System 2: Silica gel 60 F₂₅₄ (0.25 mm, Merck), 10.6% chloroform, 9.9% ethyl acetate, 9.0% dioxan, 70.6% hexane, and additional 0.5% acetic acid. System 3: POLYGRAM silica gel N-HR/ UV₂₅₄ (0.2 mm, Macherey-Nagel), light petroleum (40–60°C)–ethyl formate–formic acid (75:25:5). Developing distances: 18 cm (system 1), 8.5 cm (system 2), and 2×8.5 cm (system 3).

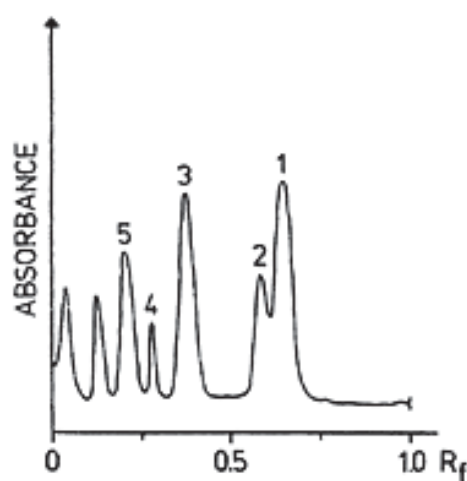


Figure 25 Separation of anthraquinone aglycones from *Rheum palmatum*.

Solvent system: light petroleum (40–60°C)–ethyl acetate–formic acid (75:25:1). Stationary phase: silica gel 60 G (Merck, lab prepared; 0.5 mm).

Developing distance: 18 cm.

Detection: absorbance at 420 nm. Peak identities: (1) chrysophanol, (2) physcione, (3) emodin, (4) rhein, (5) aloe-emodin.

6. Circular TLC of Aglycones

Circular TLC of the aglycones with carried out in the simple apparatus depicted in Fig. 1. The composition of the mobile phase is identical with the phase used in system 1 (Table 12). The extracts were applied as small spots forming an arc 1 cm from the center of the

plate. The chromatogram is shown in Fig. 26. The circular technique gave better separation of polar compounds than linear development in system 1. Rhein is less retained than aloe-emodin when laboratory-prepared plates are used, but it should be noted that the elution order is reversed when commercial plates are used. Chrysophanol and physcione move closely together in the circular system, and circular TLC is thus recommended for separation of the most polar compounds, which are barely separated when linear development is used. High separation speed and narrow bands make circular TLC a useful technique when several extracts are to be compared with respect to identical constituents.

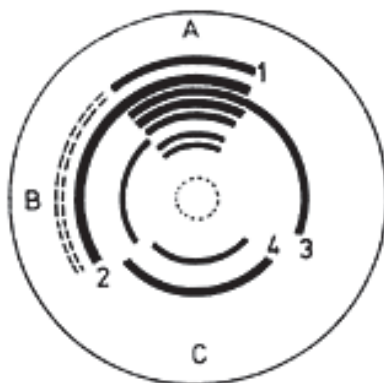


Figure 26 Circular TLC of anthraquinone aglycones from (A) *Rheum palmatum*, (B) *Rhamnus frangula*, and (C) *Aloe capensis*. Solvent system: light petroleum (40–60°C)–ethyl acetate–formic acid (75:25:1). Stationary phase: silica gel 60 G (0.7 mm, Merck) Developing distance; 4 cm. Detection: KOH reagent, visible light/UV-365. Band identities: (1) chrysophanol and physcione, (2) emodin, (3) rhein, (4) aloe-emodin.

7. Two-Dimensional TLC of Aglycones

The methanolic extract is applied in a corner on a 10×10 cm commercial glass plate and developed over 9 cm in the system described for the separation of glycosidic compounds. After development, the plate is thoroughly dried and placed in a desiccator containing a beaker with 25% HCl. The air outlet must be open, and a tube is used to lead acid fumes into a beaker containing 10% aqueous sodium hydroxide. The apparatus is placed in an oven, and the hydrolysis is completed after heating for 35 min at 90°C. The plate is

carefully removed and dried in a nitrogen stream. Second-direction development is done with petroleum ether (40–60°C)–ethyl acetate–formic acid (75:25:1) as the mobile phase.

The chromatographic pattern from two-dimensional TLC analysis of an extract of alder buckthorn bark is shown in Fig. 27. Hydrolyzed extracts of rhubarb and alder buckthorn are used as references in the second, aglycone-separating, direction. The observed pattern gives valuable data about the glycosides with respect to the aglycone moiety. The pattern obtained for alder buckthorn indicates that chrysophanol, physcione, and emodin occur in free form. Emodin derived from an emodin glycoside is located in the middle. The remaining emodin spots are due to the glycofrangulins. Diglycosides of chrysophanol and physcione give three spots at the upper right.

IX. BETALAINS

A. General

1. Structure

There are two main groups of betalains, the red-violet betacyanins and the yellow betaxanthins. The betacyanins, which are made by condensation of betalamic acid with cyclo-DOPA (dihydroxyphenylalanine), occur mainly as *O*-glycosides with a sugar attached to one of the hydroxyl groups of the dihydroindole unit (Fig. 28). Glucose and glucuronic acid are the monosaccharides most commonly present. The sugar residues may be acylated, usually with cinnamic acids. The betaxanthins result from the condensation of betalamic acid with amines or amino acids (Fig. 28). They have hitherto not been reported to be glycosylated.

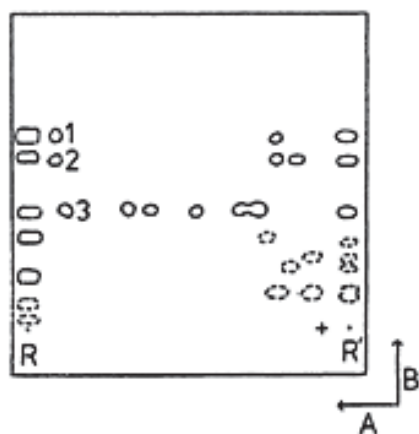


Figure 27 Two-dimensional separation of anthraquinone pigments of *Rhamnus frangula*. First direction (glycoside separation): solvent system A, ethyl acetate–methanol–water (100:13.5:10);

developing distance 9 cm. Second direction (aglycone separation): solvent system B, light petroleum (40–60°C)– ethyl acetate–formic acid (75:25:1); developing distance 8.5 cm. Stationary phase: silica gel 60 F₂₅₄ (0.25 mm, Merck). Detection: KOH reagent, visible light/UV₃₆₅. Hydrolyzed extracts used as references in the second direction: R, *Rheum palmatum*; R', *Rhamnus frangula*. Spot identities: (1) chrysophanol, (2) physcione, (3) emodin.

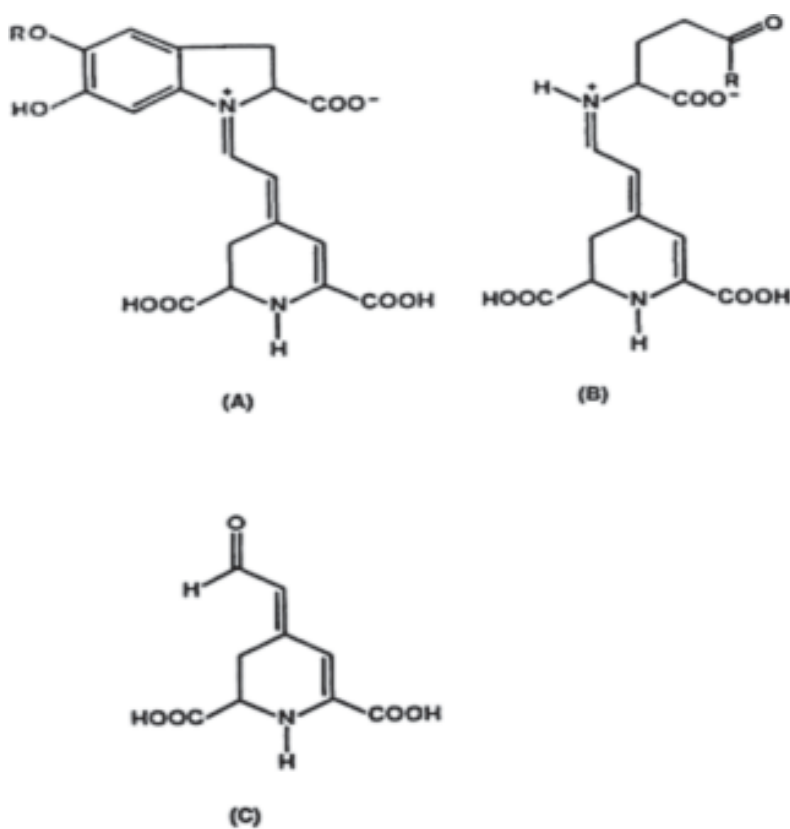


Figure 28 Structure of betalain pigments from *Beta vulgaris*. (A) Betacyanin: betanin (R=glucose). (B) Betaxanthins: vulgaxanthin I (R=OH) and vulgaxanthin II (R=NH₂). (C) Betalamic acid.

2. Distribution

The occurrence of the betalains is restricted to certain plant families of the Caryophyllales (= Centrospermae) order and to toadstools, notably *Amanita*, *Hygrophorus*, and *Hygrocybe* species (62, 63). These pigments may be present in flowers (cacti), roots (beetroot), fruits (*Rivinia* spp.), or bracts (*Bougainvillea* spp.). The betacyanins show a superficial similarity to the anthocyanins; however, the two groups seem to be mutually exclusive.

B. TLC

Betalains are highly polar water-soluble compounds. They often occur as complex mixtures and are easily decomposed during the purification steps, which renders the isolation of larger amounts of pigments difficult. The most successful methods for separating these pigments have been electrophoretic techniques, column chromatography (Sephadex and Polyamide), HPLC, and TLC.

TLC on cellulose (Avicel, Macherey-Nagel) with the solvent ethyl acetate–formic acid–water (33:7:10) was used for separation of a complex mixture of betacyanins from bracts of *Bougainvillea glabra* (64). Other solvent systems for separation of betalains on cellulose include 2-propanol–ethanol–water–acetic acid (6:7:6:1) (65). When a system using silica gel as support (66) was tested in our laboratories in order to separate betalains from beetroot, it offered no advantage over the use of the above-mentioned cellulose systems. Separation of betaxanthins has been carried out on DEAE cellulose (67). Two developments with 2-propanol–water–acetic acid (75:20:5) were required, but this system failed to separate pigments from beetroot.

A TLC system using cellulose plates (0.5 mm) was reported for the preparative separation of betalains from beetroot (65). A high sample load (200 mg of pigment) required a prerun in the polar solvent 2-propanol–ethanol–water–acetic acid (6:7:6:1). After development over 10 cm followed by extensive drying of the plate, betanin was finally separated from the betaxanthins using two successive developments (15 cm) with the same solvent components but in different proportions (10:4:4:1).

C. Practical Experiments

1. Extraction

Beetroot (*Beta vulgaris*) is a good source when testing the chromatographic properties of the betalains by TLC. Fresh beetroot (50 g) is first homogenized with 100 mL of methanol–water (1:1). The suspension is allowed to stand for 2 h at 4°C. The extract is recovered by filtration, and the process is repeated, now with water (50 mL) as solvent. The combined filtrates are concentrated below 30°C under reduced pressure.

2. Separation

The concentrated extract is applied as a streak (5 cm) on a cellulose plate (0.1 mm) (Merck) and developed twice (2×15 cm) with 2-propanol–ethanol–water–acetic acid

(6:7:6:1) as the mobile phase. The plate is dried in a nitrogen stream, and five sharply yellow bands (R_f 0.66, 0.56, 0.48, 0.43, and 0.36) are observed. The bands with R_f values at 0.48 and 0.43 are tentatively identified as vulgaxanthin I and vulgaxanthin II, respectively. The violet betanin band appears at R_f 0.27; however, some trailing may be observed. For semipreparative isolation of betalains in beetroot, homemade cellulose plates (0.3 mm) (Macherey Nagel 300) give similar results.

If a shorter developing distance (2×8.5 cm) is selected, the relatively long separation time (6 h) for the experiment given above may be reduced to about 2 h. Even though the resolution will suffer, three betaxanthine zones are observed in addition to the violet betanin band.

3. Differentiation Between Betalains and Anthocyanins

The pigment extract is administered as a small band on a cellulose plate (0.1 mm) (Merck) and developed in 1-butanol–acetic acid–water (6:1:2) for 2 h. Betalains move slowly in this solvent, whereas anthocyanins have much higher R_f values and are often separated into individual bands.

ACKNOWLEDGMENT

We thank Dr. Morten Isaksen, now of Norsk Hydro, Porsgrunn, Norway, who wrote the corresponding chapter in the first edition of this book, for unrestricted permission to adapt and adopt material from that edition. We particularly acknowledge the fact that diagrams, tables, and more especially the practical experiments are heavily based on the original edition.

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25

Nucleic Acids and Their Derivatives**Jacob J. Steinberg***Albert Einstein College of Medicine and Montefiore Medical Center,
Bronx, New York, U.S.A.***I. INTRODUCTION: HISTORICAL OVERVIEW**

Numerous studies have reviewed the technical background of thin-layer chromatography (TLC; planar or flatbed) (1), which developed under the guidance of Ismailow and Schraiber in 1938 and may date back to Friedlieb Ferdinand Runge (1850). The complexities of the early systems were refined by Stahl (1958–1960), and these breakthroughs began an immense contribution to the understanding of nucleic acid chemistry (2). The early papers of Ismailow, Schraiber, and Stahl were translated and reproduced by Pelick et al. (3).

Simultaneous with these discoveries, paper chromatography (PC) began with major discoveries in solvents and new types of paper by Consden, Martin, and Gordon (1946). The importance attached to paper chromatography resulted in the Nobel prize for Martin and Synge. Brief and compelling historical overviews of TLC and PC are given by Petrowitz (4) and Scheit (5).

One of the most important chromatographic systems for nucleic acids, ion-exchange chromatography, received great impetus with the development of polyethyleneimine-HCl prepared cellulose (PEI), which became available by 1961 (5, 6). The studies that followed laid the foundation for analytical and preparative TLC of nucleic acids. Many plates are available for TLC, but most reports are limited to the use of PEI-cellulose, octyldecasilene (ODS), and silica gel in simple unidimensional systems, typically for prescreening prior to preparative chromatography.

The systems developed for gel electrophoresis have diminished the need for TLC of large oligonucleotides. Additionally, the inability to have stable thick (≥ 2 mm) chromatographic plates has diminished the need for preparative TLC. High-performance liquid chromatography (HPLC) is important for smaller oligomer separations and is especially important for preparative chromatography. TLC and HPLC can serve for initial chemical characterization. Mass spectroscopy coupled with UV and Fourier transform infrared (FTIR) spectroscopy have added to chemical characterizations in chromatography. These systems are now available in TLC. HPLC, however, is limited

when highly radioactive molecules are used that require extensive cleaning of the whole HPLC system. Additionally, TLC offers flexibility in the hands of experimentalists that matches that of HPLC, with less labor and cost (1, 7–9).

II. CHEMICALS AND PLATES

A. Mobile-Phase Selection of Solvents

Siouffi and coworkers (10, 11) discussed specific strategy selections of solvents through the PRISMA approach. Tactically, an initial screen of unknowns or products on TLC is preparatory for HPLC. This is helpful, and the rule of thumb is that 20 solvents for multiple one-dimensional TLC runs will determine most HPLC chromatographic variables. Yet there are many important differences between TLC and HPLC.

The mode of TLC use, including ascending, descending, two- and multidimensional, circular, and drip, has been employed either to improve separations or increase the number of samples that can be run. Automated circular TLC systems in which the solvent is applied in a circular line and flows inward toward the center is another TLC flexible novelty not available in HPLC. Its advantage is that it improves the ability to examine fractions with limited mobility and R_f values near 1. Results are essentially empirical, with many advantages for most techniques based on need for slower time of analyses for a specific set of analytes. Workers have had excellent reproducibility and success with two- or multidimensional TLC, which greatly enhances the number of theoretical plates and ultimate separation. Significant progress in gradient TLC will also have an impact on nucleic acid separations. Automation would enhance the acceptability of these TLC techniques; the lack of automation has slowed TLC popularity.

Thin-layer chromatography has greater flexibility than HPLC. Yet this flexibility increases the complexity of the variables in TLC chemistry. Concentration, viscosity, polarity, pH, ionic strength, composition of the gas phase, and temperature are important and controllable variables. Educated trial and error is helpful to develop initial TLC characterization of analytes (12, 13). Regarding nucleic acid separations, the strategies of many separation techniques emphasize the differential migrations possible for deoxynucleotide monophosphates (dNMPs) with selective retention. The solvents can affect all moieties equally as a selective driving force. Further resolution of dNMPs from DNA can be accomplished by selective obliteration of, or competition among, moieties. For examples, to emphasize or diminish a specific dNMP one could consider competing with an analytically introduced analog such as using deazadeoxyguanadylate (dGMP) for the detection of naturally occurring dGMP adducts in an analyte or depurinating to emphasize pyrimidines.

In optimized planar chromatography, peak capacity in two-dimensional TLC usually exceeds that of HPLC (14). Computer programs exist that enhance choices for solvents. Solvent demixing remains a major problem in predicting R_f values and the ultimate experimental outcome vs. predicted. Again, 20 TLC chromatograms will well define experimental variables for optimum R_f values (15). Solvent selectivity is based on proton donation, acceptance, or dipole interactions. A variety of solvent mixtures exist, and a few dozen of them can reliably serve almost all purposes.

B. Sorbent (Layer) Phase

Considerations for sorbents are physical and chemical properties, pore diameter, pore volume, surface area, particle size distribution, and mean size. Most investigators use adsorption chromatography, not partition chromatography. Solutes remain in solution until they reach fresh sorbent (paper). Analyte spots remain when the layer's capacity for sorbent exceeds that for solvent. The solute undergoes repeated sorption and desorption, and migration is based on mean solvent-sorbent interaction time. Weakly adsorbing moieties migrate farther, and the degree of sorption is great enough to effect separation. Cellulose binds H_2O , yet the chemistry is ill-defined within the amorphous water-cellulose complex (grain size 2–20 μm). Weak physical interactions in TLC include van der Waals forces, dipole-dipole forces, and hydrogen bonding. Cellulose ion exchange further employs polyethyleneimine ($-\text{CH}_2-\text{CH}_2-\text{NH}_2$; 0.7 mval/g) for more specific separations. Typically, polar solvents are used for polar solutes, and reversed phase for hydrophobicity. Solvents are based on elutropic profile, and elution increases with polarity. Speed of solvents also depends on viscosity. Saturated hydrocarbons are poorly adsorbed. Adsorption is highest for $-\text{COOH} > -\text{OH} > -\text{NH}_2 > \text{C}=\text{O} > \text{O-alkyl} > -\text{CH}_3$ as the lowest. Commercially available plates were pioneered by Stahl (silica gel) and ultimately introduced in the mid-1960s (16). These contributions have made TLC accessible everywhere.

Cellulose is used when ion-exchange properties are not needed. It is most often used for the separation of sugars, amino acids, and similar compounds. Cellulose is a popular sorbent for the separation of nucleic acid derivatives and separates pyrimidines (faster R_f values) from purines. Commercial grade microcrystalline cellulose (Avicel) has been used for the retention of guanine (base or nucleoside) in either acid (HCl, formic acid) or base (ammonia) solvents. An example of its use is in the separation of thymine dimers (17).

Diethylaminoethyl cellulose (DEAE-cellulose) is the functional group incorporated into the paper. It is an anion exchanger generally used to separate proteins and enzymes but is also used for nucleic acids, nucleosides, nucleotides, and deoxynucleotides. Separation on DEAE-cellulose is not as sharp as on PEI-cellulose. However, there are considerable data (conditions, solvents, R_f values) on the separation of nucleic acids on DEAE-cellulose. TLC plate separations under various solvents demonstrate the utility of cellulose in the relative retention of amino groups regardless of purine/pyrimidine structure in either acid (HCl, isobutyric acid) or basic ammonium hydroxide mixtures. Ammonium carbonate (less so formate) does effect purine-pyrimidine separations, with R_f values greater for pyrimidines. Examples of use are purine separations (18).

ECTEOLA is named for the epichlorohydrin and triethanolamine groups, which are combined with cellulose. DEAE-cellulose and ECTEOLA-cellulose layers have about the same ability to resolve nucleic acid derivatives. ECTEOLA is especially useful for nucleic acids, nucleosides, and nucleotides as an anion exchanger. Its strength also lies in its rapid separation of pyrimidines from purines. ECTEOLA is valuable in purine analog separation (19).

ITLC (instant TLC) plates are glass microfiber sheets. The addition of silicic acid or silica gel gives the additional designation S A or SG, respectively. The strength and benefit of the paper in multiple-solvent systems allow for the retention of adenine and its associated structures. Standard purine and pyrimidine separations are applicable (1).

Silica gel has been used extensively, although it was not used in the early development of the TLC of nucleic acids. It is also used for the separation of amino acids and proteins. As an example, it is especially advantageous in separating pyrimidine from purines (18, 20, 21).

The suffix “G” is the designation for CaSO₄ binder. Silica G has been used to resolve pyridine nucleotides and UDP derivatives of hexosamines and acetylhexosamines. G is without binder.—G is used for preparation of larger quantities of bases and nucleosides and many of their derivatives. Kieselguhrs are natural geologically derived simply prepared deposits of primarily silica but may vary in both composition and size of particles. Substituted pyrimidine-pentanoic acids are separation examples (22).

Reversed phase (RP; octyldecasilene; ODS; C18) performs essentially the same as silica gel. The opportunity of developing a strategy on RP-TLC and a comparable HPLC system is possible—but not absolute. The convenience of commonly available premixed HPLC solvents (methanol, acetonitrile, tetrahydrofuran, and phosphates) for TLC is not to be denied and accelerates initial information available from TLC. RP-TLC cannot be as easily used over a wide pH range as its HPLC counterpart.

PEI-cellulose has been extensively studied and used in the separation of nucleic acid bases, nucleosides, and nucleotides, with good separation and resolution. It is also used for the separation of RNA and DNA hydrolysates and for large-scale preparations among other applications. Some believe that it remains the most versatile paper for separation of dNMPs. Guanine nucleotides and Pharmaceuticals can be separated with it (23, 24).

High-performance thin-layer chromatography (HPTLC), discovered in 1974 and continuously undergoing improvements, offers thinner layers, more uniform and smaller sorbent particles, and faster development. HPTLC can be used for nucleic acid identification but is not commonly so used. Typically, HPTLC offers too small a quantity of product for identification by GC, FTIR, or NMR. Preparative chromatography is a rapid, though apparently crude technique in which the analyte is streaked across a plate and separation commences on a thick plate (1–5 mm). The nucleic acid of interest is scraped and eluted accordingly. Papers for centrifugal layer chromatography offer an alternative preparative technique. Other circular techniques also have great value. Chiralplates provide excellent results in separating enantiomers (a generic TLC strength) and halogenated compounds and can also play a role in nucleic acid separations. An example is the separation of inositol from body fluids (25).

III. DETECTION

A. General Methods

A general problem with TLC remains the paucity of uniform guidelines for investigators. Users of TLC systems must establish rigorous and reproducible techniques. The majority of reports are of simple unidimensional systems with one unknown analyte. Typically, the analytes are well-characterized pharmaceuticals.

Success in identifying true unknowns in complicated two- or multidirectional systems [two-dimensional TLC (2D-TLC; Figs. 1A and 1B) requires uniform criteria. Some have attempted to validate TLC techniques by delineating *R_f* values and reproducibility, role of

the solvent or mobile phase, sorbent (paper) phase, quality and quantification of zones, solvent strategy, and quantification of analyte imprints (spots). Reports on TLC studies should exhibit more uniformity in stating experimental materials and methods. The list below provides a data sheet for the successful separation of deoxynucleotides (9):

1. Solvents (composition). First dimension: acetic acid (1.0 M, pH 3.5 with NaOH). Second dimension: 5.6 M $(\text{NH}_4)_2\text{SO}_4$, 0.12 M Na_2EDTA , 0.035 M NH_4HSO_4 to pH 4. Age: Stable for >2 weeks.
2. Layer (brand, grade): PEI-cellulose. Size, geometry: Square, 200×200 mm. Method of storage: Refrigerator “crisper” at 4°C. Preparation: No prerun; constant room temperature and humidity. Treatment: Cool air-dried (dehumidified) during spotting. Heterogeneity: (R_f values lower with thicker paper): >1–3% variation over each TLC run.
3. Developing tank (size): 275×275×75 mm with lid.
4. Application amount: 1.0–10.0 μL (or 20,000–100,000 cpm).
5. Drying (origin, plate, after first dimension): at 1 cm, 1 cm X -, Y axes; cold dryer.
6. Direction of development: Ascending, both dimensions.
7. Distance of origin from solvent reservoir (closer=higher R_f): 1.0 cm.
8. Depth of immersion: 5 mm.
9. Volume of solvent in reservoir: 15 mL.
10. Duration of development: First dimension, 4 h; second dimension, 15 h.
11. Temperature: 17°C; 50–60% humidity.
12. Equilibrium humidity in tank: Complete prior to TLC placement.
13. Character of solvent front: Observed as regular, linear.
14. Comparison of R_f vs. R_x : Consistency of chemical migration vs. relative standard, less than 3% variability. All R_f values given as R_x with X , Y coordinates. Note: Conversion of R_x to R_f requires all numbers to be divided by 19 cm. (R_f value multiplied by 100= hR_f .)

Most parameters in TLC are quantifiable, and all quantitative information should be listed in TLC literature or referred to if a technique paper exists (9, 12).

B. Sensitivity

In a two-dimensional TLC system for dNMPs, some attempt was made to discover and analyze altered nucleic acids (adducts) or synthetic nucleic acids typically used as pharmaceuticals (analogs). The technique can ultimately detect one radioactive adduct per 10^8 nucleotides, which is as sensitive as any analytical system available. Some ^{32}P -radiolabel 0.2 μg of DNA to 6.0×10^6 disintegrations per minute (dpm), then assay from 2.0×10^4 to 1.0×10^6 dpm, and can reliably detect as few as 50 dpm over background. This allows a minimum “mathematical” detection of one adduct per 10^5 – 10^8 nucleotides (9, 14). Yet many unique analytes can be detected up to one per 10^8 – 10^{10} dNMP (25 cpm above background). Some workers have detected adduct incorporation by inadvertently “contaminating” the dNMP reaction mixture pool with less than 1 nmol of a foreign dNMP during enzymatic incorporation. These lower values are within the range for detecting modifications by environmental, drug-related, and aging processes, e.g., methylated or deaminated dNMPs (9, 12).

C. Reliability

The use of control dNMPs with every analyte is encouraged. Many laboratories have extensive experience with this technique with alterations of R_f values ranging from 1% to 5%. Some have assessed quantitative TLC techniques and used laser densitometry, direct radiochemical detection of analytes from the TLC plate, and phosphorimager detection, which both enhances detection and reduces analytical time. Radiochemical detection has been statistically correlated with densitometry, but mean values can vary in densitometry by $\pm 6.5\%$ overall. There are also qualitative differences between densitometry and scintillation counting. Specifically, densitometry cannot account for all the analyte “spots” that it detects as analyte “smear,” though the human eye can easily distinguish analyte borders, zones, and spots. Radiochemical detection remains successful in quantifying an area by counting spot cpm. A statistical analysis of radiochemical vs. densitometric data provides a coefficient correlation of 0.93, $p < 0.001$, $n = 23$ pairs, providing the formula

$$\text{Radiochemical detection (dpm)} = 62.4 (\text{mm}^2 \text{ area from densitometry}) - 17,410$$

Both techniques have merits, though. Radiation detection directly off the TLC plate is more successful than densitometry in detecting deoxyuridylate and other less discrete dNMPs. Yet densitometry better delineates away borders between migration patterns of close dNMPs, especially methylated dNMPs. Other variations in CPM reflect quenching of radioactivity from the TLC plates, and at low dpm quenching blocks 90% of the detectable counts, but at high dpm quenching blocks only 50% of the counts. These differences are mathematically quantifiable, and the formulas generated have high predictability. TLC data must be presented in quantitative and statistical values to further increase the reliability of techniques and correlate lab-to-lab discrepancies (9, 12).

D. Data Handling—Quantification and Interpretation

Computerization has been critical in quantifying radioactivity, densitometric, or even colorimetric (chemical or immunochemical) intensities. However, no system has improved on the human eye to analyze zone configuration, which is ultimately a physicochemical phenomenon and indicates properties of the molecule or molecules present. Initial considerations of spot geometry include the following (9, 12):

1. Diffuse: Sharp, small spot at origin, fewer theoretical separation plates, and sharper spots.
2. Isotherm: Head, trailing, or streak zone.
 - a. Overloaded solute.
 - b. Solvent too fast—no equilibrium.
 - c. Irreversible change in solute.
 - d. Adsorption too strong.
3. Double tailing: Competition with contaminating substances.
4. Projections from the solvent front: Impurities.
5. Flattened zones: Close to the front.
6. Lateral spreading: Impregnated with different solvents.

7. Swerved, heart, V, arrowhead: Distorted solvent flow through initial zone.
8. Multiple
 - a. Gradient (pH or concentration).
 - b. Various ionic forms of solute.
 - c. Polymerization.
 - d. Oxidation.
 - e. cis-trans.
 - f. Overloading and precipitation with cellulose complex.
 - g. Salt complexes,
 - h. Heating origin.
9. Typically ammonium produces single spots; ammonium salts produce doublets.
10. Spot spreading by molecular diffusion or by gradient eddy diffusion through random multiple paths. Concomitantly, nonequilibrium is produced by unevenness of flow vs. slowness of uniform attainment at front.
11. Estimation of spots: Computerized laser densitometry and direct scintillation counting from plates define geometry, exactly calculate R_f (peak), and quantify zone.

IV. ANALYTE IDENTIFICATION

Analyte identification in nucleic acids (Fig. 1) resides in their functional groups and heterocyclic rings. The pioneering text on nucleic acid chemistry by Kochetov and Budovskii (26) should be available to every nucleic acid chemist. Any approach to the identification of unknown or modified nucleic acids should begin with characterizing functional groups. Further, functional groups offer sites of chemical alteration, with simple benchtop techniques, that can initially confirm structure. tRNA complexity has served as the primary impetus for developing accurate and reproducible techniques to separate methylated nucleosides (27).

In general (28), hydrophobic modifications and methylation decrease R_f values; hydrophilic modifications, e.g., succinylations, increase R_f values. Low vs. high negative charges are dependent on TLC and solvent system; glycols—borate retards and periodate oxidizes (sensitive).

Sugars are pentose-ring riboses and deoxyriboses are readily reacted with. The sugars are not charged at physiological pH and lose a proton at pH 12. The major advantage of the phosphodiester bond is that it is cleaved with extreme acid or alkali. The charge and number of the phosphates ultimately confer their mobility on chromatography. The monoester phosphate has two ionizable OH groups and is in relative equilibrium at physiological pH.

One-dimensional TLC for characterization of known purines is appropriate for screening analyses. Studies have been done on lipophilic characteristics of xanthine and adenosine derivatives. These are potentially important for large classes of drugs, including chemotherapeutic ones (chloroadenosine and numerous antiretroviral nucleic acid analogs) (Fig. 2). Criteria employed silicone and infrequently HPTLC. Methanol phosphate buffer, pH 7, is used. Methanol varies from 30% to 80%. Equations are generated to allow maximum allowable separations among 44 purines (29).

Separation of hydroxy-2'-deoxyguanosine-3'-monophosphate (30) is done with 1.5 M ammonium formate (pH 3.5) followed by 0.4 M ammonium sulfate. Though good separation of C₈hydroxy-dGMP is evident, most dNMPs remained in the midline, with a significant artifact in D2. Aside from aging, metabolism of diethylstilbesterol (DES) also forms C₈-hydroxydeoxy guanady late.

Novel separations of anomeric alpha (pharmacological) purines were done on copper acetate Chiralplates (31). Solvents were methanol-water-acetonitrile. Visualization was by UV light. Graphs were plotted based on alteration of acetonitrile percentages.

Silica gel separations of noncyclic radioactive [³H] adenosine (neuromodulators) and reduced from two- to one-dimensional analyses were reported (32). The solvents were various proportions of butanol-ammonia-water-acetic acid. Rapid separation occurred in 3-4 h. Typical separations unequivocally demonstrated cAMP inosine, adenosine, and adenine. UV sensitivity occurred at 5 nmol. Paces and Kaminek (33) separated plant cytokinins (adenine) on silica gel in ethanol-ammonium borate, butanol-ammonia, and butanol-water. Norman et al. (34) measured ATP from degraded meat via 5% cold PC A on silica gel and isobutanol-amyl alcohol-ethoxyethanol-ammonia-water. They aged solvents for 48 h.

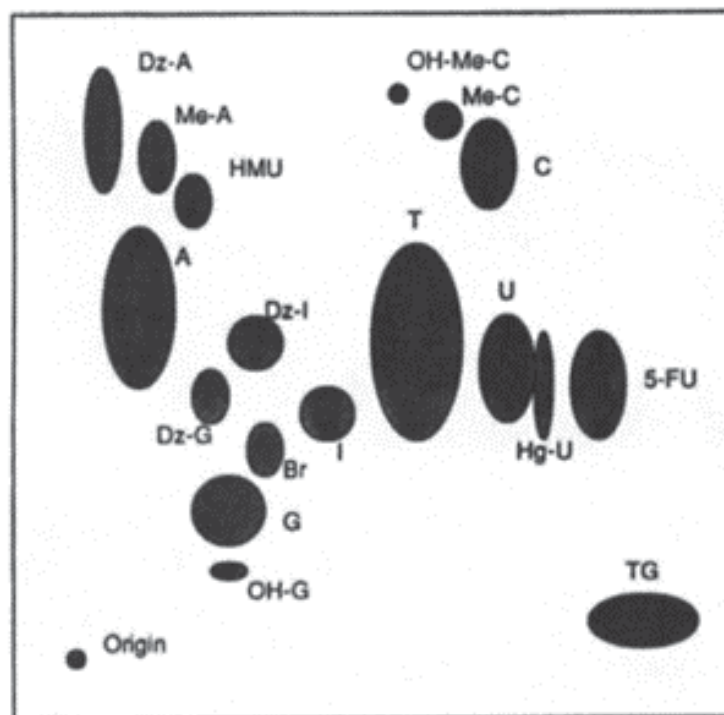
Guanine can be separated on PEI-cellulose with 0.5 M triethylammonium bicarbonate (TEAB) at pH 7.6. Good separation of cyclics, phosphates, and nucleosides is evident (35). Trifilo and Dobson (36) separated cyclic purines by PEI with ammonium acetate/hydroxide-ethanol at pH 9.0. One dimension can be used ascending, from triphosphates to nucleobases. Gulyassy and Farrand (37) separated cyclic purines with PEI in 0.4 M acetic acid, then 0.125 M LiCl. Mahandhar and Dyke (38) separated GTP with PEI and luciferase-water, and then 1.4 M LiCl for 50 min. Assay was by scintillation counting.

Alkylated deoxyuridine separation was done with RP-TLC in methanol/propanol-water-dichloroethane. Water-ethanol had the greatest effect with oligonucleotides (39). TLC gave quantitative structure-activity relationships (QSARs).

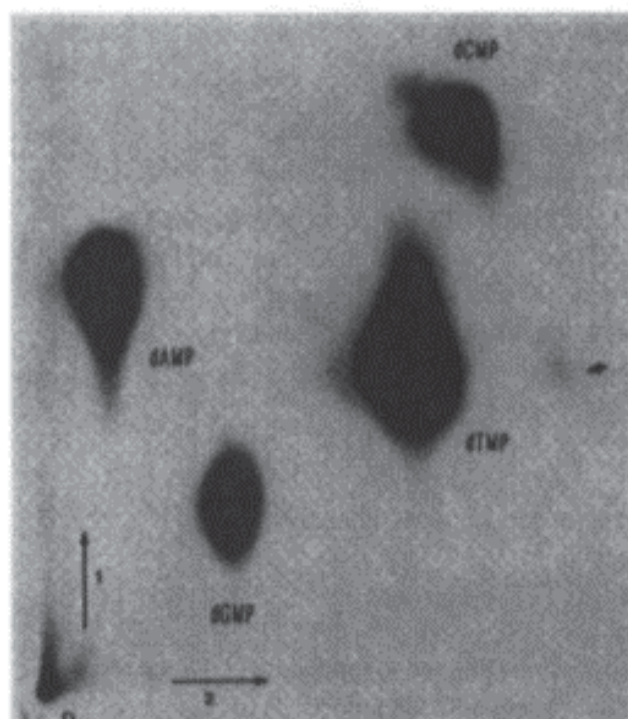
Thymine dimers were separated on silica gel with one-dimensional (1D) chloroform-methanol-water and two-dimensional (2D) ethyl acetate-propanol-water followed by spraying with cysteine-surf uric acid (40). Friedberg (41) also separated thy mine dimers but employed cellulose and *n*-butanol-water and 2D ammonium sulfate-sodium acetate-propanol. Scotch tape can be used to remove cellulose for scintillation counting.

Derivatization can be used for chemical identification of nucleic acids with dimethylaminonaphthalene-5-sulfonyl chloride (Dns-Cl). Formic acid (6%), acetate-ethanol-ammonium hydroxide, or ethyl acetate-ethanol-ammonium hydroxide was used on a polyamide sheet (42). Tritiated borohydride was also used in postlabeling reduction.

Halogenated uracils were separated on silica gel 60 HPTLC gels. Solvents were chloroform-ethanol-water with or without acetate (43). As many as 27 pyrimidine analogs have been separated. Cellulose TLC and various combinations of butanol-ammonia-ethyl acetate-formic sodium phosphate-propanol-isoamyl alcohol were used. New analogs were discovered by twodimensional TLC with PEI in isobutyric acid-water-ammonium (1D) and ammonium sulfateisopropanol-sodium acetate (2D). Hydrazine was used to destroy other pyrimidine rings. These modified nucleotides were resistant to postlabeling.



(A)



(B)

Figure 1 (A) Schematic of 20×20 cm stylized plate of dNMPs (as represented by nucleic acid

abbreviations) presently separable by 2-D TLC. (B) Control human placental DNA: Findings for human placental DNA digest include (24 hour autoradiogram): 1. Normal retention times of all radiolabeled monophosphates to cold UV-markers. Autoradiogram demonstrates, in clockwise position, the major bases of DNA as their deoxyribonucleotides: adenine (Retention factor and coordinates $\{R_f\}$ x, y- cm=2.7, 11.2), thymine (R_f =7.5, 10.0), guanine (R_f =3.6, 5.5), and cytosine (R_f =12.8, 14.9). After 72 hours of autoradiography, four additional minor spots were more clearly visible: 5-me-dAMP, two spots close to dCMP representing 5-me-dCMP (proximate to dCMP), and dUMP (arrow). Human placental DNA is essentially devoid of methylated bases and dUMP, not surprising in rapidly dividing cells, repair induced and efficient cells.

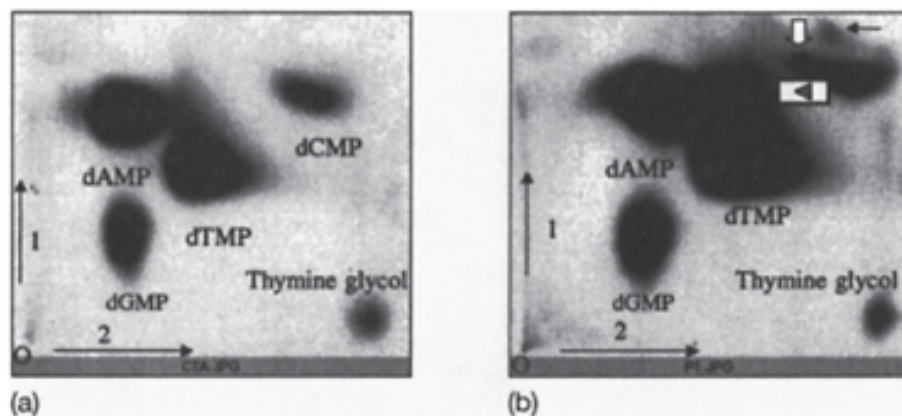


Figure 2 (a) Control human DNA compared with (b) DNA of a patient on AZT. O represents the origin of the chromatographs, and arrows 1 and 2

are the two dimensions in chromatography. $\Downarrow$ representing 5-met-dCMP; $\leftarrow$ and $\boxed{\leftarrow}$ are adducts yet to be identified.

Diol detection occurs in methyl red in ethanol–boric acid–acetone. Arabinosyl, ribosyl, and deoxyribosyl are well handled with PEI in LiCl (44). Acyclonucleosides are powerful antiviral agents; an example is, acyclovir for herpes. These analogs lack one or more atoms on the pentofuranose ring. Separation strategies can be developed to take advantage of the alterations in the sugar.

Thin TLC plates (0.1 mm thick) give no elongated spots. The separations were fast (10 min), with good theoretical plates (5000) at $R_f < 5.5$ (45). No HPLC or complicated apparatus is required, and no buildup of background radiation occurs due to TLC disposal. Chromatography required ammonium sulfate (0.2–5.0 M). Salts [many less ionized than $(\text{NH}_2)_4\text{SO}_4$] contributed little. The pH (borax, acetate, HCl, ammonium) contributed little to separations achieved with ammonium sulfate.

Thin-layer chromatography is used to separate nucleotides from cell culture (46). TLC exhibits high resolution but low load capacity, and cumbersome procedures are required to handle material. CEL 300 plates and butanol–acetic acid–water or ethanol–ammonium acetate (pH 5) effected good separations. Colorimetric quantification is possible with ninhydrin–cadmium. TLC was most effective for nucleotides below 4000 MW. Plant viral RNA has been chromatographed with cellulose TLC, *n*-butyric acid–ammonia–water in one dimension and ammonium sulfate–sodium acetate–isopropanol in two dimensions. The system easily separates 2'- and 3'-NMPs. Munns et al. (47) separated methylated RNA by two-dimensional TLC. Varying percentages of silica gel and cellulose in acetonitrile (ACN) ethyl- or acetate–propanol–butanol–water–ammonium hydroxide have been used. Many of these simpler systems delineate the NMPs in the TLC plate's midline. Pyrimidine and guanine dinucleotides are separated on PEI and 0.8 LiCl–acetic acid.

An additional challenge of biomedical applications of TLC relates to separations of cyclic nucleotides from noncyclic phosphates. Alumina TLC and ammonium acetate pH with ammonium hydroxide have been used to effect these separations (48, 49). 3',5'-cGMP uses borate-impregnated silica in butanol–methanol–ethyl acetate–ammonium hydroxide. Cyclic pyr/purines are separated on cation exchange plates pretreated with HCl, as opposed to the popular anion (PEI) systems. A run with 0.05 M oxalic acid increases separation quality.

The utility of gel electrophoresis for long-chain oligonucleotides has relegated the use of TLC to smaller chain species. Intermediate-chain oligonucleotides are nicely handled by HPLC, but many smaller ones are not. Silica gel TLC has been important in oligomer separations well up to decamers (50–53).

tRNA digests can effect separation based on nucleobases, irrespective of adenines (51–53). PEI–cellulose in butanol–methanol–water is used, followed by formic acid in water. Using TLC that is salt-sensitive, PEI plates, and 0.5% formic acid in an ascending fashion (occasionally using urea, which sharpens separation), 0.15 M lithium formate, pH 3.0, exhibited separations with low radioactivity (under 100 dpm after 3 weeks autoradiography). In two-dimensional TLC systems, one can also add urea–formic acid–

pyridine. Two-dimensional TLC is carried out in one dimension with 22% formic acid and in two dimensions with 0.1 M formic acid and pyridine to pH 4.3. Variations, can occur in TLC batches due to different binding capacities and primary, secondary, and tertiary amines of plates. The best pH is at 4.3 which is successful for up to 50 nucleotides.

Avicel cellulose can be used in two- or three-dimensional separations with isopropanol– ammonium hydroxide, isobutyric acid–ammonium hydroxide-EDTA, or ammonium acetate–ethanol (54). Up to 18-mers are nicely resolved in stepwise fashion. Silica gel in ammonium acetate separates up to 12-mer.

Practically none of the nucleic acids with altered moieties that form, whether from oxygen stress, aldehydes, or other reactive species, can be immediately chemically defined (26, 57). This is neither practical nor routinely possible. Nucleic acid adducts define a particular pattern that are specific to a chemical alteration, mutagen, or carcinogen. Steinhäuser et al. (51) discuss fingerprint analyses in tRNA TLC. One can use as much specific chemical characterization as possible (58) but ultimately rely on fingerprint analysis. Many published figures demonstrate examples of fingerprint chromatograms (55, 56, 59).

V. DETECTORS AND INSTRUMENTATION

Major techniques use colorimetry and visual inspection, zone elution (scraping) for HPLC, spectrometry, GC, or voltammetry, densitometry, and radiochemical techniques. Computerized radiochemical, laser densitometric, and phosphoroimager techniques are also successfully used (56).

Identification of an analyte requires R_f values that are reproducible to $\pm 3\%$. The geometry of the unknown must conform to that of the known under the same chromatographic conditions. Cochromatography of known and unknowns is always required.

Fluorescence remains a standard technique. TLC plates are impregnated with UV-fluorescent material at 254 nm (typically zinc silicate). Upon exposure, the nucleic acids absorb at 254 nm and therefore quench, so they appear black against a blue-green background of fluorescence. Visual error rates for quantification by UV remain high (30%). Some scanning detectors use UV light,

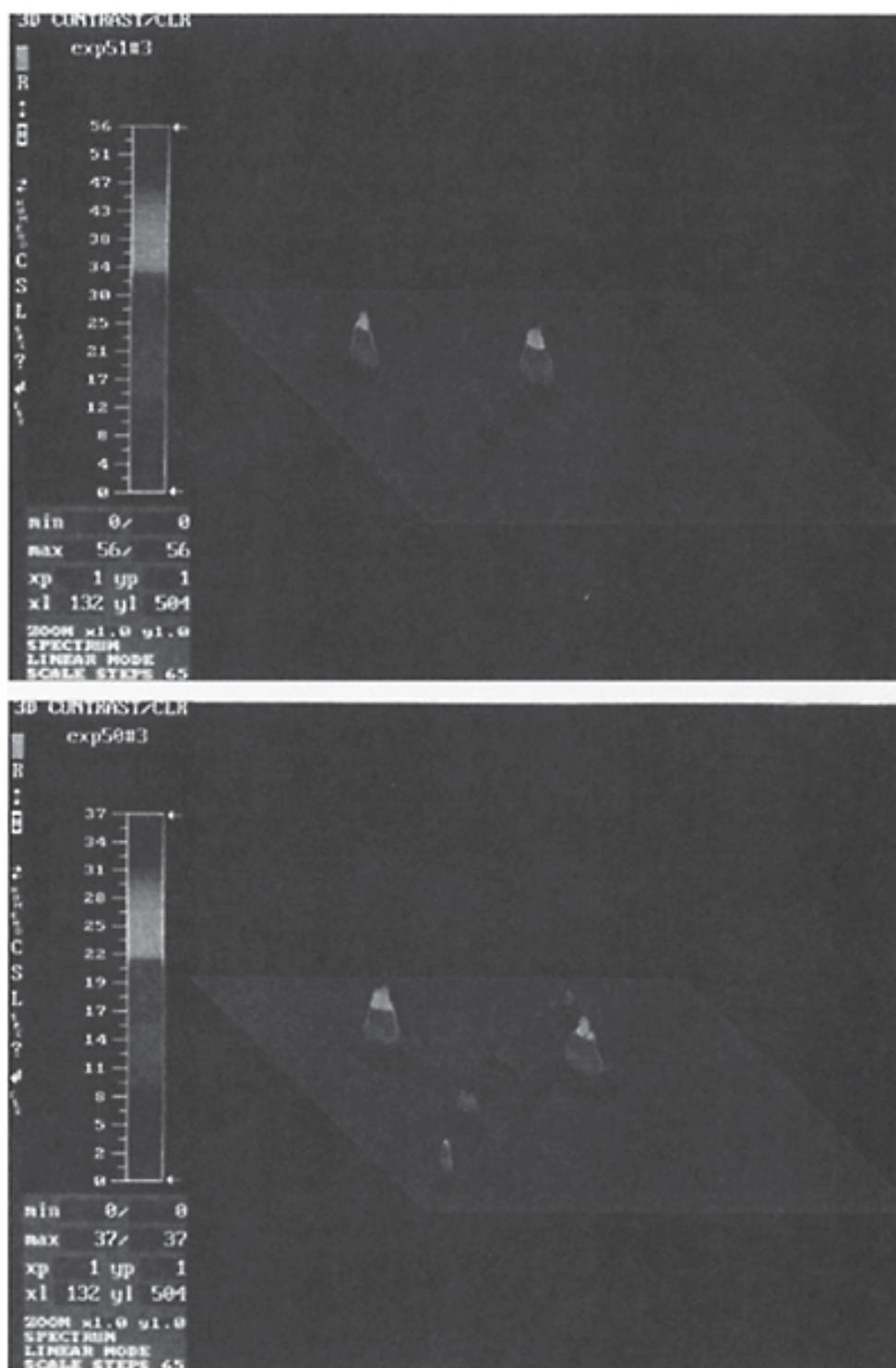


Figure 3 Three-dimensional ^{32}P computer reconstruction of DNA digest and DNA digest with chemically introduced dUMP.

which can be applied to TLC plates to improve quantitative data. Sensitivity is enhanced by fluorescent techniques over other in situ analyte quantification easily available. Typically, these techniques require derivatization (pre- or postchromatography).

Fourier transform infrared (FTIR) detection is available for TLC (60). Many chromatographic papers have high IR absorbance and are inadequate for direct IR measurement. Techniques are now available that allow direct measurement. Gas chromatography is best employed for zone elution and certainly has application to nucleic acids although lipids have been more extensively studied.

Mass spectrometry (MS) is used for nucleic acids, but typically after zonal elution to avoid interfering solutes (8, 61). Ion-coupled MS/TLC must take into account the sorbent, solvent, and analyte, which should not exceed 0.25%, (w/w) based on sample and sorbent. This requires the ability to extract, elute, or volatilize analyte directly from the TLC plate. These instruments will be a boon for the detection and characterization of analytes. Investigators have defined nucleic acid photoproducts; radical-induced products; products modified by xenobiotic biotransformation; new and naturally occurring nucleosides, particularly those found in RNA; methylated bases; stable isotopes; and interface between MS and liquid chromatography systems.

Some workers (7–9, 12, 50, 55) have accumulated a large amount of data based on laser densitometry. Some base laser densitometry on autoradiographically developed X-ray film from 2D TLC chromatograms that show the separation of radioactive dNMPs. Exposure times enhance the ability to label unknowns at high counts in as little as 2 h, but typically run 24–72 h. Present densitometric techniques can range from a few minutes for one-dimensional analyses to 2 h for complete analyses of a 20×20 cm autoradiogram. Comparisons of techniques to direct scintillation counting approach *r* values of 0.99 and similarly correlate with the best quantifying techniques (7–9, 12, 50, 55).

The coupling of sensitivity (25 dpm above background), rapidity (15 min for a 20×20 cm plate), and scintillation counting with computerization is available in TLC quantification. This allows in situ measurement of radioactivity and quantitative reconstruction of the nucleic acids in two or three dimensions. It is extremely easy to compare prior TLC plates, generate tables for comparison, and rapidly apply statistical or analytical methods by computer. This has yielded quantifiable relationships in nucleic acid and DNA chemistry that were difficult to examine previously (7–9, 12, 50, 55).

Sensitive, rapid, and very quantifiable phosphorimagers have become available. They use phosphors that are sensitive enough to capture various radiation energy sources comparably to autoradiography in less than 8% of the time. They deliver not only standard *R_f* values but also a mass of quantitative information. The better ability to subtract controls, carry out statistical analyses, and enhance minute adducts magnifies adduct and analog detection of nucleic acids. Some studies have presented data on phosphorimager use and TLC including “high power” focusing carried out by computer interaction (7–9, 12, 50, 55).

Immunoassays similar to Western blotting allow colorimetric reactions to occur after binding by antibodies that recognize nucleic acid adducts and subsequent recognition by enzyme-linked antibodies that cause colorimetric reactions with the application of the appropriate substrate. (See Table 1.) Little experience is available about these techniques, but they will increase the specificity of reaction adducts of interest.

Automated multispotters, scanning detectors, sample applicators with microprocessor control, automatic pumping, multiple development of TLC plates, automated circular TLC systems, automated scanning, and densitometric evaluation are available (CaMAG, Inc.).

VI. PROTOCOL APPLICATIONS

Thin-layer chromatography has been used in the study of metabolic diseases; illicit drug use; toxicology, including mycotoxins; environmental injury; and, particularly, analytical drug characterization and quantification for both diagnostic and regulatory purposes (7–9, 12, 50, 55, 62, 63). It has extensive applications for the pharmaceutical industry in all areas of drug development: synthesis, separation, drug screening, batch quality testing, and government monitoring and reg-

Table 1 Sensitivity and Exposures Measured for Human DNA Adduct Detection Using Immunoassays and ^{32}P Postlabeling

	Immunoassay	^{32}P -Postlabeling
Exposure	Polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines, UV light, oxidative damage, nucleoside analogs	Polycyclic aromatic hydrocarbons (PAHs), alkylation cancer chemotherapeutic agents, mycotoxins, tobacco, nucleoside analogs
Advantage	Reliable, inexpensive	Sensitive, small amount of DNA required (2–10 μg)
Disadvantage	Large amount of DNA (200 μg), antibody cross-reaction	Unidentifiable adducts, unknown and uncontrolled phosphorylation

Source: Ref. 75.

ulations. TLC is used for the detection of both licit and illicit drugs. Some workers have had success in using TLC to quantify antiviral and antineoplastic drug effects, both as a screening test and for drug discovery.

Many highly specific preparative papers have been published on the TLC of nucleic acids, especially on the preparation of products for pharmaceutical applications. These works contain TLC data on hundreds of compounds including solvent systems, paper combinations, and R_f values.

Considerable literature exists on extraction of nucleic acids and DNA from body fluids and tissue samples. The ability to detect normal dNMPs in DNA, DNA damage by aging or disease, or the effects of a drug on DNA is a primary area of TLC application. Environmental and DNA damage is caused by radiation, ozone, aldehydes, alkylating agents, and mercury (7–9, 12, 50, 55). In drug use, significant measurable effects of antiviral and antineoplastic agents are evident. Alcoholism, Alzheimer's diabetes mellitus, and a host of other diseases with metabolic consequences can alter DNA detectably. Genetic errors of metabolism can further be detected and quantified by TLC

techniques. TLC can measure enzyme activity and easily separate nucleic acids and nucleotide monophosphates from enzyme substrate mixtures (7–9, 12, 50, 55).

The application of these enhancement assays as a biomarker of damage from the direct or metabolic consequences of exposure and injury to tissue samples and biopsy specimens is feasible (7–9, 12, 50, 55, 64). Harris (65) advocated these techniques to better measure potential environmental toxicity to human populations because only nanogram amounts of DNA (and therefore milligram amounts of tissue) are required to quantify the formation of abnormal adducts. Ultimately one can correlate the deleterious effects of exposure on DNA and measure nucleotide markers of environmental disease processes and organ injury. The application of these techniques to animal and human studies may result in the recommendation of environmental guidelines that could alter the course of those at high risk of experiencing organ complications and teratogenic risk from environmental hazards. Betina (66) reviewed the application of TLC for the detection of environmental toxins but gave little information on nucleic acids.

VII. RECENT ADVANCES IN NUCLEIC ACID TLC

New achievements applicable to TLC are predominantly related to coupled instrumentation with more sensitive devices. Other applications apply to more sensitive HPTLC and refinement of ^{32}P postlabeling.

Fast atom bombardment coupled with mass spectrometry (FAB/MS) and tandem mass spectrometry (MS/MS) can be done on urine following chromatography on silica gel HPTLC plates (67). Tandem mass spectrometry uses two stages of mass analysis, one to preselect an ion and the second to analyze the fragments induced, for instance, by collision with an inert gas such as argon or helium. This dual analysis can be tandem in space or tandem in time. “Tandem in space” means having two mass spectrometers in series (68).

Biopolymer sequencing with mass spectrometry became important and accessible with the development of matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). The use of sequential digestion for the rapid identification of proteolytic fragments, in turn highlighting the general utility of enzymatic MALDI ladder sequencing and ESI tandem mass spectrometry, is improving. In general, MALDI ladder sequencing is relatively straightforward to interpret, especially for oligonucleotide (69).

An HPTLC method has been developed for the analysis of inositol mono- to hexakisphosphates on cellulose-precoated plates where plates are developed in 1-propanol-25% ammonia solution-water (5:4:1) and quantities as low as 100–200 pmol can be detected by molybdate staining. Charcoal treatment is employed to separate nucleotides from inositol phosphates with similar R_f values prior to HPTLC analysis (25).

A substantial improvement exists for the assay of enriching 8-oxodeoxyguanine by TLC prior to ^{32}P labeling. Unmodified nucleotides are removed by one-directional polyethyleneimine (PEI)-cellulose TLC in 0.2 M formic acid. 8-Oxodeoxyguanine is eluted in 2 M triethylammonium acetate (pH 7.0), lyophilized, ^{32}P -labeled, resolved by TLC, and quantified. The 8-oxodeoxyguanine signal is found to increase linearly with increases in the amount of DNA (70). A ^{32}P postlabeling method can be used for the sensitive detection of 1,*N*(2)-propanodeoxyguanosine adducts of the lipid peroxidation

product *trans*-4-hydroxy-2-nonenal in vivo. The adducts are enriched by nuclease P1. They are subsequently reacted with [γ - ^{32}P]ATP to give the respective 3',5'-bisphosphates, which are two-directionally separated by PEI-cellulose TLC and quantified by autoradiography. This method can be applied to DNA from colon and brain tissue of untreated Fischer 344 rats and humans (71).

7*H*-Dibenzo[*c,g*]carbazole (DBC) is a potent environmental liver carcinogen. Liver DNA from mice treated with DEC exhibited seven distinct 7*H*-dibenzo[*c,g*]carbazole-DNA adducts as detected by ^{32}P postlabeling using multidimensional TLC. To improve quantification and chemically characterize the adducts, hydrolyzed DNA samples were ^{32}P -postlabeled, and the adducts were separated from the unadducted normal nucleotides on TLC by using 0.65 M sodium phosphate (pH 6.8) as mobile phase in the first direction. Adducts were eluted from the TLC plates with 4.0 M pyridinium formate, concentrated, resuspended in 50% aqueous methanol, and injected onto the HPLC; five individual adduct peaks were resolved and collected by this method (72). DNA isolated from livers of rats receiving tamoxifen was analyzed by the ^{32}P -postlabeling method. The postlabeled DNA hydrolysis mixture was analyzed both by reversed-phase HPLC with ^{32}P on-line detection and by TLC on polyethyleneimine plates followed by autoradiography. Using the HPLC method, five well-separated adduct peaks were detected, whereas with the TLC method two groups of adduct spots were observed. The detection limit of the TLC assay was lower (0.5 adduct per 10 nucleotides) than that of the HPLC assay (3 adducts per 10 nucleotides). The TLC assay is more sensitive but also more laborious. The advantages of the HPLC assay were, in addition to better resolution, the ease of quantification and operation (73).

A combination of TLC and HPLC was used to achieve separation of ^{32}P -postlabeled 7-methylguanine and 7-(2-hydroxyethyl)-guanine adducts. The levels of these two adducts were determined in human total white blood cells (mean values 0.7–1.5 adducts per 10 or 7, respectively, normal nucleotides) and isolated lymphocytes (mean values 1.1–12 adducts per 10 or 7, respectively, normal nucleotides). The separation of the two adducts revealed that the level of 7-(2-hydroxyethyl)guanine was twice the level of 7-methylguanine adducts in total white blood cells, whereas in isolated lymphocytes it was at least four times more. The combined level of these two adducts in the lymphocytes of nonsmokers was 1.1–8.4 adducts per 10 (or 7, respectively) normal nucleotides, and in the lymphocytes of smokers, the level was 5.6–12 adducts per 10 (or 7, respectively) normal nucleotides. We also reported the detection of three unidentified adducts in the samples analyzed, and at least one of these adducts was related to smoking. The chromatographic behavior and depurination at neutral pH indicated the probable 7-alkylguanine or 3-alkyladenine nature of these unidentified adducts (74).

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Peptides and Proteins

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I. INTRODUCTION

Thin-layer chromatography has found extensive application in protein chemistry. This includes recovery of peptides in microgram and nanogram quantities for further primary structural analysis; identification of peptides in partial hydrolysates, in correlating the chromatographic properties of the intact peptides with those of individual amino acids; peptide mapping to characterize or identify a protein available in very small quantities; resolution of diastereomeric and enantiomeric peptides without any derivatization; fractionation of proteins on the ultramicro scale; testing the optical homogeneity of synthetic peptides; and determination of molecular weights of peptides and proteins. However, TLC has been practiced to a lesser extent for the analysis of peptides, especially the proteins, because several other high-resolution techniques are available, e.g., HPLC; column liquid chromatography involving size exclusion, ion-exchange, and affinity phenomena; SDS-PAGE; capillary electrophoresis; and mass spectrometry as a detector.

Optimization of chromatographic separations of peptides and proteins means a complete resolution of all components in minimum time, on a preparative scale, and with the recovery of bioactivity. Therefore, a knowledge of the behavior of peptides and proteins with both mobile and stationary phases is required; this includes information about kinetics of diffusion, adsorption and desorption, denaturation, and conformational changes. Various principles of liquid chromatography have successfully been applied to TLC resolution of peptides and proteins, e.g., reversed phase, size exclusion, and ion exchange. The thin-layer materials used for the purpose include silica gel, cellulose, mixtures of silica gel and cellulose, hydroxyapatite, and cross-linked dextran gel filtration media such as Sephadex (various grades from Pharmacia, Uppsala, Sweden). The ordinary porous silica-based stationary phases containing chemically bonded alkyl chains of varying lengths have several disadvantages, including low stability at alkaline pH values (pH>8), secondary equilibria caused by low diffusion kinetics within the pores,

and ion-exchange effects due to ionized underivatized silanol groups. Therefore, alternative stationary phases are being developed, e.g., coated silica phases, polymer-based phases, and nonporous materials. The separation and purification of peptides and proteins by an ion-exchange approach offers advantages because the mild separation conditions provide higher recovery of bioactivity.

The various peptides and proteins have been located on thin-layer chromatograms by using ninhydrin, fluorescamine, orthophthaldialdehyde, iodine vapors, or UV. Quantification has been

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performed by densitometric scanning or by spectrophotometry after eluting the peptides. Immunochromatography using monoclonal antibodies has also been used for quantification.

II. PREPARATION OF SAMPLES

Depending on their nature and source, various methods have been reported in the literature for digesting proteins before applying them to thin-layer plates. Two of these methods are described below.

1. Proteins are dissolved in ammonium bicarbonate (0.5%, pH 8.0) and digested with trypsin (1% w/w) for 4 h at 37°C. Chymotrypsin (1% w/w) may be added for trypsin-chymotrypsin digest and the digestion continued for a further 4 h. The peptides are recovered by freeze-drying (1).
2. Burns and Turner (2) subjected proteins to either alkylation with iodoacetic acid (3) or performic acid oxidation (4) to render them susceptible to enzymatic digestion. The treated proteins were then dissolved in ammonium bicarbonate buffer (0.05 M, pH 8.4) to a concentration of 2 mg/mL, and TPCK-treated trypsin [L-(1-tosylamide-2-phenylethyl chloromethyl ketone)] was added to give a final enzyme/substrate ratio of 1:75. The digest was incubated for 5 h at 30°C, freeze-dried, and redissolved in 10% isopropanol for application to the plates.

For TLC of smaller peptides, the samples have been either synthesized (5) or obtained commercially. The stock solutions (0.025 M) are prepared in aqueous 2-propanol (10%) and are kept refrigerated when not in use. For proteins, solutions can be prepared in dilute saline solutions or in an appropriate buffer.

Some recent applications of TLC to peptides and proteins are included in Table 1. Experimental studies of solute retention and support matrix effects in RP-TLC of peptides were published (71). A rapid thin-layer immunochromatography method using monoclonal antibodies of two distinct specificities was used for quantification of protein antigens (75). The following reports were also made: Certain nonstoichiometric models for theoretical treatment of the chromatographic process on ion-exchange phases (77–79); a review on the peptides and proteins (80); microscale synthesis of a dipeptide from its component amino acids and its analysis by chiral phase TLC (81); TLC on Chiralplates with MeCN–MeOH–H₂O (4:1:1) for the determination of amino acid configuration of

synthetic peptide analogs prepared starting from the racemic aromatic amino acids (82); studies reporting dependence of the silanophyl effect on the chemical structure of peptides and on the type of mobile phase (83); HPTLC and electroblotting for detection of antiganglioside antibodies in human sera (84); study on the salting-out behavior of some peptides with aromatic groups by adsorption TLC on cellulose (85); methods for the separation of peptides on Empore TLC sheets and blotting onto polyvinylidene difluoride (PVDF) membranes with subsequent gasphase sequencing (86); and a method for the analysis of peptide and protein hydrolysates by 2D cellulose TLC and densitometry and its application to luteinizing hormone (87).

III. PREPARATION OF THIN-LAYER PLATES

Most workers have used commercially available precoated silica (1, 5, 6) or cellulose plates. Sometimes even commercially available plates have uneven coatings. This can be checked by holding the plates against a lightbox and looking for dark streaks or patches that indicate uneven thickness. Such plates should be rejected or used for initial trial runs. However, the method of Heathcote et al. (8) for making plates with cellulose powder has been used widely and is described below. Preparation of thin-layer plates from Sephadex is also described.

A. Thin-Layer Plates from Cellulose

Cellulose powder is slurried with methanol–water (4:1, 200 mL). The slurry is poured into a Büchner funnel and is washed successively with 2-propanol–water–acetic acid (3:1:1, 300 mL);

Table 1 Solvent Systems, Support Materials, and Detection Procedures for TLC of Proteins and Peptides

Sample	Protein/peptide	Developing system	Detection	Ref.
1.	Carbazo peptides	(a) <i>n</i> -Butanol–acetone–acetic acid–5% NH ₄ OH– water (9:3:2:2:4) (b) <i>n</i> -Butanol–acetic acid–5% NH ₄ OH (11:6:3) (c) <i>n</i> -Butanol–acetic acid–pyridine–water (15:3:10:12) (d) <i>n</i> -Butanol–acetic acid–5% NH ₄ OH– H ₂ O two-phase mixture (6:1:1:2)	0.2% Ninhydrin spray (<i>n</i> -butanol–2 N acetic acid, 95:5) followed by a spray of a saturated solution of K ₂ Cr ₂ O ₇ in conc. H ₂ SO ₄	32
2.	Leu-Trp-Leu	CHCl ₃ –(34%)	Dried for 2 h at 60°C	6

		NH ₄ OH–ethanol (2:1:2)	sprayed with 1% (v/v) triethylamine in acetone followed by 0.1% fluorescamine in acetone	
3.	Np-S-L-Met-DL-Ala-O-Np, Np-S-L-Met-L-DL-Ala-O- Np, Np-S-Met-Ala-Met-O- Me	Solvents and R_f given in Table 3	Iodine	5
4.	<i>N</i> - <i>t</i> -Butoxy peptides	Ethanol–pyridine– water–acetic acid (5:5:3:1)	Exposure to chlorine followed by spraying with <i>o</i> - toluidine reagent or dicarboxidine reagent	35
5.	Lysino alanine	(a) Butanol–anhyd. acetic acid–water (4:1:1) (b) Phenol–water (3:1)	Ninhydrin	37
6.	Peptides from proteases	(a) Chloroform– methanol (3:1) (b) Chloroform– methanol–acetic acid (45:4:1)	(a) Aq. 2% sodium nitroprusside (b) 4% Ninhydrin in acetone– pyridine–acetic acid (97:3:2) (c) 1-Naphthol–NaBrO (Sakaguchi reagent)	38
7.	Insulin	(a) 0.1 N HCl–96% ethanol (1:1) (b) 0.5 N HCl– ethanol–acetone (5:3:0.5)	0.5% Ninhydrin in acetone	39
8.	Pepsin, trypsin, and α - chymotrypsin	(a) 0.1 N HCl– ethanol (1:1) (b) 0.5 N HCl– ethanol–acetone (5:3:0.5)	0.5% Ninhydrin in acetone, heated at 100°C	40
9.	Angiotensin	(a) <i>n</i> -Butanol–acetic acid–water (4:1:1) (b) <i>n</i> -Butanol– pyridine–water (15:10:3)	Ninhydrin	41
10.	Bradykinin	Isopropanol–methyl acetate–conc. ammonia (9:7:4)	UV	42
11.	Dansyl peptides from ATP- creatine phosphotransferase	Methyl acetate– isopropanol–conc. ammonia (9:7:4)	UV after exposure to dioxane vapors	43
Sample	Protein/peptide	Developing system	Detection	Ref.

12.	Glycopeptides from <i>E. coli</i> and <i>B. megaterium</i>	<i>n</i> -Butanol–pyridine–acetic acid–water (130:20:6:24)	Chlorine–toluidine	44
13.	Lipopeptides from <i>Nocardia asteroides</i>	Chloroform–methanol (9:1)	P ₂ O ₅ , 130°C	45
14.	Cyclotriptides from <i>Aspergillus ochraceus</i>	Five different solvent systems	Chlorine–toluidine, KMnO ₄	46
15.	Basic polypeptides	(a) <i>n</i> -Butanol–25% ammonia–water (34:3:3) (b) Chloroform–methanol (9:1)	Ninhydrin–chlorine–toluidine	47
16.	Lipase, diastase, papain, emulsin, invertase	96% Ethanol–0.1 N HCl (1:1)	0.5% Ninhydrin in acetone, heated at 100°C for 10 min	48
17.	Lipopeptides from <i>E. coli</i>	Chloroform–methanol–water (68:25:4)	Radioactivity	49
18.	Angiotensin antagonists	(a) Chloroform–acetone (5:1) (b) <i>n</i> -Butanol–acetic acid–water (4:1:1)	Ninhydrin	50
19.	Vasopressin and oxytocin, tripeptide amides	Butanol–acetic–acid–water (4:1:1)	UV	51
20.	<i>N</i> -Methyl oligopeptides	(a) Chloroform–methanol (9:1) (b) Chloroform–acetone (8:2 or 9:1)	Chlorine–toluidine, UV, iodine UV, iodine	52 53
21.	Dansyl peptides of lipoproteins	Three different solvent systems	Ninhydrin	53
22.	Glycoprotein-derived oligosaccharides	Propanol–acetic acid–water (3:3:2)	Orcinol	54
23.	Various peptides	Chloroform–methanol–aq. 17% ammonia (2:2:1)	Sprayed with 0.1% solution of 4-chloro-7nitrobenzofuran in ethanol, followed by 50% methanol (adjusted to pH 11 with NaOH)	55
24.	Enantiomeric and diastereomeric dipeptides (on Chiralplate)	Solvents and <i>hR_f</i> given in Table 5	0.1% Ninhydrin	19, 62
25.	Boc-β-Ala-Trn-Met-	Chloroform–methanol–acetic	Ninhydrin Ehrlich's	63

Asp-Phe	acid (18:2:1); <i>sec</i> -butanol–3% ammonia (25:11 and 3:1); ethanol–acetic acid–water (20:6:11); <i>n</i> -butanol–acetic acid–water (4:1:5, upper phase)	reagent, fluorescamine reagent	
26. Oligopeptides (8 standard samples)	(a) Isoamyl alcohol–ethanol–anhyd. acetic acid–pyridine–water (175:50:13:175:150)	Ninhydrin	4
27. Lysino alanine	(b) Isopropyl alcohol–ethanol–aq. ammonia (4:4:3) (a) Isopropyl alcohol–1 N HCl (3:1) (b) <i>tert</i> -Butyl alcohol–acetone–methanol–water–conc. ammonia (4:4:1:14:5); electrophoresis in glacial acetic acid–98% formic acid–water (17:5:28, pH 2)	Ninhydrin	37
28. Ribonuclease B, globin, R17-bacteriophage coat protein, carboxymethylated protein, subunits from BI component of TYMV; tryptic digest of glyceraldehyde 3-phosphate dehydrogenase of yeast	(a) <i>n</i> -Butanol–pyridine–acetic acid–water (15:12:3:12) (b) Isobutanol–pyridine–water (7:7:6) (c) Isoamyl alcohol–pyridine–water (7:7:6) (d) Butanol–acetic acid–water (5:1:4), upper phase)	Cd-ninhydrin spray followed by heating at 60°C	2
29. Dipeptides with Leu/Ile as N-terminal residues	Solvents and hR_f given in Table 4	Cd-ninhydrin spray followed by densitometry	8
30. Several di- and tripeptides (see Table 2)	Solvents and hR_f given in Table 2	Cd-ninhydrin spray followed by heating at 60°C for 15 min	34
31. <i>S</i> -Carboxymethyl insulin chain A, Ala-Leu-Gly, Leu-Leu-Val-Tyr, Glu-Gly-Phe	(a) Pyridine–acetic acid–water–acetone (2:4:79:15) at pH 4.4, electrophoresis (b) <i>n</i> -Butanol–pyridine–acetic acid–water (15:10:3:12)	(a) 0.3% Ninhydrin in collidine–acetic acid–ethanol (3:100:87) (b) 1% Fluorescamine (w/v) in acetone (c) 0.05% <i>o</i> -Phthalaldialdehyde in methanol (w/v) containing 0.2% β -mercaptoethanol and 0.09% Brij-35; sheets pre- and postsprayed with 10% triethylamine in meth-vlene	

			chloride	
32.	Tryptic peptides from polyoma virus	(a) Acetic acid–0.5% pyridine, pH 3.5 (25 V/cm) for electrophoresis (b) <i>n</i> -Butanol–acetic acid–water–pyridine (5:1:4:4)	Ninhydrin spray	56
33.	Ribonuclease S protein	(a) 1.25 M Pyridine acetate buffer, pH 6.45, 400 V for electrophoresis (b) Butanol–acetic acid–pyridine–water (30:6:20:24), TLC	Cd-ninhydrin	57
34.	Actinomycin hydrolysate	(a) Formic acid–acetic acid, pH 6.0, 10 mA, electrophoresis (b) Butanol–water–acetic acid (5:4:1) TLC	Cu-ninhydrin, fluorescamine	58

Sample	Protein/peptide	Developing system	Detection	Ref.
35.	Acid hydrolysates of collagen	(a) Isopropanol–butanone–1 M HCl (12:3:5) (b) 2-Methyl-2-butanol–butanone–acetone–methanol–conc. ammonia (5:4:7:1:4)	Cd-ninhydrin, <i>o</i> -phthalaldialdehyde	59
36.	Cystine peptides	Butanol–acetic acid–water (71:7:22)	Ninhydrin	60
37.	Streptococcal peptides	Aq. 10% trichloroacetic acid	Ninhydrin	61
38.	Enantiomeric dipeptides, Trp-Trp, Ala-Ala, Phe-Phe, Tyr-Tyr, LysAla, Asp-Ala (synthesized)	Pyridine–water (2:1 or 4:1) at –10°C	Ninhydrin	62
39.	Bovine serum albumin, bovine γ globulin, α -chymotrypsin, cytochrome c, hemoglobin, lysozyme, myoglobin, ovalbumin, ovomucoid, pepsin, ribonuclease, thyroglobulin, trypsin (Sephadex G100, G-200)	Solvent and R_{Fb} given in Table 6	Staining in 0.2% Ponceau S in 10% aq. acetic acid for 30 min followed by washing with water	9
40.	Ornithine carbamoylphosphate transferase (Sephadex G-200, G200 superfine)	0.5 M NaCl	Amido Black 10 B in methanol–water–glacial acetic acid (5:4:1)	33
41.	α -Chymotrypsin aldose	6 M guanidine	On paper by spraying	30

	catalase, bovine serum albumin, ovalbumin, lysozyme, phosphorylase b, β -galactosidase, alkaline phosphatase, lactate dehydrogenase, glyceraldehyde phosphate dehydrogenase (Sephadex G-75, G-100)	hydrochloride (descending chromatography)	with diazotized sulfanilic acid	
42.	Small peptides on aluminum-backed thick silica gel	—	Fluorescence	64
43.	Cystine and its peptides on polyamide or silica gel	—	Spraying with dil. 2-nitro-5-sulfthiobenzoate	65
44.	Peptides from soybean meals on KHSO ₄ -coated silica gel plates	Butanol-dioxane-water (4:5:1)	Treated with saturated thiobarbituric acid solution, 85% H ₃ PO ₄ (1:1), then with ethanolic naphthoresorcinol-20% H ₂ SO ₄ (10:1), heated at 105°C	66
45.	Phenylalanine peptides on ammonium tungstophosphate-coated silica gel	0.1 M nitric acid-1 M ammonium nitrate		67
46.	Small peptides	Suspended over aq. 2,4,6-trimethylpyrylium tetrafluoroborate made alkaline with 0.5 mL of triethylamine then adjusted to pH 3 by acetic acid (chamber temp. 80°C)	SIMS	68
47.	Peptides on silica gel 60 H	Water or aq. methanol	Ninhydrin	69
48.	Oleoyl-L-alanyl-L-alanine	Water-MeCN (7:3), pH 5.0	Ninhydrin	70
49.	Peptides on cellulose, impregnated cellulose, or alumina layers (reversed phase)	Propan-1-ol as organic component	—	71
50.	Enantiomeric peptides on CHIR plate	—	—	72
51.	Peptide from <i>Schumannia phyton magnificum</i> with anti-cobra venom activity on silica, or silica impregnated with 5% liq. paraffin in petroleum ether	Acetone-methanol-10% ammonia (3:1:1); butanol-methanol-10% ammonia (3:1:1); chloroform-methanol (1:1)	Spraying with Dragendorff's reagent, 5% ferric chloride, or 2% ninhydrin in acetone	73

52. HBsAG (hepatitis B virus) segments in solution on silica	Ethyl acetate+stock soln. of pyridine–acetic acid–water (20:6:11) in various proportions	Spraying with toluidine–KI after chlorination	74
53. Protein antigens	A “sandwich” assay format using monoclonal antibodies of two distinct specificities, one covalently immobilized to a defined detection zone on a porous membrane while the other serves as a label. Sample is mixed with the coating, and the mixture is then passed along a porous membrane in the detection zone.	Monoclonal antibodies immobilized to a defined detection zone on a porous membrane; blue color	75
54. Thyrotropin hormone, on silica	Chloroform–methanol (9:1), or ethyl acetate+ stock soln. of pyridine–acetone–water (20:5:11) in 6:4).	—	76

Layer material for samples 1–25, 42–45, 51, 52, 54, silica gel; for samples 26–38, cellulose; for samples 39–41, Sephadex; for sample 49, cellulose or alumina; and for sample 50, CHIR plate.

methanol–water (1:3, 200 mL); methanol–1 N HCl (3:2, 200 mL); water (200 mL), and finally methanol (200 mL). The powder is dried overnight in vacuo before use. The purified cellulose powder (15 g) is spread as a slurry over five plates (20×20 cm) at an initial thickness of 400 μm . The coated plates are allowed to dry overnight in a horizontal position before use.

B. Thin-Layer Plates from Gel Filtration Media

Sephadex G-100 (6 g) or Sephadex G-200 is suspended in 100 mL of the solvent (e.g., 0.5 M NaCl solution). Care should be taken to ensure that no aggregates are present in the final gel suspension. The dextran gels usually take 48 h to proceed to complete swelling. Thoroughly cleaned and dry glass plates (10×20 cm) are coated with a 0.9 mm thick layer of a suitable thin-layer spreader (e.g., spreader from Camag, Muttenz, Switzerland). The plates are kept in a closed vessel containing a dish of the solvent and stored in the horizontal position for at least 18 h before use. The plates may be stored for fairly long periods in a wet chamber; if they dry out or show cracking, a very mild spray with buffer solution is applied to regenerate the layers.

IV. THIN-LAYER CHROMATOGRAPHY

The silica gel- or cellulose-based chromatograms are developed in the usual manner. The development of gel plates is carried out as shown in Fig. 1 (9).

Various solvent systems, support materials, and detection procedures for the TLC of a variety of proteins and peptides are summarized in Table 1. The hR_f values for some of these systems are recorded in Tables 2–6.

For two-dimensional peptide mapping, conventional chromatography follows electrophoresis. The plate is dampened with electrophoresis buffer, taking care not to

smudge the applied sample, and run at 1000 V for 40–90 min for 20×20 cm plates. This is, however, a potentially dangerous procedure. The electrophoresis plate is removed (1) from the apparatus (Fig. 2) and dried overnight in a fume hood. There should be no smell of acetic acid on the plates. The composition of the solvent for chromatography is critical for the mobility of peptides. More organic solvent in the mixture tends to increase the relative mobility of the hydrophobic peptides, because the stationary phase is hydrophilic. Some of the solvent systems used for two-dimensional work are mentioned in Table 7 (10, 11).

Most of the earlier reports on protein separations by TLC were limited to size-exclusion chromatography on homemade swollen gel layers, but a few recent studies report adsorption TLC separations. These include a process for separation and analysis of hydrophobic proteins using TLC on a modified silica matrix and immunostaining for detection (11a) and thin-layer ion-exchange chromatography to separate four model proteins using silica gel layers, 0.01 M bicine, pH 8.5, and a three-step elution process with 0.01, 0.025, and 0.10 M NaCl (11b).

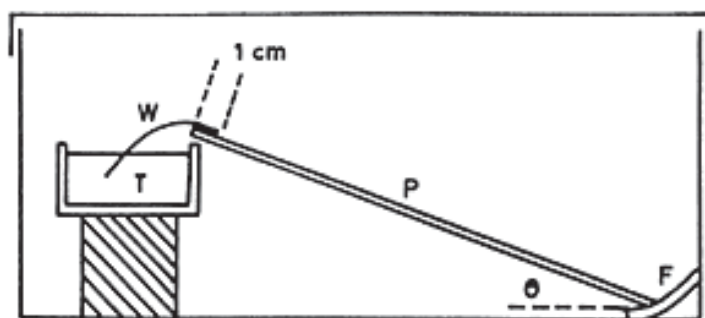


Figure 1 Apparatus for thin-layer chromatography of proteins. Solvent (0.5 M NaCl) is led to plate P by means of Whatman No. 3 filter paper wick W. The wick should overlap about 1 cm onto the gel layer. T is the solvent trough. (From Ref. 9.)

Table 2 hR_f Values of Peptides from L-Amino Acids After One-Dimensional TLC on Cellulose

Peptide	Solvent system				
	A	B	C	D	E
Ala-Ala	65	26	55	68	58
Ala-Asp	56	1	45	44	19
Ala-Glu	64	5	58	56	29
Ala-Gly	50	17	36	46	46

Ala-Phe	94	52	86	84	85
Ala-Ser	52	17	36	41	49
Gly-Ala	52	18	37	43	46
Gly-Asp	43	0	30	29	13
Gly-Gly	34	13	22	29	34
Gly-His	7	16	5	16	32
Gly-Ile	81	49	65	80	75
Gly-Leu	82	51	67	87	80
Gly-Lys	14	11	2	21	27
Gly-Phe	75	50	65	76	67
Gly-Pro	47	17	39	45	44
Gly-Ser	32	13	22	28	26
Gly-Tyr	68	28	45	64	51
Gly-Val	72	36	56	73	62
Leu-Ala	97	56	88	90	89
Leu-Gly	82	52	67	84	83
Leu-Val	100	77	98	96	95
Val-Gly	67	38	56	70	69
Val-Leu	100	80	98	100	95
Ala-Gly-Gly	47	13	34	39	43
Glu-Cys-Gly	16	0	7	7	0
Gly-Gly-Gly	32	8	20	31	30
Val-Gly-Gly	65	28	52	65	61

Solvent systems: A, 2-Propanol–butanone–1 N HCl (60:15:25); B, 2-methyl butan-2-ol–butanone–propanone–methanol–water–ammonia (10:4:2:1:3:1); C, 2-propanol–water (3:1); D, 2-propanol–water–acetic acid (15:4:1); E, 2-propanol–water–ammonia (15:4:1).

Source: Ref. 34.

V. DETECTION OF PEPTIDES AND PROTEINS ON THIN-LAYER PLATES

A. Detection on Cellulose or Silica Gel Plates

The plates can be viewed using a long-wavelength (366 nm) UV source or stained with a suitable reagent as described in Table 1. Certain other detection methods are described below.

a. Morin Reaction. The dried chromatograms are sprayed with a 0.05% solution of morin (3, 5, 7, 2',4-pentahydroxyflavone) in methanol and heated for 2 min at 100°C. The N-protected amino acids and peptide derivatives give yellowish-green fluorescence on a green fluorescent background or dark absorption spots under UV. The detection limit is just about 2 µg/spot (12).

b. Iodine-Starch Reaction. The chromatogram is placed in a strong iodine vapor atmosphere for 5 min. The excess iodine is removed by leaving the plate in the open air, then the

Table 3 hR_f Values for Dipeptides and Tripeptides

Peptide	hR_f	Solvent
Np-S-Met-Ala-O-Np		A
L,L	77	
L,D	67	
Np-S-Met-Met-Ala-O-Np		B
L,L,L	58	
L,L,D	66	
Np-S-Met-Ala-Met-O-Me		C
L,L,L	56	
L,D,L	52	

Solvents: A, acetic acid–diethyl ether (1.5:20, v/v); B, acetic acid–diethyl ether (0.2:20, v/v); C, diethyl ether– isopropanol (20:0.25, v/v).

Np=*p*-nitrophenyl-.

Source: Ref. 5.

Table 4 Chromatographic Behavior of Dipeptides from L-Amino Acids on Thin Layers of Cellulose

Peptide	hR_f			Color yield ($\text{mm}^2/\mu\text{mol}$) $\times 10^{-4}$	
	A	B	C	405 nm	490 nm
Ile-Ala	90	68	56	3.5	7.8
Ile-Gly	84	53	46	3.7	5.2
Ile-Glu	94	13	13	10.1	23.0
Ile-Leu	100	88	86	1.6	14.6
Ile-Lys	58	50	41	86.6	22.5
Ile-Met	94	79	80	3.7	8.4
Ile-Phe	100	81	89	4.5	13.6
Ile-Pro	89	60	63	2.9	3.5
Ile-Ser	87	53	38	5.8	13.7
Ile-Trp	100	83	83	2.6	10.8
Ile-Val	100	80	83	5.8	14.5
Leu-Ala	97	58	—	2.9	5.1
Leu-Gly	82	52	—	2.9	5.6
Leu-Leu	99	66	83	3.8	8.5
Leu-Met	100	71	75	9.6	15.1
Leu-Phe	99	73	79	6.9	10.5
Leu-Ser	88	61	41	4.5	9.8
Leu-Trp	100	73	77	7.7	13.0
Leu-Tyr	97	67	66	8.9	10.8
Leu-Val	100	77	—	3.1	5.0

Solvents: A, 2-propanol–butanone–1 N HCl (12:3:5); B, 2-methyl–2-butanol–butanone–propanone–methanol–water–0.88 NH₃ solution (10:4:2:1:3:1); C, *n*-butanol–butanone–water–0.88 NH₃ (80:5:17:3).

Source: Ref. 8.

Table 5 Resolution of Enantiomeric and Diastereomeric Dipeptides

Enantiomeric dipeptide	hR_f value	
	A	B
Gly-L-Phe } Gly-D-Phe }		57 63
Gly-L-Leu } Gly-D-Leu }		53 60
Gly-L-Ile } Gly-D-Ile }		54 61
Gly-L-Val } Gly-D-Val }		58 62
Gly-L-Trp } Gly-D-Trp }		48 55
D-Leu-L-Leu } L-Leu-D-Leu }	19 26	48 57
D-Ala-L-Phe } L-Ala-D-Phe }	21 26	59 65
D-Met-L-Met } L-Met-D-Met }	29 33	64 71
Diastereomeric dipeptide	hR_f in eluent B	
L-Leu-L-Leu } L-Leu-D-Leu }		45 53
L-Ala-L-Ala } L-Ala-D-Ala }		64 70
L-Ala-L-Phe } L-Ala-D-Phe }		59 65
L-Met-L-Met } L-Met-D-Met }		62 72

Solvent system: A, methanol–water–acetonitrile (1:1:4); B, methanol–water–acetonitrile (5:5:3).
Length of run 13 cm. Detection: 0.1% ninhydrin reagent.

Source: Ref. 19.

layer is sprayed with 1% aqueous starch solution. The peptides (and amino acids as well) give blue spots (13).

B. Detection on Gel Layers

The gel layer is covered with dry filter paper (Whatman 3 mm), avoiding bubble formation, and is carefully smoothed down over it. The layer and the paper are dried together at 120°C, or the paper is carefully peeled off the layer and dried at 110°C.

The proteins on the paper are detected by the usual paper chromatographic methods. The dried paper is immersed for 15 min in Amido Black 10 B (0.6 g) dissolved in a mixture of methanol or ethanol (750 mL), water (450 mL), and glacial acetic acid (100 mL) and destained three times with 1% acetic acid for 30 min each time. Alternatively, the paper is stained in 1%

Table 6 R_m Values of Proteins on Sephadex Plates

Protein	Mol wt $\times 10^{-2}$	R_{Hb}	
		A	B
Cytochrome c	13.0	0.68	0.74
Ribonuclease	13.6	0.68	0.74
Lysozyme	14.5	0.65	0.70
Myoglobin	16.9	0.79	0.80
α -Chymotrypsin	22.5	0.87	0.87
Trypsin	23.8	0.83	0.86
Ovomucoid	27.0	0.94	1.03
Pepsin	35.0	0.99	1.04
Ovalbumin	45.0	1.03	1.04
Hemoglobin	68.0	1.00	1.00
Bovine serum albumin	65.0	1.28	1.54
Bovine γ -globulin	180.0	1.28	1.54
Thyroglobulin	650.0	1.33	1.83
Macroglobulin	1000		1.86

$R_{Hb} = d_p/d_{Hb}$, where d_p and d_{Hb} are the distances traversed by the test protein and by hemoglobin, respectively.

A, Plates of Sephadex G-100, developed in 0.5 M NaCl solution; B, plates of Sephadex G-200, developed in 0.5 M NaCl solution.

Source: Ref. 9.

solution of bromophenol blue saturated with mercuric chloride for 5 min and then destained five times with 0.5% acetic acid for 30 min each time.

C. Recovery of Peptides

After detection, the peptide spots on the cellulose or silica gel thin layers are carefully scraped (6) and are transferred to Pasteur pipets (150 mm long) that have been tightly plugged with one-fourth of a glass fiber membrane filter (20 mm diameter, Sartorius SM 13400) and prewashed with 2 mL of 6 N HCl. Two hundred microliters of 6 N HCl containing 0.02% 2-mercaptoethanol is added to each of the Pasteur pipets, and the peptide is extracted at room temperature for 15 min. The HCl is then forced through the filter with nitrogen (1 atm). A video densitometric method was proposed for quantitative determination of short peptides in biological fluids with appropriate mobile-phase selection (13a).

VI. TLC OF DIASTEREOMERIC DIPEPTIDES

The successful separation of dipeptide diastereomers was reported by Wieland and Bende (14), Taschner et al. (15), and Pravada et al. (16) either as the free peptides or as the N-protected methyl esters. Hubert and Dellacherie (5) separated diastereomeric *p*-nitrophenyl (Np) esters of N-protected di- and tripeptides; starting from pure L-methionine and DL-alanine, they synthesized Np-S-L-Met-DL-Ala-O-Np and Np-S-L-Met-L-Met-DL-Ala-O-Np. The separation was achieved on silica gel F₂₅₄ precoated (Merck) plates. TLC separation of diastereomeric dipeptides has been well documented by Arendt et al. (17) and Lepri et al. (18).

Günther et al. (19) reported for the first time the separation of enantiomeric dipeptides on Chiralplate (20). Typical examples of these separations are given in Table 5. It was observed that the antipodes with C-terminal L-configuration always gave a smaller *R_f* value than the correspond-

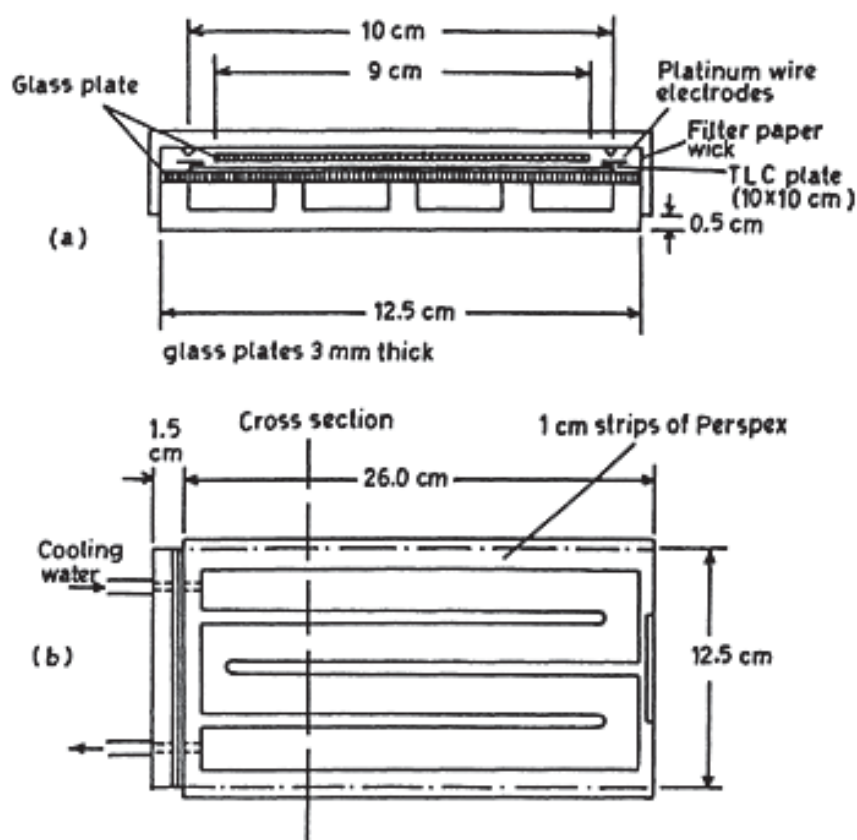


Figure 2 Simple apparatus for electrophoresis of 10×10 cm thin-layer plates, (a) Section; (b) plan. The cooling plate is constructed from a 12.5×26 cm glass sheet (0.3 cm thick) and a Perspex sheet (0.5 cm thick) glued together. Cold water is run through the cooling plate. A glass plate is placed on top of the silica plate to minimize solvent evaporation. (From Ref. 1.)

ing enantiomeric dipeptide with C-terminal D-configuration. The method (19) also resolves diastereomeric dipeptides. Wang et al. (62) compared the resolution of four isomeric Trp-Trp, Ala-Ala, Phe-Phe, Tyr-Tyr, Lys-Ala, and Asp-Ala mixtures on Chiralplate and on microcrystalline cellulose plates. The separation of L,L and D,D pairs of all tested dipeptides was better on microcrystalline cellulose plates, while L,D, and D,L pairs separated to a better degree on Chiralplate. TLC methods for separation of peptides containing epimers and enantiomers of β -alkylamino acids were reviewed (19c).

Table 7 Conditions for Fingerprinting Tryptic Peptides on Silica Gel G Thin-Layer Plates (20×20 cm×0.1–0.25 mm)

First dimension: electrophoresis
 pH 3.5, pyridine–acetic acid–water (2:20:978), 1000 V, 45 min
 pH 6.5, pyridine–acetic acid–water (100:3:897), 1000 V, 40 min
 pH 4.7, *n*-butanol–pyridine–acetic acid–water (2:1:18)
 Second dimension: chromatography at 25°C
 Chloroform–methanol–ammonium hydroxide (2:2:1)
n-Propanol–ammonium hydroxide (7:3)
n-Butanol–pyridine–acetic acid–water (97:75:15:60), pH 5.3

Source: Refs. 10 and 11.

VII. SEPARATION OF PEPTIDES ON REVERSED-PHASE PLATES IMPREGNATED WITH ION-PAIR REAGENTS

Separation of a large number of di- and tripeptides and some tetra- and pentapeptides on homemade silanized silica gel plates (21) and reversed-phase (RP) TLC (RP-2, RP-8, RP-18) plates impregnated with dodecylbenzene sulfonic acid (22, 23), anionic and cationic detergents (24) such as triethanolamine dodecylbenzene sulfonate (DBS), sodium dioctyl sulfosuccinate (Na-DSS), and *N*-dodecyl pyridinium chloride (N-DPC), and on ammonium tungstophosphate layers (25) was carried out successfully by Lepri et al. (21–26). The separation conditions and R_f values of some polypeptides of moderate size and of similar structure on homemade layers of silanized silica gel and RP-2 plates (21) are recorded in Table 8; the separation of such peptides is, however, a difficult problem of analytical importance (27). The different solvent systems and chromatographic conditions used by Lepri et al. are summarized in Table 9. The description of the peptides and their structures is omitted in view of the length of the article. These systems may thus provide helpful guidance for choosing or developing a solvent system according to the actual requirement of the experiment.

The use of RP-TLC and HPLC for isolation and characterization of peptides was reviewed (27a). Rapid screening of synthetic peptides was carried out by use of personal OPLC systems with silica gel layers and 1-butanol–pyridine–acetic acid–water (12:4:1:4) mobile phase; OPLC was shown to be used orthogonally with HPLC or CE for multimodal separations of closely related peptides (27b).

VIII. DETERMINATION OF MOLECULAR WEIGHT OF PROTEINS

Thin layer chromatography of proteins and polypeptide chains on cross-linked dextran gel (such as Sephadex) as described by Determann and Michel (28), Jaworek (29), and Heinz and Prosch (30) provides a method that allows estimation of molecular weights of

polypeptides in a much shorter time, because Andrews (31) found a close correlation between the logarithm of the mo-

Table 8 hR_f Values of Polypeptides on Homemade Layers of Silanized Silica Gel and on RP-2 Plates

Compound	Silanized silica gel		RP-2	
	A	B	B	C
Angiotensin III inhibitor	47	76	75	81
Angiotensin III	16	55	53	71
Angiotensin II	37	75	73	79
Angiotensin I	22	63	59	75
Melittin	00	00	00	52
Glucagon	00	33	26	72
Insulin B chain	00	36	31	72
Actinomycin C ₁	00	03	02	25
Actinomycin V	00	04	03	22
Actinomycin I	00	08	05	32

A, 1 M acetic acid in 30% methanol; B, 1 M acetic acid+3% potassium chloride in 50% methanol; C, 3% potassium chloride in water-methanol-tetrahydrofuran (4:3:3). Migration distance was 11 cm for homemade layers and 6 cm for RP-2 plates. Actinomycins were yellow; other compounds were located by 1% ninhydrin in pyridine-acetic acid (5:1). Actinomycins I and V differ from C₁ by having one of the two prolines replaced by 4-hydroxy- and 4-ketoproline, respectively.

Source: Ref. 21.

Table 9 TLC Conditions for the Separation of Peptides on Impregnated and RP Silica Gel Plates

1. RP-2, RP-8, RP-18 plates impregnated with 4% HDBS
 - a. 1 M acetic acid in methanol-water (1:1)
 - b. 1 M acetic acid+0.2 M HCl in methanol-water (1:1); at high methanol percentage, i.e., methanol-water (4:1), the R_f values increased and more polar peptides gave elongated spots. The RP plates cannot be used with aqueous organic eluents containing more than 30% water.
2. Untreated thin layers of silanized silica gel or impregnated with different detergents
 - a. Water-methanol-acetic acid (64.3:30:5.7), for untreated plates
 - b. 0.1 M or 0.05 M HCl+1 M acetic acid in 30% methanol (pH 1.25 or 1.55); 0.1 M NaCl+ 1 M acetic acid in 30% methanol (pH 2.75 or 3.30); 0.1 M sodium acetate+0.1 M acetic acid in 30% methanol (pH 5.10); 1 M sodium acetate in 30% methanol (pH 8.15), for plates impregnated with 4% HDBS.

- c. Water–acetic acid (7:3 or 1:1), for plates impregnated with 4% HDBS.
- d. 0.1 M acetic acid+0.1 M sodium acetate in 30% methanol; 1 M acetic acid in 30% methanol; 1 M sodium acetate in 30% methanol, for plates impregnated with 4% N-DPC.

Alkaline elements, which cannot be used in RP column chromatography, can be used here. Separation on layers impregnated with N-DPC is better with an eluent of pH 5.10 than with an eluent of pH 2.75.

3. Layers of ammonium tungstophosphate + $\text{CaSO}_4 \cdot \frac{1}{2} \text{H}_2\text{O}$ in the ratio 4.2

- a. Aqueous solutions of ammonium nitrate (1 M, 2 M) These plates provided compact spots and good resolutions. Mainly di-, tri-, tetra-, and homopeptides were resolved by the above methods. The peptides were detected by spraying the wet layers a solution of 1% ninhydrin in pyridine–glacial acetic acid (5:1) and then heating the layers 100° for 5 min.

HDBS, dodecylbenzenesulfonic acid; N-DPC, *N*-dodecylpyridinium chloride.

Source: Refs. 21–26.

molecular weight of a protein and its chromatographic behavior (the distance covered in a constant time on a given layer). Heinz and Prosch (30) successfully estimated molecular weights of several polypeptides on Sephadex G-75 and G-100 plates developed with 6 M guanidine hydrochloride (Table 10). Sephadex G-75 is used for chromatography of polypeptide chains with molecular weights less than 100,000, and Sephadex G-100 is used for high molecular weight proteins. The solutions of proteins are prepared in guanidine hydrochloride (10 mg/mL), and cytochrome c (10 mg/mL) is used as an internal standard. Descending chromatography is carried out at room temperature under an inclination angle of 25° to the horizontal line. After 3 h of development, the

Table 10 Molecular Weights of Proteins on Sephadex Plates

Protein	M.W.	No. of PCs	M.W. of PC	M.W. results (S.D.)
Lysozyme	14,500	1	14,500	17,500 (520)
Lactate dehydrogenase	126,000	4	31,500	31,000 (500)
Glyceraldehyde phosphate dehydrogenase	140,000	4	35,500	35,500 (1200)
Alkaline phosphatase	41,000	1	41,000	41,500 (800)
Phosphorylase b	188,000	2	94,000	98,000 (500)
β -Galactosidase	135,000	1	135,000	132,000 (400)

S.D., standard deviation; PC, polypeptide chain.

Results are mean of 15 runs.

Source: Ref. 30.

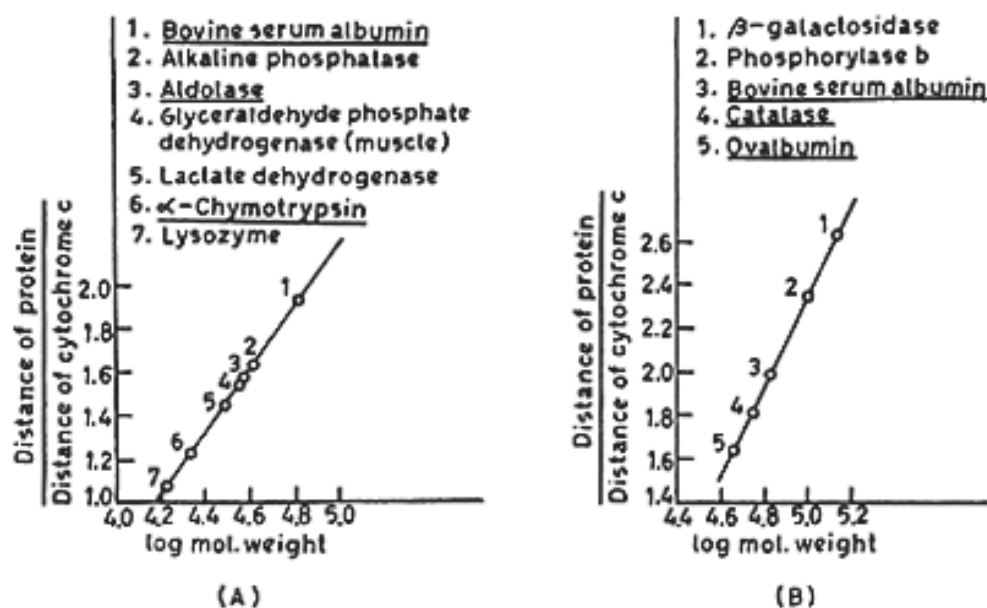


Figure 3 Calibration line from thin-layer gel chromatographic experiments on (A) Sephadex G-75s and (B) Sephadex G-100. The averages of the quotients R_s protein/ R_s cytochrome c (R_s =distance of protein to starting line) were plotted as a function of logarithm of molecular weight. Proteins used as standards are underlined. (From Ref. 30.)

quotient R_s protein/ R_s cytochrome c is calculated for each protein–cytochrome c combination. A standard calibration line is obtained (Fig. 3) by plotting the log molecular weights of standards against protein/cytochrome c quotient, and the molecular weights of unknown proteins are calculated from this plot.

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Pesticides

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I. INTRODUCTION

Because of the growing awareness of the detrimental influence of pesticides and their residues on the environment and human health, there has been an increase in the number of papers that analyze their separation and isolation from water, soil, food, and biological material. Numerous analytical techniques have been developed with chromatography in the forefront. Although gas chromatography (GC) and high-performance liquid chromatography (HPLC) are still the leading chromatographic techniques, thin-layer chromatography (TLC) is being used more frequently in the analysis of pesticides, thanks to modified stationary phases, optimization of mobile phases, and modern apparatus for developing chromatograms and for quantification. The growing use of efficacious techniques for the separation and isolation of pesticides from complex sample matrices is contributing to the success of thin-layer chromatography.

Because the second edition of this Handbook gives a detailed and instructive review of sample preparation and pesticide identification (1), and because of the large number of references over the last 10 years or so, this chapter reviews the topic only for the period 1990–2001.

Numerous useful reviews, both general ones and those devoted to specific determinations, were published over this period. General reviews of pesticide analysis by TLC, including theory, chromatographic systems, methods of detection and quantification, and applications, have been published (2–13). Separation and determination of nonionic surfactants used as pesticide additives were reviewed (14). TLC methods for determining the octanol/water partition coefficient with data for 221 pesticides and metabolites were published (15).

Chromatographic methods, including solid-phase extraction (SPE), supercritical fluid extraction (SFE), and TLC determination of pyrethrin and pyrethroid pesticide residue in crops, foods, and environmental samples were reviewed (16). A paper was published on the determination of herbicide residue in these sample matrices (17). A selective review was given of TLC methods of pesticide residue analysis (18). Papers were published on

chromatographic pesticide residue analysis and advances in the techniques and application of TLC (19, 20).

Pesticide residue analyses in environmental samples (21, 22), food and agricultural samples (23–26), and water (27, 28) were reported. Extraction methodology and chromatography for determination of pesticide residues in water were reviewed (29), as were the high-performance separation and determination of triazine herbicides and their residues (30, 31).

Two interesting reviews discuss matrix solid-phase dispersion (MSPD), a patented process for conducting simultaneous disruption and extraction of solid and semisolid samples that can be successfully applied to pesticide analysis (24, 32). The application of luminescence methods for determining pesticides in various sample matrices were reviewed (33). The color reactions of 178 pesticides with six detection reagents were tabulated to form a rapid screening system for forensic analysis (34). A review of modern HPTLC pesticide analysis using automated multiple development (AMD) was presented (35).

II. SAMPLE PREPARATION

There is growing interest in sample preparation in pesticide analysis to optimize the usefulness of extraction while ensuring the lowest possible environmental pollution. Therefore, in the recent literature one encounters new extraction techniques such as solid-phase extraction (SPE) (16, 36) and supercritical fluid extraction (SFE) (9, 16). It is expected that microwave-assisted solvent extraction (MASE), which was applied for the efficient determination of triazines in soil samples with aged residue (37), or matrix solid-phase dispersion (MSPD), which permits complete fractionation of the sample matrix components as well as the elution of a single compound or several classes of compounds from the same sample (32), will also find broad application in TLC. Ultra-sonic extraction (USE) has also proved to be a reliable extraction technique that successfully replaces classical procedures. Therefore, the emphasis in this review is placed on modern methods of sample preparation; nevertheless, Table 1 includes certain classical extraction methods.

There is no doubt that SPE is the method of choice for the simultaneous extraction and concentration of organic contaminants in water samples (38). Six phenylurea herbicides in food were extracted with acetone and purified by solid-phase extraction (39). An SPE method using aminopropyl cartridges was developed to remove interference in HPTLC analysis of pesticides (40). Recoveries of metribuzin from soil and water samples using SPE were 73–86% and 89–92%, respectively (41). Factors influencing the SPE (the ratio between sorbent and sample volume, flow rate, and concentration of the eluate) were investigated (42). Carbamate insecticides in water were determined by C-18 SPE and HPTLC (43). RP C₁₈ silica was used as a single cartridge in the analysis of carbamates, phenylureas, and triazines (44) and of organophosphorus (OP) and organochlorine (OC) insecticides (45–47) in water. SPE on graphitized carbon black (GCB) cartridges was applied for extraction of 27 polar pesticides and in a multiresidue method (48, 49). C-18 SPE cartridges were used in pesticide residue analysis of water samples (50–53).

Solid-phase extraction was also used on CN-bonded silica gel (54). Multiresidue analysis of a number of pesticides required SPE pretreatment (55). A systematic study of seven pesticide residues in soil samples using solid-phase extraction disks was reported (56). Residues of hexazinon and its metabolites were extracted from soil samples (57).

In classical solvent extraction, efforts were made to reduce the influence of organic solvents on human health and to reduce the cost of analysis. Solvent quality was investigated for extraction of OC compounds from environmental samples (58).

The efficiency of the cyclodiene pesticides and the extraction of metabolites from aqueous media was found to be between 60% and 80% using the method with Mixxor reservoirs (59). More than 80 pesticides and 12 PCBs were examined using *n*-hexane extraction (60).

Supercritical fluid extraction has the advantage of reducing the amount of coextracted contents, which can cause serious errors in the results. This extraction technique is mainly used for complicated systems such as soils, sediments, food, and biological matrices.

Degradation of chlorpyrifos in relation to soil pH was evaluated by SFE and TLC (61). The efficacy of SFE in the recovery of cyanazine herbicide from soil was investigated. Several SFE parameters were optimized for maximum (>90%) recovery (62). Pesticides in soil samples and sediment were extracted and analyzed (63, 64).

Organophosphorus (OP) and organochlorine (OC) insecticides were extracted from diverse soil samples by SFE, either with CO₂ or with 3% methanol containing CO₂ gas (65). Some chlorobenzenes and HCH isomers were extracted from soil samples with high recovery (66). An SFE method for screening of pesticide-contaminated soil has been developed. The extraction was carried out by using supercritical CO₂ with methanol as a modifier (67). This method, using only CO₂ or organic solvent containing CO₂, was done for extraction from animal tissue (68). The recovery of thiocarbamates extracted from food samples was 80% (69). There has been a review of SFE extraction of diverse insecticides and herbicides in environmental samples (70).

Ultrasonic extraction and video densitometric quantification was reported as an efficient method for determining pesticides in soil. The method was tested and validated for the determination of a six-component mixture of pesticides from spiked soil using USE with various solvents (71). Recoveries of agrochemicals from spiked soils were about 79% for propham, 90% for

Table 1 Sample Preparation

Compound	Sample	Extraction	Cleanup	Recovery (%)	Ref.
Pyrethrin and pyrethroid pesticide residues	Crops, foods, and environmental matrices	SPE, SEE			16
Herbicides, pesticides	Animal tissues, fruits, vegetables	MSPD			32
Pesticide residues	Soil	SPE		80	36

	contaminated by petroleum derivatives				
Pesticides		SPE: aminopropyl cartridges	<i>tert</i> -Butyl methyl ether-methanol (99:1)		40
Metribuzin	Soil and water			Soil: 86 Water: 89-92	41
Carbaryl, carbofuran, methiocarb	Water	SPE C-18			43
Carbamates, phenylureas, triazines	Water	C-18	Acetone, chloroform		44
Azinphos-ethyl, diazinon, ethyl parathion, fonafos, malathion, methyl parathion	Water	C-18	Ethyl acetate	83.5-96	45
OC, OP insecticides	Water	C-18	Ethyl acetate	73-104	46
Insecticides	Water	SPE C-18			47
Carbamates, phenol, phenoxyacids, phenylureas, phosphorothionates, triazines	Water	GCB; Carbopack; C-18	Ether-hexane (1:1); petroleum ether-toluene (1:1); CH ₂ Cl ₂ - CH ₃ CN (6:4); CH ₂ Cl ₂ -methanol (8:2); CH ₂ Cl ₂	65-98	49
Atrazine, esfenvalerate, metolachlor, metribuzin	Runoff water	C-18	Methanol, ethyl acetate	87-107	50
Diflubenzuron	Water	SPE C-18	Acetonitrile	97-100	51
Pesticides	Water	C-18			52
2,4-D, atrazine, bentazone, diuron, fluazifop acid, linuron, MCPA, metobromuron, metox- uron, monolinuron, simazine	Water	SPE C-18	Methanol	70-90	53
OC pesticides	Water	CN-bonded silica	Pentane	95	54
Compound	Sample	Extraction	Cleanup	Recovery (%)	Ref.
Carbamates; OC, OP pesticides; triazines	River water, sea water drinking	Porapak Q, P C-18	Methanol, acetone diethyl		55

	water, tap water, surface water, agricultural water, lake water	GCB; Tenax- GC; PRP; XAD-s; Poly sorb S; polyurethane foam; Worfarit Y77	ether, light petroleum, acetonitrile, dichloromethane, hexane		
Hexazinone (metabolites)	Soil			94±3	57
OC pesticides	Environmental	<i>n</i> -Hexane (AR), methanol			58
Aldrin; aldrin epoxide and metabolites; Endosulfan I, II; cyclodiene pesticides	Aqueous media	Hexane– acetone– methanol– water (15:5:2:2) in Mixxor reservoir		60–88	59
Pesticides	Olive oil, butter, lyophilized fish tissue, potato, carrot	<i>n</i> -Hexane, H ₂ SO ₄	Florisil	90–99	60
Chlorpyrifos	Surface and subsurface clay loam soils	SFE: Methanol			61
Cyanazine	Silty clay loam soil	SFE: CO ₂ - [methanol– water (1:1)]		>90	62
OP insecticides	Soil	SFE: CO ₂ , CO ₂ –acetone, CO ₂ –ethyl acetate, CO ₂ – methanol		90	63
Phenylureas, triazines	Sediment	SFE: CO ₂ , CO ₂ –acetone			64
DDT, decachlorobiphenyl, diazinon, dichlorvos, endrin, endrin aldehyde, methiodathion, para- thion, Ronnel, tetrachloromethaxylene (TCMX), tetrachlorvinphos	Soil, sand, furnaced top soil	SFE: CO ₂ , CO ₂ –3% methanol		94	65

14 Chlorinated hydrocarbons	Soil	SFE: CO ₂ , CO ₂ –toluene, CO ₂ –toluene–cyclohexane	60–85	66
Pesticides	Soil	SFE: CO ₂ –methanol		67
Dieldrin, endrin, heptachlor, heptachlor epoxide	Animal tissues	SFE: CO ₂		68
Thiocarbamates	Apples	SFE: CO ₂	80	69
OC pesticides, phenoxy-carboxylic acids, <i>s</i> -triazines	Environment	SFE: CO ₂ , N ₂ O, CO ₂ –methanol, CHClF ₂	65–100	70
Atrazine, chlorpropham, cypermethrin, diflubenzuron, propham, tetramethrin	Soil	USE with acetone	83.4–97.2	71
Atrazine, chlorpropham, cypermethrin, diflubenzuron, propham, tetramethrin	Soil	USE with acetone	79.3–102	72
Altrazine, chlorpropham, cypermethrin, diflubenzuron, propham, tetramethrin	Soil	Shaker-flask extraction, Soxhlet extraction, USE	79.7–103.5	73
Azadirachtin, salannin	Seeds	Dichloromethane	Biotage flash column	74
OC pesticides	Milk, milk powder	C-18	Methanol, <i>n</i> -hexane, light petroleum ether, <i>n</i> -hexane–petroleum ether (1:1)	80–100 270
Cymiazole, pentachlorophenol	Water and honey	SPE C-18	Water: 89.5–100 Honey 91.9–96.1	272

chlorpropham, 98% for diflubenzuron, and 100% for atrazine and α -cypermethrin (72). A comparison was made between USE with shaker-flask and Soxhlet extraction. The extraction procedure was optimized with regard to the amount of solvent, the duration of sonication, and the number of extraction steps (73).

Rapid separation of triterpenoids from neem tree seed extracts using the Biotage™ flash chromatography system was examined. After a second pass through the Biotage flash column, pure compound traces could be extracted from a complex sample (74).

III. CHROMATOGRAPHIC SYSTEM

A. Optimization of the Stationary and Mobile Phases

Certain stationary phases have been investigated. Particular organochlorine pesticides were separated on mixed oxide layers (75). Hydrated zirconium oxide layers were used for thin-layer chromatographic separation of pyrethroid insecticides (76). Application of Florisil (magnesium silicate) in TLC analysis of pesticides, environmental contaminants, mycotoxins, drugs, and other substances was reviewed (77). Herbicides were separated on mixed silica gel–calcium sulfate plates (78). Retention of 4-cyanophenyl herbicides on water-insoluble β -cyclodextrin support was examined (79). Charge transfer reversed-phase TLC (RP-TLC) of various pesticides and their interaction with water-soluble β -cyclodextrin polymer was worked out. Relationships were established between the relative strength of interaction and the polar sorbent and the polar surface energy of the pesticides examined (80, 81). Aminoplast (carbamide-formaldehyde polymer) was used as a support in triazine herbicide separation and quantification (82, 83).

Organophosphorus warfare agents in the presence of 22 pesticides were separated by 2-D overpressured layer chromatography (OPLC) using mobile-phase optimization according to the PRISM A model (84). A comprehensive approach for mobile phase optimization, including an optimum graphic system, a multifactor optimization system, and computer simulation diagram optimization was developed (85). A computer-assisted method for optimization of two mobile phase compositions and the selection of development distance in two-step development TLC was presented (86). The optimization of chromatographic separations of pesticide mixtures using the Windows diagram method was described. The criteria for comparison were minimum R_f difference, ΔR_f product, separation, and multispot response function (87). Determination of the relationships between R_f values and mobile phase composition for 11 moderately polar pesticides was chemometrically characterized for the selection of a suitable chromatographic system (88, 89). Chemometric characterization of the R_f values of 20 pesticides for the TLC system silica gel–nonpolar diluent+polar modifier was also carried out (90). A new weighted chromatographic response factor containing first- and second-degree terms was demonstrated for optimization of a ternary solvent system composed of benzene, petroleum ether, and chloroform (91). A computer-assisted optimization of two mobile phase selections for separation of a mixture of eight pesticides in 2-D TLC was presented. Using the distance between two spots as the selection criterion with a two-factor statistical scanning technique, excellent agreement was obtained between predicted and experimental results (92). Theoretical investigations of hydrophobicity and the specific hydrophobic surface area of 12 pesticides by RP-TLC and RP-HPLC showed that the biological activity of the compounds could not be attributed to these parameters alone (93). By using multivariate regression analysis, the

relationship between the R_f values of OP insecticides and a series of topological descriptors was obtained (94).

B. Development Techniques

1. Automated Multiple Development

Automated multiple development (AMD) is a very efficient technique that is used largely in pesticide residue and multiresidue analysis. Explanations of the AMD principles have been given in two reviews mentioned earlier (28, 35). HPTLC separation of 24 pesticides using the AMD technique increased the sensitivity and speed of the procedure (95). Multiple and stepwise development combined with gradient elution is a suitable method for the determination of crop protection agents in drinking water (42, 96, 97). Optimized mobile phase gradients were designed for the AMD separation of OC pesticides and phenols (98). Organochlorine pesticides were separated and detected on silica gel with AMD gradient based on dichloromethane–heptane (99). HPTLC-AMD has been used for identification and determination of pesticides in water (100). The same technique was used to screen water samples for pesticides. A universal gradient based on dichloromethane was used to check for a variety of pesticides (101). AMD development was used in pesticide multiresidue analysis in water, with a software program used to facilitate pesticide recognition (102). A study related to the AMD-TLC determination of pesticide multiresidues in water was described (103). Thermolabile benzoyl urea insecticides were analyzed in food plant products from fractions of the S-19 cleanup multimethod, with detection by light remission at 260 nm to reduce interference (104). Application of AMD on-line coupling with reversed phase in environmental pesticide analysis was reviewed. The method was demonstrated by the analysis of a surface water sample spiked with pesticides. The procedure is very effective (105, 106). The AMD technique has become the German standard in the field of water analysis. The suitability of the method was proved for 283 pesticides, and the corresponding ISO standard was applied for (28). HPTLC of 32 pesticides and herbicides using a universal gradient by means of an AMD system was reported (107). A description was given of the determination of iprodione residues in vegetable food samples using on-line coupling of RP-HPLC followed by AMD-TLC (108). Microbial release and degradation of nonextractable anilazine residues was investigated using AMD-TLC (109). Bioactivity-based analysis in HPTLC-AMD detection of bioactive environmental compounds was carried out (110). AMD-TLC was used in the analysis of pesticide-contaminated soils (36, 67).

2. Overpressured Layer Chromatography

Overpressured layer chromatography (OPLC), which enhances separation power, has been used in the separation of phenylurea and triazine herbicides (111).

3. Soil Thin-Layer Chromatography

Soil TLC is a development technique in which the examined soil serves as the stationary phase. It is frequently used for the investigation of pesticide mobility and adsorption in soils, which can have a serious influence on the pollution of groundwater. The effect of soil type and pH on the adsorption, mobility, and efficacy of imazaquin and imazethapyr was investigated. Clay silt loam and sandy clay served as the stationary phase. Both pesticides were more strongly adsorbed at lower pH (112). Reference was made to the dissipation of [^{14}C]glufosinate in two of the soils (113). The effect of 25 soil characteristics on the sorption and mobility of [^{14}C]diazinon was examined. On the basis of the R_f values obtained, the pesticide was found to be slightly mobile in 80% and immobile in 20% of the soil studied (114). The adsorption and mobility of acephate in soils were studied by the use of soil TLC and soil-packed columns, respectively (115). A microextraction technique combined with GC-NPD was developed to estimate the relative mobility of seven coapplied pesticides (alachlor, atrazine, carbofuran, cyanazine, ethoprop, metolachlor, and metribuzin) on soil TLC plates. The results showed that the mobility of none of the pesticides was affected by the presence of the other coapplied pesticides (116). The effect of various surfactants on the mobility of selected non-ionic pesticides in soil was reported. The pesticide mobility depended on the chemical nature of the surfactant, its concentration, and the pesticide hydrophobicity (117).

The effect of soil amendment using urban compost, agricultural amendments, and surfactants on the mobility of two sparingly soluble pesticides (diazinon and linuron) was studied. The results indicated that the organic carbon content of soils and the amendment content in the soluble fraction played important roles in pesticide mobility (118). The mobility of emamectin was also assessed in six soils using the soils as a sorbent (119). An evaluation was made of sulfentrazone adsorption and mobility as affected by soil and pH. Adsorption decreased in response to increasing pH, and the greater decrease occurred above the $\text{p}K_a$ of sulfentrazone (120). Batch equilibrium and soil TLC were compared. It was suggested that the soil TLC gives results under nonequilibrium conditions and can provide information relevant to herbicide partitioning in the field environment that is not provided by batch equilibrium (121). Soil TLC with water or water-methanol as solvent allows measurement of the mobility of labeled pesticides through soil microstructure. Eleven sieved matrices were studied; these included pure humus, pure clays, schists, and soils. An equation was suggested to describe the pesticide movement in soil microstructures under the action of rain (122). The mobility of linuron in soils as influenced by soil properties, organic amendments, and surfactants was reported (123). The significance of soil properties in the adsorption and mobility of the fungicide metalaxyl in vineyard soils was investigated (124). Assessment of pesticide mobility by packed soil columns and soil TLC was reported (125). Movement of pesticides using successive elutions has been assessed (126). A study was made of the use of thin-layer chromatography to investigate pesticide mobility (127). The mobility of the herbicides alachlor, metolachlor, simazine, and atrazine was determined by soil TLC and GC (128).

IV. DETECTION AND IDENTIFICATION OF ZONES

Various chemical, physical, and biochemical methods have been used in the detection and identification of chromatographic zones. A useful handbook containing theory and application in TLC detection was published in 1990 (129).

A. Visualization by Color Reactions

Computer-aided pesticide identification is enhanced by a database containing data on corrected hR_f values in three solvent systems and colors formed with a number of detection reagents (130).

A number of color reactions for OP and OC insecticide detection were cited. The new OP pesticide BAY 93820 was detected by two different methods, with 4-aminoantipyrine and $K_3Fe(CN)_6$ and with KIO_4 -starch (131). The Griess reaction can be used for aromatic amino- containing pesticides such as ethyl parathion, methyl parathion, and fenitrothion. After reduction with $SnCl_2$, these insecticides were diazotized and coupled with 1-naphthylamines (132). Endosulfan and phosphamidon residues were detected by cobalt acetate and *o*-toluidine with a sensitivity of 10 μg (133). A new spray reagent for selective detection of dichlorvos in biological materials was described. Dichlorvos in the presence of moisture breaks down to dichloroacetaldehyde to give a yellow-red color. The sensitivity of detection increases in acidic media. Other OP insecticides failed to give colored spots without the interference of other pesticides (134). Selective and sensitive dichlorvos and dimethoate detection by 0.5% orcinol solution was used. After heating at 100°C for 10 min the detection limit under UV light at 365 nm was 1 μg and 15 μg for dichlorvos and dimethoate, respectively (135). Dichlorvos was detected after drying at room temperature by spraying with 2% NaOH solution, then with 2% 2-thiobarbituric acid solution, and heating at 90°C for 10 min (136). Pink spots were obtained on the white plate backgrounds in the investigation of dialkyl phosphate degradation products in OP pesticides. Detection was done by dipping into a 5% methanolic solution of $MgCl_2$, air drying, dipping into a hexane solution of 0.3% *N*,2,6,-trichlorobenzoquinoneimine, and heating at 110°C (137). The OP pesticide detection reagent, 0.05% ethanolic solution of 9-methylacridine, was successfully used (138). A 1% 4-(4-benzyl)pyridine spray reagent followed by 10% triethylenetetramine or polyethylene polyamines in acetone was used for metaphos visualization (139). The OP insecticide monochlorophos on alkaline hydrolysis yields *N*-methylacetoacetamide, which reacts with diazotized sulfanilamide or sulfanilic acid to give a red color (140). The herbicide bialaphos was detected using a mixture of 0.3% ninhydrin solution in acetone and acetic acid (97:3) (141). Some toxic metabolites of disulfoton, phorate, and terbufos were detected by spraying with $PdCl_2$ reagent and exposure to iodine vapor. An alternative method was Ackermann's esterase inhibition technique (142). Crystal violet was used as a selective reagent for OC insecticide visualization in toxicological material (143). OP pesticides in apples were detected by the oxidation of *o*-dianisidine with H_2O_2 . Detection limits were 0.6–0.7 μg (144).

Various visualization reagents were examined for detection of carbamate insecticides, herbicides, and fungicides. Several detection reagents for carbaryl were referred to: formation of the oxime, treatment with β -naphthol, and oxidation with nitric acid (145); diazotized *p*-nitroaniline and diazotized *p*-aminoacetophenone (146); *p*-nitrobenzenediazonium fluoborate (147); alkaline phenylhydrazine hydrochloride, with a detection limit of about 100 ng/spot (148); specific reagent ammonium cerium(IV) nitrate in 20% HCl, which reacts with 1-naphthol hydrolysis product of carbaryl and forms a violet complex (149); diazotized 6-amino-1-naphthol-3-sulfonic acid and then NaOH solution (150).

Visualization of phosphorothionate and phosphorothiolothionate pesticides was carried out by using chromogenic reagent containing 0.3 g of 4-amino-*N,N*-diethylaniline dihydrochloride in 20 mL of ethanol and exposing the plate to bromine vapors (151). The reaction between thiocarbamate herbicides and 2,6-dichlorobenzoquinone-*N*-chloroimine or 2,6-dibromobenzoquinone-*N*-chloroimine is very sensitive (152). Fungicidal ethylenebisdithiocarbamates and ethylenethioureas in plants were detected by spraying with 2% aqueous sodium nitroferrocyanide solution (153). The same reagent as well as iodine vapor and Ehrlich's reagent were used in the detection of oxidative degradation products of ethylenethiourea (154).

A color reaction based on the formation of black PbS by treating the dithiocarbamate fungicide mancozeb with alkaline plumbite solution was developed. The test can also be used for the detection of mancozeb in soil extracts (155). Visualization of carbaryl, propoxur, and carbofuran was carried out by spraying successively with 5% aqueous NaOH solution and 4-aminoantipyrine reagent and with $K_3Fe(CN)_6$ reagent. The detection limit of red spots was approximately 1 μ g (156). Tetraacetonitrilocupper(I) perchlorate is an appropriate reagent for visualization and quantification of some dithiocarbamate fungicides (ziram, ferbam, and thiram), giving yellow spots (157). Carbamate insecticides were detected by drying the plate and spraying with 5% NaOH, heating at 100°C for 5 min, and spraying after cooling with freshly prepared zinc(II) hexacyanoferrate reagent. The detection limit was 1–2 μ g/spot (158).

N-Methyl-(2-isopropoxyphenyl)carbamate was visualized by spraying with 6% ethanolic KOH solution and 4-nitrobenzene-diazonium fluoborate with 10% polyethyleneglycol in ethanol (159). A solution of 1% KI in ethanol was used for mipcin detection in groundwater (160). Detection by exposure to pyridine vapor gave detection limits in the range of 0.2–1.6 ng for five carbamate pesticides (161). The detection reagent zinc chloride diphenylamine for OP and carbamate insecticides was reported (162). Dapsone reagent was used to detect the carbamate insecticides baygon, carbaryl, and carbofuran. Detection was based on the alkaline hydrolysis of the carbamate, forming the corresponding phenols, which can be reacted in the para position with the diazotized arylamines (163).

The visualization reagent 2-(trichloromethyl)benzimidazole was used in heteroaromatic pesticide TLC analysis (164). Simultaneous determination of *o*-phenylphenol, imazalil, and thiabendazole residues in citrus fruit is possible by spraying plates from the origin with Dragendorff's reagent and from 10 cm beyond the solvent front with Fast Blue B reagent (165).

Certain reagents were used for pyrethroid insecticide detection. Pyrethroid insecticides containing a nitrile group were detected using sodium hydroxide–cupric acetate–

phosphomolybdic acid solution (166). Phosphomolybdate chromogenic reagents and 2,4-dinitrophenylhydrazine have also been used (167). Visualization of deltamethrin in formulations and surface residues in wheat using $\text{KMnO}_4\text{--H}_2\text{SO}_4$ reagent was carried out (168). Pyrethroids yield an intense blue color on bromination and treatment with *o*-toluidine. Limits of detection are 0.25–1.0 μg (169). Fenvalerate, cypermethrin, and deltamethrin were detected after drying by successively spraying with 1% cobalt acetate and 5% NaOH followed by spraying with 0.1% *o*-toluidine after 5 min. The detection limit was 10 μg (170). Chromogenic detection of synthetic pyrethroids containing a nitrile group is based on alkaline hydrolysis to give HCN, which reduces 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride to formazin, a pink product, in the presence of phenazonium methosulfate. The detection limit is about 1 μg , and OC and OP insecticides and carbamates do not interfere (171). Synthetic pyrethroids containing the nitrile group were selectively detected after alkaline hydrolysis. The liberated HCN reduced 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium (INT) to formazin, which in reaction with phenazonium methosulfate (PMS) gave a stable pink zone (172).

In the TLC determination of monensin pesticide in feeds, visualization was carried out by spraying with vanillin and *p*-anisaldehyde, respectively, and heating at 120°C for 1–5 min (173).

B. Physical Methods of Detection

1. Visualization by Luminescence

As mentioned in the introduction, current luminescence-based methods for determining pesticides in various sample matrices have been reviewed. The use of fluorescence detection in TLC, HPLC, and FIA and its application to environmental samples has been described (33).

Among the various existing stationary phases, fluorometric detection is used mostly on silica gel plates (174). Theoretical examination has shown that in fluorogenic labeling a fluorophor molecule is fixed to the nonfluorescent analyte, which can be determined fluorometrically (175). Absciscic acid produced by cyanobacteria was determined by fluorescence labeling (176). A luminol-based chemiluminescence flow-injection method for the determination of dichlorvos pesticide has been developed (177).

Detection of fluorescent spots under UV light at 366 and 254 nm is widely applicable. Spraying with $\text{AsCl}_3/\text{HIO}_4$ reagent and UV detection were used in TLC determination of plant growth regulator (178). Rhodamine 6G is commonly used as a reagent before UV detection (179). A biologically active congener of koningin A from *Trichoderma koningii* was visualized under UV light at 254 and 366 nm or by spraying with anisaldehyde and heating (180). HPTLC of about 150 insecticides and fungicides has been carried out. Chromatographic spots were detected by AgNO_3 and UV irradiation or by cholinesterase inhibition (181). TLC of biphenyloxide and metabolites on silica gel using various solvent systems and UV detection at 254 nm has been reported (182). Chlorpyrifos and its by-products were detected with various reagents, and the technique was compared to fluorescence quenching detection (183). In qualitative TLC screening of nitrofur residues in food, detection was performed by spraying with pyridine and exposing to UV

light of 366 nm. Visual comparison was made against a standard equivalent to the maximum residue level (MRL) set by European countries (184). The microbial transformation of prosulfuron was investigated on silica plates with UV (254 nm) detection followed by consecutive spraying and heating with cerie ammonium sulfate and Dragendorff's reagent (185). Numerous hydrolysis products of pyrethroids were detected under UV light and by exposure to iodine vapors (186). In chemical reduction of zoalene to ANOT (3-amino-5-nitro-*o*-toluamide), detection was done under UV light at 366 nm and by exposure to nitrous acid vapors. The plate was then sprayed with a Bratton-Marshall solution (0.4% naphthylethylenediamine dihydrochloride solution in methanol) (187). Detection with 254 nm UV light and spraying with 2% 4-(4-nitrobenzyl)pyridine in acetone, with heating at 110°C, of the OP insecticides in human serum after acute poisoning gave satisfactory results (188).

2. Autoradiography and Related Techniques

Over the last decade, detection by autoradiography was used often, in parallel with UV irradiation and chemical color reactions in agricultural and environmental pesticide analysis. 3-Phenoxybenzaldehyde and its 11 metabolites were detected (189); the metabolism of the insecticidally active GABA A receptor antagonist was investigated (190); radioactive cinmethylin and its degradation products were detected (191); ¹⁴C-8-hexachlorocyclohexane was identified after TLC separation (192); and the chromatographic behavior of chlorotoluron and its metabolites was investigated (193). After scraping and elution, quantification was carried out using liquid scintillation counting (LSC) (194–196). The aerobic and anaerobic soil metabolism of dicamba and 2,6-dichlorosalicylic acid were also investigated in this way (197). Structural characterization of [¹⁴C]propargite metabolites in goat urine was detected by autoradiography and LSC, followed by FT-NMR and MS (198). In toxicological analysis of food, radioactive zones of moxidectin and metabolites were located by autoradiography (199). In metabolism investigation of pentyl 2-chloro-4-fluoro-5-(3, 4, 5, 6-tetrahydrophthalimido)phenoxyacetate in rats, visualization was done under UV or by spraying with bromocresol purple or 2,6-dichlorophenol sodium salt. Radioactive compounds were detected by autoradiography (200). Degradation of commonly used pesticides in Malaysian soils was controlled in the same way (201). Qualitative identification by autoradiography served in the determination of radiochemical purity by elution in preparative TLC of S 53482 and its metabolites (202).

Radio-TLC was used to measure the chemical and biological release of ¹⁴C-bound residues from soil treated with [¹⁴C]*p,p'*-DDT (203) and microsomal oxidation of the herbicides EPTC and acetochlor and of the safener MG-191 in maize (204). Detection by radioscanning was done in the investigation of the degradation of [¹⁴C]tebupiriphos under anaerobic aquatic conditions (205) and for TLC separation of soil-bound residues of cyprodinil (206).

C. Biological Methods of Detection

The use of biological methods in TLC is justified because they are highly specific and detection limits are lower than with other methods.

Enzyme inhibition methods include fluorescent detection, because cholinesterase, which is used as a substrate after hydrolysis, gives a fluorescent background. Such identification was investigated for about 150 pesticides (181). In the quantitative determination of OP insecticides the esterase was obtained from *Bacillus subtilis* cultures. The plate was sprayed with 1-thionaphthyl acetate and with a solution of 2,2'-azo(1-naphthol-8-chloro-3,6-disulfonic acid)-4,4'-diphenyl disulfide. The limit of detection was 0.1–5.0 ng (207). A variety of methods for the separation, activation, and quantification of OP insecticides using TLC with enzyme inhibition were examined, with special attention to activation with bromine, hypochloric acid, and *m*-chloroperbenzoic acid (208). Coupling chemical and physical methods with enzymatic inhibition tests allows for the detection of toxicologically active substances in situ (209). 4-Methylumbelliferone esters were used for low-level detection of OP pesticides and warfare agents. The limit of detection for OP compounds was 0.01–1.00 ng (210).

Herbicides having an inhibitory effect on photosynthesis can be detected by inhibition of the Hill reaction, which is highly selective and sensitive (1). The sensitivity of thiazafluoron determination in water by inhibition of the Hill photosynthesis reaction was 20 times greater than that of standard TLC methods. Chloroplast homogenate preparation and the chromatographic procedure were described (211). *Colletotrichum fragariae* was used as the indicator species for direct bioautographic assay of some natural fungicides (212). A spore suspension of *Botrytis cinerea* in 1% potato dextrose agar was used for inhibition bioassay of antifungal activity (213).

D. Detection by Liquid Crystals

The liquid crystal method, which involves mapping the chromatogram by transferring organic substances from a TLC plate to a liquid crystal layer, was applied to warfare agents and pesticides separated on carbon layers. Liquid crystalline structure was disturbed by the pesticides incorporated, and light transmittance changed the pesticide spots, which enabled quantitative determination (214).

V. QUANTITATIVE DETERMINATION AND VALIDATION OF METHOD

Although thin-layer chromatography can still not compete with GC and HPLC in the quantitative determination of pesticides, more recent technologies such as video densitometry offer certain improvements in comparison with traditional slit-scanning densitometry. Validation was carried out on the video densitometric and slit-scanning determination of propham, chlorpropham, atrazine, diflubenzuron, tetramethrin, and α -cypermethrin for linearity, precision, and detection limit. A comparison of results showed that slit scanning is more sensitive and precise than video densitometry, but the RSD of 3.5–5.3% for the charge-coupled device (CCD) camera was acceptable. The main advantages of video technology were speed, excellent archiving capability, and the fact that data can be stored together, edited, and used for many tasks (215). A mixture of 10 pesticides was separated by two-dimensional (2-D) chromatography on cyano HPTLC plates with a polar mobile phase in the first dimension and a nonpolar mobile phase in the

second dimension. Chromatograms were recorded with a color CCD camera and evaluated with Camag VideoScan software. In video densitometry, the parameters that should be adjusted to achieve maximum quantitative precision include camera settings and track settings. Two quantification modes were compared: scanning of the whole plate and scanning of manually defined bent tracks. Evaluation of a 2-D chromatograph with a CCD camera is suitable for routine quantitative analysis (216). The influence of the instrumental settings of a video-imaging system on the quality of captured images was studied. The effects of different camera settings on background response, baseline noise, and sensitivity and reproducibility of detection were studied for different TLC and HPTLC plates. Dark or moderately luminous video images gave more repeatable results than very bright images (217). Reversed-phase TLC in conjunction with video densitometry was used for the quantitative determination of a six-component mixture of pesticides. Video densitometric quantification was validated for linearity, precision, and detection limit. All results were satisfactory according to the validation requirements (71). TLC of OP insecticides, carbamates, pentachlorophenol, etc., on silica was carried out. Quantification was done by slit-scanning densitometry and video densitometry (218). To determine compliance with Swiss legislation, the results of organotin residue determination in vegetables, fruit, and tap water were validated by comparing TLC results with data obtained by AAS (219). Quantification and validation of the HPTLC propham determination were carried out. The detection limit was 20–30 ng, repeatability 2.54–3.23%, and reproducibility 6.78–10.20% (220). Practical experience with the TLC pesticide determination according to DIN 38407 norms has been reported. The step wise confirmation of positive results and thus the flexibility of the method have been shown (221).

An optimized screening system for 170 pesticides that is useful in forensic and toxicological analysis was based on TLC in combination with GC and UV spectroscopy (222). The successful transfer of chromatographic conditions from TLC to HPLC columns was demonstrated for 62 pesticides on C₁₈-, C₈-, diol-, amino-, and cyano-bonded silica gel sorbent (223). Quantification by coulometric titration after TLC detection was reported (224). Fenvalerate was determined in emulsifiable concentrate formulation by acetone extraction. After scraping and elution of the spots, Fourier transform infrared (FTIR) spectrometric measurement of the carbonyl band at 1775 cm⁻¹ was done (225). Computer-assisted TLC-HPLC coupling for iprodione determination was investigated (226). The advantages of HPLC-TLC coupling for pesticide quantification in food, wash additives in sewage plants, and surface waters are discussed in Ref. 227. Heterocyclic pesticides in organic materials were determined using TLC coupled with surface-enhanced Raman spectroscopy (228). Thin-layer chromatography coupled with matrix-assisted laser desorption ionization mass spectrometry (MALDI) was used for the determination of cationic pesticides in the picogram range (229).

VI. APPLICATION

Because of its speed and simplicity, TLC is often used in research on various pesticides and their residues, degradation, and toxicity. Along with the examples mentioned herein, the reader will also find data on the use of TLC in the foregoing sections.

A. Residue and Multiresidue Analysis

Because many pesticides are persistent in the environment and degradation and chemical reaction with media can produce highly detrimental compounds, residue analysis, particularly multiresidue analysis, is being done more frequently.

Insecticides, acaricides, and fungicides were simultaneously determined on fresh and processed fruits with 2-D TLC (230). The fate of monocrotophos in the environment was investigated by using 2-D TLC of ^{14}C -labeled residues on silica (231). Thirteen 2-nitro-4-cyanophenyl esters and 10 trisubstituted s-triazine derivatives were investigated on silica gel layers impregnated with silicone oil of variable vinyl content. The retention of the solution compounds increased with increasing vinyl content (232).

Modern multiresidue TLC methods are based on sample preparation and cleanup by SPE and determination with computer-assisted AMD-HPTLC. These procedures allow the determination of 30–50 or more pesticides on one TLC plate.

Quantitative HPTLC determination of chlorpropham, propham, and thiabendazole residues in potatoes on C_8 layers has been reported (233) as well as that of benomyl, carbendazim, ethoxyquin, and thiabendazole residues in apples and pears (234). Application of modern TLC techniques to confirm results in pesticide multiresidue analysis has been examined (235, 236). A selective review was presented that focused on stationary and mobile phases, and TLC techniques were used for detection, separation, determination, and quantification of pesticide residues in various environmental samples (18). Carbofuran, atrazine, metolachlor, and their by-products were separated on HPTLC plates. The quantification of the compounds was done by densitometric scanning (183). The lipophilicities of 31 commercial pesticides were investigated by RP-TLC using water–methanol mixtures. The R_m values of the compounds decreased linearly with increasing concentration of the methanol (237). Examples of applying AMD to the determination of pesticide residues in groundwater and drinking water were presented (238–240). More than 20 pesticides in water samples were investigated by the AMD-HPTLC method using multiple and stepwise development combined with UV/Vis detection and determination (96). The optimization of the AMD-HPTLC method was investigated, and it was concluded that this method offers very sensitive quantitative determination of pesticide residues in water matrices (97).

A review of advances in the residue analysis of *N*-methylcarbamate pesticides was published in 1996 (241). Pesticide synthesis residual products in commercial chlorpyrifos on silica with hexane–ethyl acetate were studied (242). The binding mechanism of soil-bound residues of cyprodinil with humic substances in soil was investigated (243). A GC/NPD method and a rapidscreening TLC method were developed for the simultaneous determination of uracil herbicide residues (244). A review was published on environmental pollutants and the application of the adsorption phenomena for their analyses (245). HPTLC and HPLC with a conductometric detector were applied to determine chlormequat residues in pears after extraction with methanol and purification by formation of ion pairs with sodium tetraphenylborate (246). Diflubenzuron residues (51) and residues of 12 insecticides (47) in water samples were analyzed.

Food was qualitatively screened for nitrofurans residues (184). Pesticide residues in soil were determined (203, 204). An AMD-TLC method of pesticide multiresidue analysis in water was described (102, 103). A procedure for analyzing pesticide residues in drinking water by TLC became a German standard; the suitability of this method was

proved for 283 pesticides (28). Analyses of pesticide residues in grossly contaminated soil samples were reviewed (36).

The on-line coupling of RP-HPLC followed by AMD-TLC in determination of iprodione pesticide analysis was described (108). The soil-bound anilazine residues were determined, and bondings between soil organic acids and pesticide residues were investigated (109). TLC of ^{14}C labeled cloransulam-methyl residues and metabolites was carried out (247). The use of SPE as a sample preparation technique for multiresidue analysis of organic contaminants in water was described (38). An overview of chromatographic methods for the determination of pyrethrin and pyrethroid pesticide residue in crops, foods, and environmental samples was published (16). Extraction and a comparison of HPLC, HPTLC, and GC use in pesticide residue analysis in raspberries and lettuce have been reported (248). Quality control in textile mills was implemented using TLC pesticide analysis. Sixteen pesticides were detected using AgNO_3 for detection of halogenated compounds and an enzymatic test for P- and S-based pesticides (249).

The procedures for residue and multiresidue analysis are listed in Table 2.

B. Degradation, Biotransformation, and Toxicology

Numerous contributions discuss the application of TLC for the examination of pesticide degradation products. Transformation of [^{14}C]2,4-dichlorophenol and its derivatives in Saskatchewan (Canada) soils was investigated (250). Dissipation of alachlor was detected in four soils as influenced by degradation and sorption processes using hexane–dichloromethane–ethyl acetate (6:1:3) as the mobile phase and silica gel GF plates. Quantification was made by GC after elution of spots (251). The biotransformation of the pesticide metabolite 4-[U- ^{14}C]-nitrophenol was investigated (252). Chemotaxis and biodegradation of 3-methyl-4-nitrophenol by *Ralstonia* sp. SJ98 was reported (253). A study was made of the mobility of the fungicide penconazole in vineyard soils. If the fungicide degradation rate is low, its accumulation may lead to pollution of surface water (254). Dichlorvos, trichlorfon, and their metabolites and degradation products have been determined in environmental waters and human urine on silica gel (255).

Table 2 Residue and Multiresidue Analysis

Compound	Stationary phase	Mobile phase	Detection	Sample	Extraction	Ref.
Pesticide residues	HPTLC	AMD		Drinking water	SPE	28
Pesticides	HPTLC	AMD: universal gradient based on dichloromethane	UV	Water	SPE C-18, liquid/liquid extraction with dichloromethane	101
Pesticides		AMD	In situ reflectance spectra	Water		102

Pesticide multiresidues		AMD		Water	103
Iprodione		AMD	UV	Food samples	108
Anilazine		AMD		Soil	109
Imazalil, <i>o</i> -phenylphenol, thiabendazole	Silica gel	Acetone–formamide (95:5), toluene	Imazalil: UV 450 nm <i>o</i> -Phenylphenol: UV 500 nm Thiabendazole: Dragendorff's reagent and Fast Blue B reagent	Citrus fruits	165
Atrazine, carbofuran, metolachlor and by-products	HPTLC F ₂₅₄ , HPTLC G ₂₅₄	Hexane–CH ₂ Cl ₂ –EtOAc (6:3: 1); benzene–propanol–butanol–glacial acetic acid–H ₂ O (1:1:1:0.5:0.5); <i>n</i> -hexane–CHCl ₃ –MeOH–glac. acetic acid (5:2:1:0.3); CHCl ₃ –acetone (3:2)	UV, 1-pyrene carboxaldehyde dansyl–Cl, NBD–Cl, DPH, Rhodamine 6 G		183
Furaladone, furazolidone, nitrofurantoin, nitrofurazone	Silica gel (with concentration zone)	Dioxane–chloroform (1:1)	Pyridine, UV (366 nm)	Food	184
Pendimethalin (PROWL herbicide)		One-dimensional: chloroform–acetone–acetic acid (12:6:2 and 18:9:3) Two-dimensional: (1) benzene, (2) heptane–triethylamine (95:5) Preparative: 1,2-dichloroethene	Autoradiography	Tissues in rats	196
Acaricides, fungicides	Silica gel	2D: (1) cyclohexane–	UV (254 nm, 366 nm): 0.1%	Fresh and processed	230

insecticides	GF ₂₅₄	acetone (10:1); (2) petroleum ether– benzene– ethanol (65:30:5)	bromophenol blue; iodine	fruits	
Monocrotophos	Silica gel	2D: dichloromethane– acetone–acetic acid (60:40:5) acetonitrile–water– NH ₃ (40:9:1)	Autoradiography	Environment	231
Chlorpropham, propham, thiabendazole	HPTLC C-8		Densitometric scanning	Potatoes	233
Fungicides, insecticides	HPTLC silica gel; HPTLC RP-18	Dichloromethane, hexane–acetone (4:1), ethyl acetate, methanol–water (3:2, 7:3), acetonitrile–water (7:3)	AgNO ₃ , UV, chlorine–o– toluidine	Fruits and vegetables	236
31 Pesticides	HPTLC	Methanol–water (gradient elution)	UV, I ₂		237
Chlorpyrifos	Silica gel	Hexane–ethyl acetate (9:1, 4:1)	UV (254 nm)		242
Bromacil, lenacil, terbacil				Roots of <i>Echinacea</i> <i>angustifolia</i> Moench (Asteraceae)	Acetone, chloroform, water–methanol (5:1), cyclohexane Florisil: dichloromethane– acetone (9:1) 244
Cloransulam– methyl	Silica gel	Toluene– acetonitrile– acetic acid (10:9:1)	Radio scanning, UV (254 nm)	Lactating goats	247
Cymoxanil, iprodione, vinclozolin	HPTLC, silica gel	Hexane–acetone (7:3, 4:1, 3:2); cyclohexane– ethyl acetate– acetic acid (90:10:1, 8:2:1); ethyl acetate– cyclohexane (9:1)	UV (210 nm, 268 nm)	Raspberries and lettuce	248

There has been an increase in the number of papers concerned with the application of TLC in toxicology during the last few years. The deoxynucleotide composition of strawberry samples was used to demonstrate a chromatographic method for quantifying the difference between pesticide- and toxin-exposed strawberries. The samples were analyzed by ^{32}P labeling and 2-D TLC (256). TLC has been used as a rapid screening method for the detection of 46 common pesticides in serum and gastric lavage solutions (257). To elucidate the insecticidal activity of spider toxins, metal ions in venoms and in the body were determined by TLC, MS, ion-chromatography, and ICP. It was suggested that metal chelates play an important role in the intoxication and detoxification of spider toxins (258). A simple HPTLC method for the simultaneous determination of eight anticoagulant rodenticides in liver samples was reported (259). Identification, confirmation, and distribution of toxic pesticides that can cause the poisoning of domestic animals or wild fauna was carried out with TLC and HPLC techniques (260). An investigation was made of a rapid screening method for the identification of pesticides in the case of toxicosis using TLC. Thirty common pesticides were selected and analyzed using hexane–acetone (4:1) and chloroform–acetone (9:1) as the mobile phases and fluorescent silica gel as the sorbent (261). The metabolism of 2,4-dichlorophenoxyacetic acid (2, 4-D), the exposure to which results in an increased risk for certain malignant disorders, was investigated with TLC followed by NMR and IR spectroscopy (262). A new HPTLC method for the analysis of liver and crop samples in suspected poisoning cases was reported; the toxicity of imidacloprid in wild birds was evaluated (263). Three cases involving acute poisoning fatalities due to benfuracarb ingestion and forensic toxicological application were described. Benfuracarb, a carbamate insecticide, and its main metabolite, carbofuran, were detected using TLC and GC/MS (264). Toxicological interactions of chlorpyrifos and methylmercury in the amphipod *Hyaella azteca* were investigated (265). The carbamate insecticides furathiocarb (266) and carbofuran (267) were detected in gastric contents after poisoning by use of TLC and GC/MS.

C. Organochlorine Insecticides

Organochlorine (OC) insecticides are very stable and persistent compounds. Their capability to accumulate in the environment makes them very toxic. Therefore, numerous research projects have been devoted to their identification and determination.

HPTLC on silica with toluene-acetone (8:2) was used for pentachlorophenol determination in leather (268). Optimized mobile phase gradients were designed for the AMD separation of OC pesticides and phenols (98, 99). A method was described for the determination of 2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane isomer in the insecticide methoxychlor by using TLC (269). A report was published on a TLC method that provided 80–100% recovery for 26 OC pesticides in milk and milk powder (263). Isolation and identification of endosulfan in biological materials on silica gel plates were reported (270). Determination of pentachlorophenol and cymiazole in water and honey by RP-TLC was also reported. Recoveries from water were 97.7–100.0% for pentachlorophenol and 89.5–94.9% for cymiazole, and those from honey were 94.0–96.1% and 91.9–93.7%, respectively (272). Separation of certain OC insecticides on mixed oxide sorbents was mentioned earlier (75). The *Mucor thermo-hyalospora* MTCC 1384 fungus was found to bring about the transformation of endosulfan, whose

metabolites were identified by TLC (273). The research was aimed at optimizing chromatographic conditions for simultaneous separation and identification of OC and OP insecticides. The OC insecticides examined were DDT and methoxychlor, lindane, chlordane, and endosulfan (274).

An approach to insect control using sodium trichloroacetate to inhibit synthesis of the hydrophobic cuticular lipids that protect insects from dehydration was tested on *Triatoma infestans*. TLC and scanning electron microscopy showed disruption of the cuticular lipid layer in treated insects (276). Certain insect repellents in cosmetic products were determined using HPTLC (277). Time-dependent sorption of various insecticides in two different soils was investigated (278).

Chromatographic systems for OC pesticide determination are listed in Table 3 and Fig. 1.

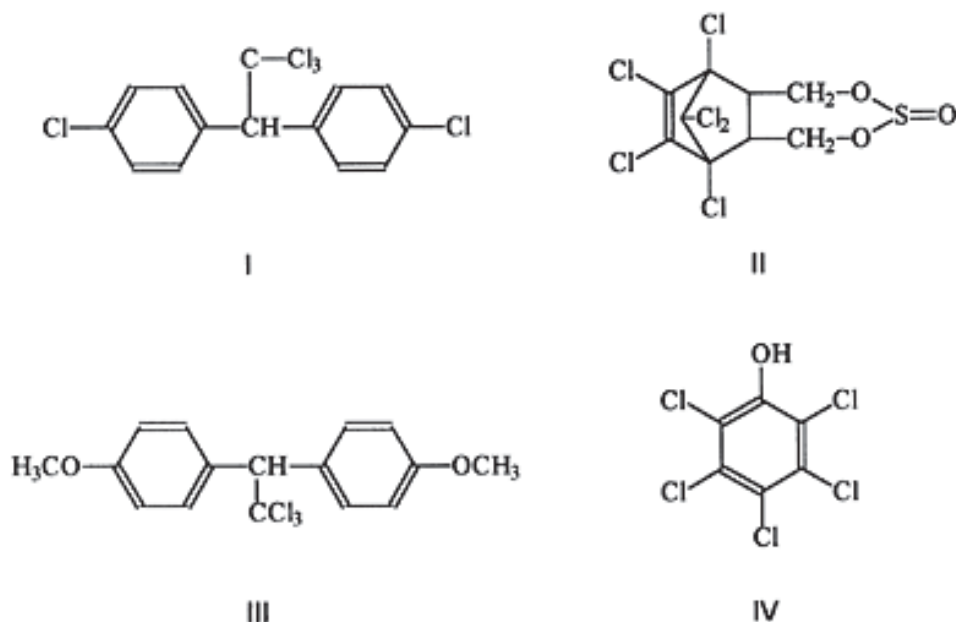


Figure 1 Organochlorine insecticides. I, DDT; II, endosulfan; III, methoxychlor; IV, pentachlorophenol.

D. Organophosphorous Insecticides

Residual organophosphorus (OP) insecticides were determined in crude herbal drugs (279). In contributions mentioned earlier, OP insecticides were determined in water (45) and in complex samples (84) using 2-D TLC and PRISMA optimization. The limit of detection was 15–100 pg. Various detection methods reviewed in Section IV were applied for chemical (130–136, 139), physical (177), and enzymatic (207, 208) detection of OP insecticides. ^{14}C -labeled tebupirimphos and metabolites were separated on silica gel with various solvent systems (205). TLC separation and determination of metrifonate and DDVP in rat blood, brain, and liver homogenates were achieved (280).

Quantification of terbufos and its metabolites in the lower microgram range was examined on silica gel with various solvent systems (281).

The degradation of isazofos was studied in soil samples under field and laboratory conditions. The pH of the soil had significant influence on the degradation of isazofos (282). The fate of ^{14}C -labeled diazinon during the composting of yard trimmings was examined (283). Seven TLC systems were investigated to determine their usefulness for separation of 19 pairs of *E-Z* geometrical isomers of pyrazole, pyrimidine, and purine derivatives with potential cytokinin activity (284). TLC analysis of chlorpyrifos and methylmercury reaction mixture was done in a study of their toxicological interactions (265). Novel protein targets for OP compounds were analyzed using TLC (285). TLC was used together with GC/MS in a chronological study of diazinon in putrefied viscera of rats (286). The OP pesticides malathion, dimecron, chlorpyrifos, monocrotophos, dimethoate, quinolphos, and methyl demeton were separated on hydrated stannic oxide layers (287). The chromatographic systems and the methods of detection and quantification of the cited OP pesticides and others are listed in Table 4.

E. Carbamates and Urea Derivatives

The thin-layer chromatographic behavior of carbamate pesticides and related compounds was investigated with several sorbents. Good separations of phenol from carbamate insecticides were reported using a variety of solvents (288).

Carbaryl is frequently examined in forensic analysis. Some methods of carbaryl, carbofuran, and propoxur detection were reviewed in Section IV (145–149, 156). A reagent for phosphoro-

Table 3 Organochlorine Insecticides

Compound	Stationary phase	Mobile phase	Detection	Limit of detection	Sample	Ref.
Endrin	Silica gel	Heptane and polar modifier (ethyl acetate, tetrahydrofuran, dioxane, diisopropyl ether)	UV (254, 366 nm), iodine vapor			90
DDT	Radio-TLC				Soil	203
2,4-Dichlorophenol	Silica gel	Benzene	Radio-TLC		Soil	250
Pentachlorophenol	HPTLC silica gel	Toluene–acetone (8:2)	UV (215 nm)	2.7 mg/kg	Leather	268
2,2-Bis-(<i>p</i> -methoxyphenyl)-1,1,1-trichloroethane	Silica gel	<i>n</i> -Pentane–ether (9:1)	UV (245 nm)			269

isomer in the
insecticide
methoxychlor

Endosulfan	HPTLC silica gel				Biological materials	271
Pentachlorophenol	HPTLC RP-18	Toluene– methanol (9:1); hexane– acetone– methanol– acetic acid (35:10:5:0:1)	UV 254 nm		Water	272
Chlordane, DDT, diazinon, endosulfan, ethion, lindane, malathion, methylparathion, methoxychlor, parathion, phorate	Silica gel 100 F ₂₅₄	<i>n</i> -Heptane– acetone (4:1)	UV (254 nm, 366 nm)			274
Chlordimeform	Silica gel	Benzene– chloroform– ethyl acetate (5:5:1)	5% <i>N</i> - (1Naphthyl)ethylenediamine dihydrochloride	10 ng	Honey	275

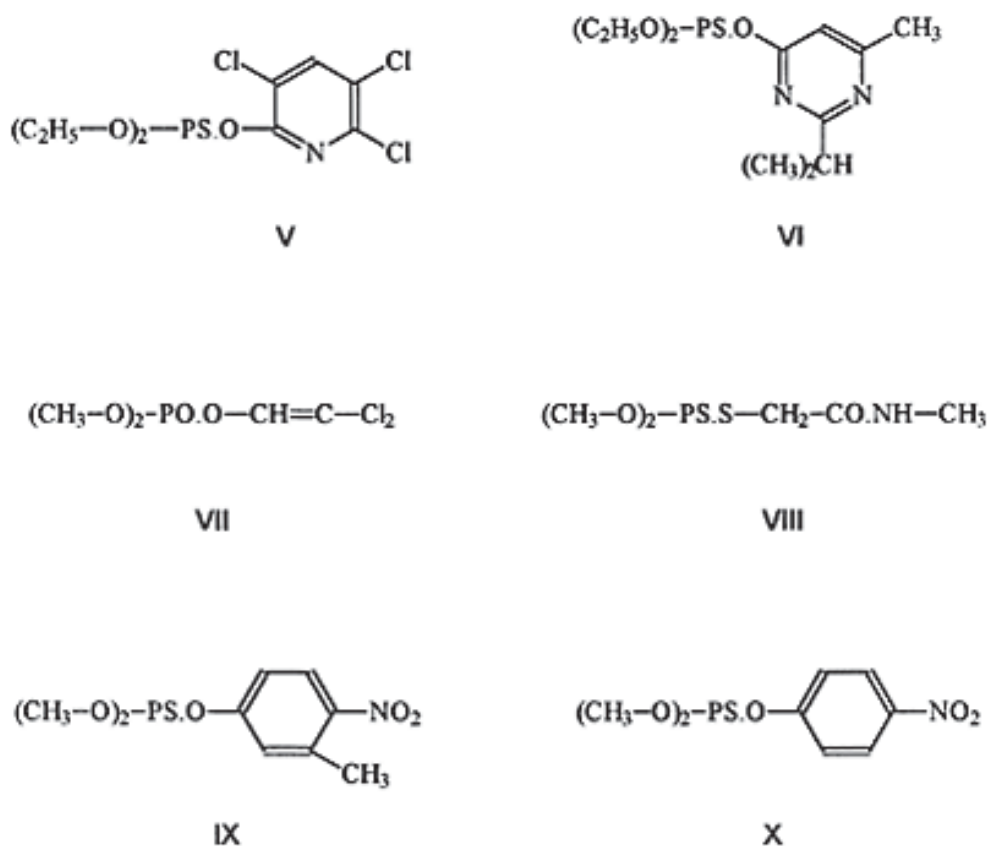


Figure 2 Organophosphorus insecticides. V, Chlorpyrifos; VI, diazinon, VII, DDVP; VIII, dimethoate; IX, fenitrothion; X, parathionmethyl.

thionate and phosphorothiolothionate pesticide detection was also mentioned (151). The TLC of carbaryl and related compounds on silica sequentially developed with benzene, CCl_4 , chloroform, distilled water, 1,4-dioxane, ethyl acetate, etc., was reported (289).

An organonitrogen insecticide, *N'*-(2, 4-dimethylphenyl)-*N'*-methylformamidine, was determined (290). Methods used in the pharmaceutical research of a carbamate pesticide mixture were compared (161). TLC of degradation products of ethylenebisdithiourea on silica with acetone, acetone-water, and ethanol was reported (154). Thifensulfuron insecticide synergism in soybeans and corn was examined (291). Chlorotoluron and its metabolites were chromatographed on silica gel (193). Development of a selective enzyme-linked immunosorbent assay for 1-naphthol, the major metabolite of carbaryl, was reported (292).

The chromatographic systems examined are listed in Table 5 and Fig. 2.

F. Herbicides and Growth Regulators

1. Herbicides

Because of the frequency of their use, triazines were the most frequently analyzed herbicides over the 1990s. In vitro studies were conducted of the metabolism of atrazine, simazine, and terbutryn in vertebrate species (293). A TLC study of triazine herbicide lipophilicity and an investigation of the effect of different solvent systems on R_m values were reported (295). Various TLC systems were used for s-triazine herbicide quantification (82), and the influence of mobile-phase pH on retention was examined (83). TLC studies on 24 new chlortriazines on silica gel were done with 14 different solvents to monitoring their synthesis and determine their stability (298). Reversed-phase HPTLC was used for the separation and detection of atrazine and its major metabolites and for the analysis of their residues in two surface soils and five subsoils. Recoveries were between

Table 4 Organophosphorus Insecticides

Compound	Stationary phase	Mobile phase	Detection	Limit of detection	Sample	Ref.
OP insecticides	Silica gel	Hexane–acetone (4:1)	5% MgCl_2 , 0.3% <i>N</i> ,2,6-trichlorobenz oquino neimine	100 ng	Water	45
Chlorfenvinphos, fenitrothion	Silica gel	Ethyl acetate–heptane	UV (254 nm, 365 nm), iodine vapor			88
Dimethoate	Silica gel	Heptane and polar modifier (ethyl acetate, tetrahydrofuran, dioxane, diisopropyl ether)	UV (254, 366 nm), iodine vapor			90
Ethoprop	Soil TLC		Autoradi ography		Silty clay soil	116
Glyphosate	Soil TLC	Water; water–methanol			Pure humine, clays, schists, and soils	122
Fenitrothion, methylparathion	Silica gel	Hexane–acetone (9:1)	5% Stannous chloride 5%	1 μg		132

			NaNO ₂ , 1-naphthylamine			
Dichlorvos			Phenylhydrazine hydrochloride	10 µg	Biological materials	134
Dichlorvos, dimethoate	Silica gel	<i>n</i> -Hexane–acetone–methanol (16:6:1); benzene–ethyl acetate–methanol (9:1:1)	2% NaOH; 0.5% orcinol solution	UV 365 nm; dichlorvos: 1 µg/spot; dimethoate: 15 µg/spot		135
Dichlorvos	Silica gel	<i>n</i> -Hexane–acetone–methanol (16:6:1)	2% NaOH; 2% 2-thiobarbituric acid	Semiquantitative		136
Azinophosmethyl, bensulfide, chlorfenvinphos, dimethoate, disulfoton, ethephon, ethion, fenitrothion, fenthion, malathion, methidathion, parathionethyl, parathionmethyl, phosalone, phosmet	1. Silica gel R 2. Aminobonded silica gel R	1. Petroleum ether–chloroform–ethyl acetate (13:6:1); petroleum ether–tetrahydrofuran (9:1) 2. Petroleum ether–tetrahydrofuran (19:1)	0.05% Ethanol solution of 9-methylacridine			138
Basudin (Diazinon), metaphos (Parathion-methyl), phosphamide (Dimethoate)		1% 4-(4-Benzyl)pyridine followed by 10% triethylenetetraamine or polyethylene polyamines in acetone		0.2 µg		139
Monocrotophos			Alkaline hydrolysis	1 µg	Biological materials	140
Bialaphos	Silica gel	1-Propanol–25% aqueous NH ₃	0.3% Ninhydrin		Fermentation broth	141
Disulfoton, phorate, terbufos	Silica gel	Toluene; toluene–acetone (17:3); toluene–acetone–	PdCl ₂ , iodine vapor			142

		methanol (18:1:1); chloroform– methanol (46:1)				
Bromophos, chlorpyrifos, diazinon, ethion, fenitrothion, fenthion, malathion, parathion, parathion- methyl, phorate, pirimiphos- methyl, quinalphos	Silica gel	2,2,4- Trimethylpentane– acetone– chloroform (12:5:1)	4-Amino- <i>N,N</i> - diethylaniline dihydrochloride	0.05– 0.50 μ g		151
Dichlorvos		Luminol-based chemiluminescence flow-injection method		0.008 mg/mL	Vegetable sample	177
Chlorpyrifos	HPTLC silica gel		Rhodamine 6G, Nile red, Merocyanine 540, 1-pyrene- carboxaldehyde, TNS-chloride, dansyl-chloride, NBD-chloride, 1,6-diphenyl- 1,3,5-hexatriene			183
OP insecticides	1. HPTLC silica gel 2. RP- 18 silica gel	1. Hexane–acetone (4:1); toluene 2. Methanol–water (7:3)	UV (254 nm), 2% 4-(4-nitrobenzyl)- pyridine, PdCl ₂		Human serum	188
Tebupirimphos	Silica gel	Hexane–acetone (4:1); chloroform– methanol (3:1)	Radioscanning			205
OP pesticides	HPTLC silica gel	Hexane–acetone (3:1, 9:1, 4:1); benzene–acetone (33:17)	Enzyme inhibition			207
OP insecticides			Enzyme inhibition			208

Dichlorvos, mevinphos, naled, parathion	Silica gel	Tetrahydrofuran- <i>n</i> - hexane (7:25)	NBD-reagent, cholinesterase inhibition			209
OP insecticides	HPTLC silica gel	Tetrahydrofuran- hexane (7:25); hexane-ethyl acetate (3:2)	In situ enzymatic and biological detection			218
Monocrotophos	Silica gel	Dichloromethane- acetone-acetic acid (60:40:5); acetonitrile-water- NH ₃ (40:9:1)	Autoradiography	Environment		231
Chlorpyrifos	Silica gel	Hexane-ethyl acetate (9:1 and 4:1)	UV (254 nm)			242
Chlorpyrifos	Silica gel	Hexane-acetone (7:3)	Iodine vapor	Amphipod <i>Hyalella</i> <i>axteca</i>		265
OP pesticides	Silica gel	Ethyl acetate- hexane (3:2)	EL method	8 ng	Crude herbal drugs	279
Terbufos	Silica gel	Toluene-acetone (85:15)	PdCl ₂ , iodine vapor			281
Isazofos	Silica gel	Chloroform-ethyl acetate-hexane- acetic acid (12:12:6:1)		Soil		282
Pyrimidme	Silica gel	Hexane-ethyl acetate (1:9)				284

Table 5 Carbamates and Urea Derivatives

Compound	Statio nary phase	Mobile phase	Detection	Limit of detection	Sample	Ref.
Carbaryl, carbofuran, methiocarb	HPTLC silica gel	Carbaryl, carbofuran, methiocarb: toluene- acetone (4:1) Propoxur: hexane- acetone- chloroform (75:15:10)	<i>p</i> -Nitrobenzene- diazonium fluo-borate; diethylene glycol		Water	43
Diffubenzuron	HPTLC silica	Ethyl acetate- toluene (1:3)	Ethanollic HCl, 1% NaNO ₂ in ethanolic	0.1 µg	Water	51

	gel		HCl, 1% ethanolic <i>N</i> (1-naphthyl)ethylenediamine dihydrochloride			
Chlorpropham, diflubenzuron, propham	RP18 F ₂₅₄ S	Methanol–water (4:1)	UV (254 nm)	Chlorpropham, 150 ng/spot; diflubenzuron, 30 ng/spot; propham, 150 ng/spot		71
Carbaryl, diuron	Silica gel	Ethyl acetate–heptane	UV (254 nm, 365 nm); iodine vapor			88
Lenacil, linuron	Silica gel	Heptane and polar modifier (ethyl acetate, tetrahydrofuran, dioxane, diisopropyl ether)	UV (254 nm, 366 nm); iodine vapor			90
Diflubenzuron	HPTLC	AMD	UV (260 nm)		Plant food products	104
Carbaryl	Silica gel	Hexane–acetone (4:1)	Alkaline phenylhydrazine hydrochloride	100 ng/spot		148
Carbaryl, propoxur	Silica gel	Acetone–hexane (1:4)	Diazotized PNA			150
Mancozeb	Silica gel		Alkaline plumbite	0.45 µg/spot	Soil extracts	155
Carbaryl, carbofuran, propoxur	Silica gel	<i>n</i> -Hexane–acetone (4:1)	5% NaOH, 4-aminoantipyrine, potassium ferricyanide	1 µg		156
Ferbam, thiram, ziram		Benzene–chloroform (9:1)	Tetraacetonitrilocupper(I) perchlorate			157
Baygon (propoxur), carbaryl, carbofuran		Hexane–acetone (4:1)	5% NaOH; zinc(II)hexacyanoferrate(III)	Baygon, 1.5 µg/spot; carbaryl, 1 µg/spot; carbofuran, 2 µg spot		158
<i>N</i> -Methyl-(2-isopropoxyphenyl) carbamate	Silica gel RP-18	Hexane–ethyl acetate (4:1); hexane–ethyl	KOH, 4-nitrobenzenediazonium fluoroborate 10%			159

		acetate–acetone (8:2:2); methanol– water(3:1)	polyethyleneglycol			
Mipcin	Silica gel	Acetone–ethyl acetate (1:1)	1% KI, ethanol		Groundwater	160
Carbadox, furaltadone, furazolidone, nitrofurantoin, nitrofurazone	Silica gel	Chloroform– acetonitrile– formic acid (87:10:3); chloroform– acetone (1:3)	UV (366 nm) pyridine vapor	0.2–1.6 ng		161
Ethylenethiourea, ethylenebisdithiocarbamates	Silica gel	Chloroform– butanol– methanol–water (200:10:2:1); chloroform–ethyl acetate– methanol (3:2:1)	UV (254 nm); 2% sodium nitroferricyanide		Plants	163
Chlorotoluran	Silica gel	Chloroform– methanol–acetic acid (8:2:1); chloroform– methanol–water (65:25:4)	UV (254 nm); autoradiography		Rats and Japanese quail	193
Carbofuran	Silica gel	Petroleum ether– chloroform– ethanol (2:2:1)	Autoradiography, liquid scintillation		Rice	194
Pendimethalian		One- and two- dimensional: chloroform– acetone–acetic acid (12:6:2 and 18:9:3)	Autoradiography		Tissues in rats	196
Aldicarb, butocarboxim, butoxycarboxim, carbaryl, oxamyl	Silica gel	Tetrahydrofuran– <i>n</i> -hexane (7:25)	NBD-reagent, cholinesterase inhibition			209
Chlorpropham, diflubenzuron, propham	RP-18	Methanol–water (4:1)	UV (254 nm)	Chlorpropham, 150 ng/spot; diflubenzuron, 30 ng/spot; pro pham, 150 ng/spot		215
Carbamate pesticides	Silica gel	Tetrahydrofuran– hexane (7:25); hexane–ethyl	Enzymatic and biological			218

		acetate (3:2)			
Propham	HPTLC silica gel	Dichloromethane	UV (228 nm)	20–30 ng	220
Organonitrogen insecticides	Silica gel	Benzene– cyclohexane– methanol (1:1:1)	Bismuth hyponitrite+KI		290
Carbaryl	Silica gel	Chloroform– methanol (98:2, 95:5)			292

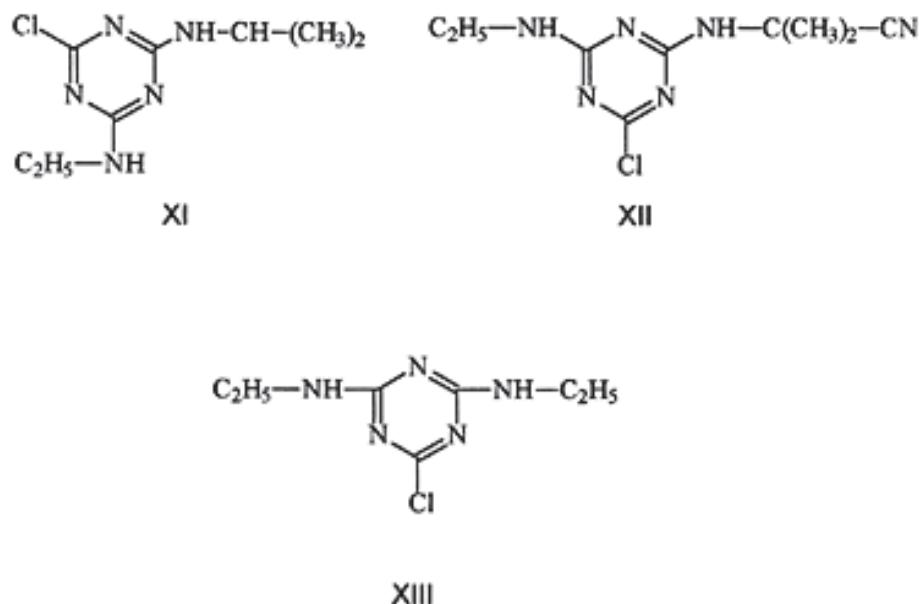


Figure 3 Herbicides. XI, Atrazine; XII, cyanazine; XIII, simazine.

87% and 97%, and the LOD was 20 ng/spot (17). Triazines and phenylurea herbicides were separated by OPLC with a binary mobile phase (111).

The sorption and sorption kinetics of the diphenyl ether herbicide acifluorfen in soils were investigated (299). Benfluralin, ethafluralin, and trifluralin were chromatographed using alumina sorbent with hexane as the mobile phase (300). The metabolism of [^{14}C]quizalofop-ethyl in soybean and cotton plants was examined (301).

One- and two-dimensional TLC were used to study the absorption and metabolism of ^{14}C -labeled pendimethalin and its residues in tissues treated with it (196). Substituted phenylurea herbicides are widely used as selective herbicides. Diuron, isoproturon, linuron, metoxuron, monolinuron, and neburon were isolated from crops, food, and environmental samples and determined using AMD-TLC (39).

Photolysis of imazapyr herbicide in aqueous media was examined by TLC on silica gel with seven solvent systems (195). The metabolism of the herbicide diflufenican in wheat field soil was studied (302). Two-dimensional TLC was used to study radioactive

cinmethylin and degradation products (194). A summary is given in Table 6 of additional TLC systems for analysis of triazines and other herbicides. Structure formula are given in Fig. 3.

2. Growth Regulators

Absciscic acid (ABA) was determined by HPTLC on silica gel plates with fluorescent labeling (303). Sumilarv (pyriproxyfen) was studied by TLC. The enantiomers were separated on cellulose tris(4-methylbenzoate)-coated silica as the stationary phase and hexane–hexanol (9:1) as the mobile phase (304). A new type of plant growth regulator, jasmonates, was separated and identified using TLC, GC, HPLC, and some other purification procedure. Quantification was done using GC/MS (305). The effects of cultivar, nitrogen level, presence of growth regulators, and retting process on the lipids and pigments of flax fiber were examined. The lipids were extracted and analyzed by TLC (306). As listed in Table 7, some growth stimulators were detected using normal-phase and reversed-phase TLC (307).

G. Fungicides

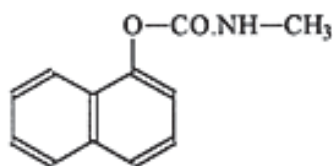
Thin-layer chromatography is a useful technique for detection of fungicides and for testing their biological activities. Acetylated triadimenol fungicide was identified during agricultural and food analysis (308). Over 100 pesticides, mostly fungicides and insecticides, were determined in stan-

Table 6 Herbicides

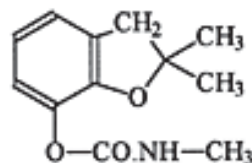
Compound	Stationary phase	Mobile phase	Detection	Limit of detection	Sample	Ref.
Metribuzin	RP-18	Methanol–water (45:55)	UV (290 nm)	30 ng	Soil and water	41
Atrazine	RP-18	Methanol–water (4:1)	UV (254 nm)	80 ng/spot	Soil	71
Ametryne, atrazine, aziprotryne, prometryne, simazine	1. Aminoplast 2. Cellulose 3. Acetylated cellulose	1. Cyclohexane–acetone (9:1) 2. Water–acetone (7:3) 3. Water–methanol (1:1)	UV (254 nm)			82
s-Triazines	Aminoplast, cellulose	Methanol–aqueous acetic acid (3:2); methanol–acetic acid–acetonitrile (6:4:1); methanol–NH ₃ (3:2)				83
Atrazine	Silica gel	Ethyl acetate–heptane	UV (254 nm, 365 nm), iodine			88

			vapor			
Propazine, simazine, trifluralin	Silica gel	Heptane and polar modifier (ethyl acetate, tetrahydrofuran, dioxane, diisopropyl ether)	UV (254 nm, 366 nm); iodine vapor			90
Atrazine, propazine	HPTLC	AMD	UV		Drinking water	97
Triazines	HPTLC	AMD: universal gradient based on dichloromethane	UV		Water	99
Atrazine	Soil TLC	Water				121
Atrazine	Soil TLC	Water; water-methanol				122
Imazapyr	TLC silica gel	Dichloromethane-ether (1:1), one- and two-dimensional	Autoradiography, UV		Aqueous media	195
Atrazine	RP-18	Methanol-water (4:1)	UV (254 nm)	80 ng/spot		215
Pentachlorophenol	HPTLC RP-18	Toluene-methanol (9:1); hexane-acetone-methanol-acetic acid (35:10:5: 0.1)	UV (254 nm, 215 nm)		Water and honey	272
Atrazine, simazine	Silica gel	2D: hexane-isoamyl alcohol (8:2)	Radio scanner		Vertebrate species	293
Atrazine	RP-18	Methanol-water (7:3)	UV (222 nm)	20 ng		294
Triazines	RP on silicone-impregnated silica	Acetone-methanol or acetonitrile as the organic modifier				295
Atrazine, simazine	HPTLC silica gel	Nitromethane-tetrachloromethane (1:1)	UV		Drinking and surface water	296
Atrazine	HPTLC RP-18	Methanol-water (85:15, 70:30)	UV (220 nm)		Polymeric microcapsules	297
Acifluorfen	HPTLC silica gel	Toluene-ethyl acetate-acetic acid-water	Densitometry		Soil	299

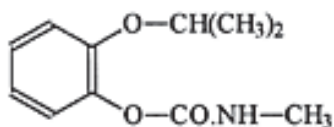
(100:100:2:1)				
Diflufenican	TLC silica gel	Dichloromethane–hexane (1:1); etherhexane (1:2); ethyl acetate–hexane (1:2); butanol–NH ₃ (30%) (6:1)	Wheat field soil	302



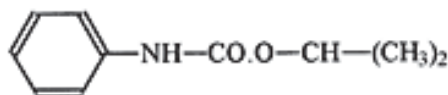
XIV



XV



XVI



XVII

Figure 4 Carbamates. XIV, Carbaryl; XV, carbofuran; XVI, propoxur; XVII, propham.

dard solutions by HPTLC (181). Some detection reagents used in the analysis of fungicide residues as well as in the study of their degradation products, were mentioned earlier (155, 165). The extraction and assay of agrochemicals in acrylic antifungal formulations were examined (309). Carbendazim and imazalil were chromatographed in fruits (310). The fungicide action of some vegetal extracts and their chromatographic separation and identification, including the TLC of some chemicals isolated from those extracts, have been researched (311). Photodegradation of the azole fungicide briadimefon was investigated (312). A comparative detection of fluorinated xenobiotics and their metabolites through NMR or ¹⁴C labeling in plant cells was done. One- and two-dimensional TLC were used (313). Seed treatment using preinfiltration and biocontrol agents to reduce damping-off of corn caused by pythium and fusarium was monitored with TLC (314).

RP-TLC investigation of the hydrophobicity and biological activity of new fungicidal compounds was reported, as well as the characterization of potential fungicides (315, 316). An optimal chromatographic system for separation and detection of some thiazole

derivatives was investigated (317). A lipophilicity study for certain 2-hydrazinothiazolic derivatives with antifungal activity was done by TLC on an RP-8 stationary phase and various methanol–water solvent systems (318). The relationship between fungistatic activity of thiobenzanilides and their lipophilicity was determined by TLC (319). Certain triazole derivatives that can be used as fungicides, herbicides, and insecticides were chromatographed by RP-TLC. A good correlation was found between the retention constants and log P, the new isocratic chromatographic hydrophobicity index, and the biological activity of the compounds investigated (320).

Certain dithiocarbamate fungicides were detected and quantified using TLC (157). Degradation products of fungicidal ethylenebisdithiocarbamates after oxidation with KMnO_4 were studied. The optimum conditions for degradation were found to be an acidic medium, a temperature of 20°C , and a fungicide/ KMnO_4 ratio of 1:7 (153). After the oxidation process mentioned, residues of these fungicides and ethylthiourea in plants were analyzed by using TLC. A 2-D TLC method for the analysis of new natural fungicides was described (321). The antagonism and structural identification of antifungal compounds from *Chaetomium cochliodes* were studied, and a direct inhibition bioassay of antifungal activity on TLC plates was carried out (213).

Chromatographic systems for the fungicides mentioned and some others are listed in Table 8.

Table 7 Growth Regulators

Compound	Stationary phase	Mobile phase	Detection	Limit of detection	Sample	Ref.
Metoxuron	HPTLC	AMD	UV		Drinking Water	97
Metoxuron Ethephon	1. Silica gel R 2. Amino-bonded silicagel R	1. Petroleum ether–chloroform–ethyl acetate (13:6:1); petroleum ether–tetrahydrofuran (9:1) 2. Petroleum ether–tetrahydrofuran (19:1)	0.05% Ethanolic solution of 9-methylacridine		Drinking water	128
Absciscic acid	HPTLC silica gel	Toluene–ethyl acetate–acetic acid (25:15:2)	Fluorescence labeling			176
Plant growth regulators			$\text{AsCl}_3/\text{HIO}_4$, UV			178
Growth regulators	TLC			Qualitative	Retted and scutched flax fiber	306
Growth stimulators	1. TLC silica gel	1. Methanol–MEK–benzene–acetic acid	UV (254 nm)	1.5–20 ng/spot		307

2. HPTLC
RP18
(7 different
proportions)
2. Methanol–water
and acetone– water

Table 8 Fungicides

Com pound	Statio nary phase	Mobile phase	Detection	Limit of detection	Sample	Ref.
Captan	Silica gel	Ethyl acetate– heptane	UV (254 nm, 365 nm); iodine vapor			88
Benomyl, dodine, hexachlo robenzene	Silica gel	Heptane and polar modifier (ethyl acetate, tetrahydrofuran, dioxane, diisopropyl ether)	UV (254 nm, 366 nm); iodine vapor			90
Benzanilide, procymidone, vinclozolin	HPTLC	AMD	UV		Drinking water	97
Iprodione	Silica gel	AMD	UV	30 µg/kg	Food samples	108
Anilazine		AMD			Soil	109
Degradation products of ethylenebisdithiocarbamate (ethylenethiourea, ethyleneurea, urea, and ethylenediamine)	Silica gel	Acetone; acetone–water (99:1, 95:5, 90:10); ethanol	Sodium nitr oferricyanide, iodine vapor, Ehrlich's reagent			154
Mancozeb	Silica gel		Alkaline plumbite	0.45 µg/spot	Soil	155
Ferbam, thiram, ziram		Benzene– chloroform (9:1)	Tetraacetonitr ilocopper(I) perchlorate			157
Imazalil, <i>o</i> -phenylphenol, thiabendazole	Silica gel	Acetone– formamide (95:5) for 10 cm and after drying with toluene for 19 cm	UV (300 nm); Dragendorff's reagent; Fast Blue B reagent		Citrus fruit	165
Cyprodinil	Silica gel	Toluene– methanol (9:1); chloroform– ethanol–acetic acid (90:10:1)	Radioscanning			206

Fungal pathogens		2D: 1. Methanol– dichloromethane (1:9) 2. Ethyl acetate– hexane (1:1)	Bioautographic assay: <i>Colletotrichum fragariae</i>			211
Antifungal compounds	Silica gel G	Cyclohexane– ethyl acetate (1:1); cyclohexane– methylene chloride–acetone (0.5:8:2)	Inhibition bioassay			213
Fungicides	Silica gel	2D: 1. Cyclohexane– acetone (10:1) 2. Petroleum ether–benzene– ethanol (65:30:5)	UV (254 nm, 366 nm); 0.1% bromophenol blue	Fruits		230
Fungicides	HPTLC RP-18	Dichloromethane; ethyl acetate; hexane–acetone (4:1); methanol– water (3:2, 7:3); acetonitrile–water (7:3)	AgNO ₃ , UV, chlorine- <i>o</i> - toluidine	Fruits and vegetables		236
Cymoxanil, iprodione; vinclozolin	HPTLC silica gel	Hexane– acetone (7:3, 4:1, 3:2) cyclohexane– ethyl acetate– acetic acid (90:10:1, 8:2:1); ethyl acetate– cyclohexane (9:1)	UV (210 nm, 268 nm)	Cymoxanil, 0.5 ppm; iprodione, 0.2 ppm; vinclozolin, 0.43 ppm	Raspberries and lettuce	248
Pentachlorophenol	HPTLC silica gel	Toluene– acetone (8:2)	UV (215 nm)	Leather		268
Pentachlorophenol	HPTLC RP- 18	Toluene– methanol (9:1); hexane– acetone– methanol– acetic acid (35:10:5: 0.1)	UV (254 nm)	Water and honey		272

Pyrimidine	Silica gel	Hexane–ethyl acetate (1:9)			284
Triadimenol	Silica gel	Benzene–ethyl acetate (4:1)	UV		308
Briadimefon	Silica gel	Hexane–acetone (19:1)	Iodine		312
Fluorinated fungicide	Silica gel	One- and two-dimensional: 1. Toluene–ethanol (4:1) 2. Ethyl acetate	UV (254 nm)	Plant cells	313
Droxythiobenzanilides	RP-18	Water–acetone; water–methanol	UV (325 nm)		315
2,4-Dihydroxythiobenzanilides	HPTLC RP 18	Water–methanol; methanol–water–10 mM acetate buffer (pH 4)	UV (325 nm)		316
Thiazole	HPTLC silica gel, normal silica gel, rice starch, cellulose	Hexane–ethyl acetate (4:1)	UV (254 nm)		317
2-Hydrazinothiazolic derivatives	RP-8	Methanol–water, with methanol concentrations ranging from 90% to 75% in increments of 5%	UV (254 nm)		318
Triazoles	RP-TLC silica gel impregnated with paraffin oil	Water–methanol	UV (254 nm)		320
Fenpropathrine, fluvalinate	Silica gel	Hexane–acetone (9:1)	KMnO ₄ , H ₂ SO ₄	Commercial formulations	322
Thionhanate-methyl	Silica gel	Hexane–ethyl	UV		309

thiram	acetate– acetone (4:4:1)	(254 nm)

H. Pyrethroids

A few chromogenic reagents described in Section IV and listed in Table 9 have been examined and used for pyrethroid pesticide detection (166–171). Development of immunoassays for Type II synthetic pyrethroids has been reported, as well as UV detection or exposure to iodine vapor for some pyrethroids (186). A hydrated zirconium oxide layer was used as a sorbent in TLC of cypermethrin, deltamethrin, and fenvalerate (76). Fenpropathrin and fluvalinate were determined

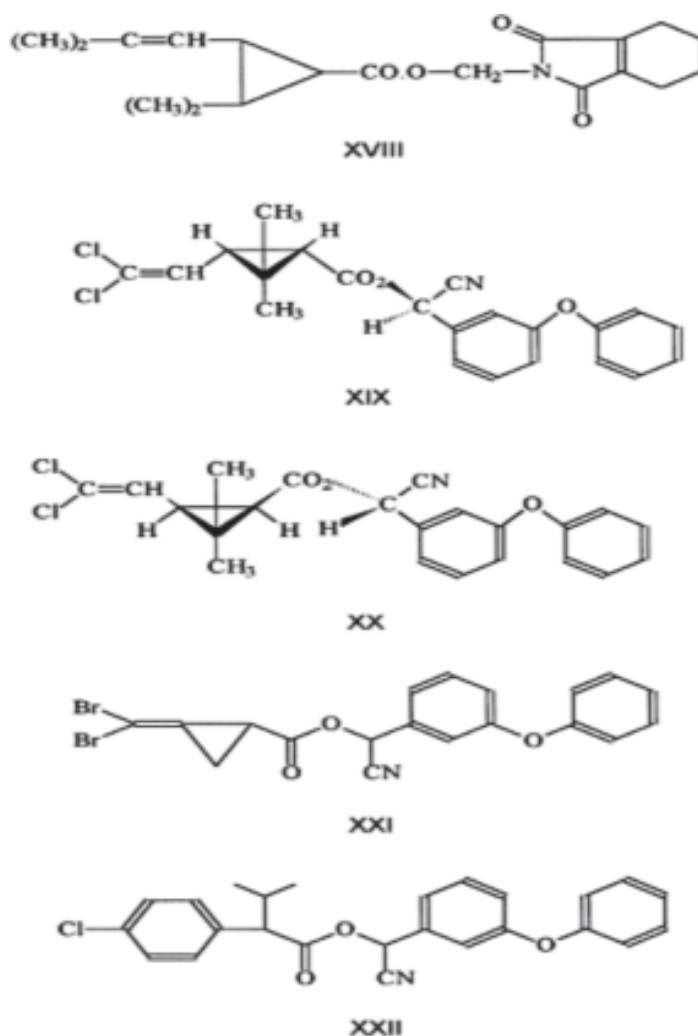


Figure 5 Pyrethroids. XVIII, Tetramethrin; XIX, (*R*,1*S*)-*cis*-cypermethrin; XX, (*S*,1*R*)-*cis*-

cyperaiethrin; XXI, deltamethrin;
XXII, fenvalerate.

Table 9 Pyrethroids

Compound	Stationary phase	Mobile phase	Detection	Limit of detection	Sample	Ref.
Cypermethrin, tetramethrin	RP-18 F _{254S}	Methanol–water (4:1)	UV (254 nm)	Cypermethrin, 200 ng/spot; tetramethrin, 450 ng/spot	Soil	71
Cypermethrin, deltamethrin, fenvalerate	Hydrated zirconium oxide	Methyl acetate–formic acid (4:1); ethanol–hexane (1:1); acetone–cyclohexane (3:2)	Iodine vapor, <i>o</i> -toluidine reagent	1 µg		76
Cypermethrin, deltamethrin	Silica gel	Heptane and polar modifier (ethyl acetate, tetrahydrofuran, dioxane, diisopropyl ether)	UV (254 nm, 366 nm); iodine vapor			90
Cypermethrin, deltamethrin, fenvalerate	Silica gel	Hexane–acetone–ethyl acetate	2,4-Dinitrophenylhydrazine, phosphomolybdate			167
Pyrethroid insecticides			<i>o</i> -Toluidine	0.25–1.0 µg		169
Cypermethrin, deltamethrin, fenvalerate	Silica gel	Petroleum ether–ether (9:1)	1% Cobalt acetate, 5% NaOH, 0.1% <i>o</i> -toluidine	10 µg		170
Pyrethroids	Silica gel	<i>n</i> -Hexane–ethyl acetate (1:1); ethyl acetate–petroleum ether (4:6, 3:7); ethyl acetate–petroleum ether–acetic acid (20:79.9:0.1, 20:79:1, 30:69:1)	UV, iodine vapor, 0.03% KMnO ₄ /H ₂ SO ₄ , 1% AgNO ₃			186
Cypermethrin	RP18 F _{254S}	Methanol–	UV (254 nm)	Cypermethrin		215

tetramethrin	water (4:1)	200 ng/ spot; tetramethrin, 450 ng/spot	
Fenpropathrin, Silica gel fluvalinate	Hexane– acetone (9:1)	KMnO ₄ /H ₂ SO ₄	322

spectrophotometrically after TLC separation (322). Validation of video densitometric quantification methods for cypermethrin and tetramethrin determination was reported (71, 215). Structure formulas of common pyrethroids are in Fig. 5.

I. Miscellaneous Determinations

A rapid TLC method for the determination of chlordimeform residues in honey was developing that uses silica gel with benzene–chloroform–ethyl acetate (5:5:1) as mobile phase. Chromatograms were sprayed with 5% N-(1-naphthyl)ethylenediamine dihydrochloride. The detection limit was 10 ng (275). The acaricide ivermectin was separated on silica with ethyl acetate–chloroform (1:3) and quantified by densitometry at 365 nm (323). Another chromatographic system for ivermectin detection uses silica gel as the sorbent and hexane–acetone–decane–methanol (59:30:10:1) as the mobile phase (324).

Screening methods for identification of rodenticides and lipids in animal feed using HPTLC–AMD determination was developed using hexane–ethyl acetate (7:3) on silica gel sorbent. Methanol–water–acetic acid (75:25:0.6) and methanol–ammonium acetate–triethylamine buffer (4:1) were used as mobile phase on RP-18 layers (325). The chemical reduction of zoalene to ANOT and primary metabolites was studied. Chromatograms were developed with chloroform–ethyl acetate–methanol (5:5:1) as solvent (187). Determination of 4-hydroxy-3-(1-tetrahydronaphthalenyl)-coumarin raticide was done on silica with chloroform–methanol (99:1) (326).

The HPTLC of bifonazole in cream and lotion was carried out on silica with hexane–ethyl acetate–acetone–diethylamine (45:45:10:4) as mobile phase and densitometric quantification. The RSD values for cream and lotion were 4.6% and 5.1%, respectively (327). Synthesis of a phthaloylglycine-derived strigol analog was monitored by TLC on silica with hexane–ethyl acetate as mobile phase and visualization under UV light (328). Lupine seed extracts have been shown to possess pesticide activity. Using HPLC and TLC, researchers found that systemin, a polypeptide defense signal in plants, is one of the components of lupine extracts (329). TLC and GC/MS were used in the determination of cloning and sequencing of the 2,5-dichlorohydroquinone reductive dehalogenase gene whose product is involved in degradation of γ -hexachlorocyclohexane by *Sphingomonas paucimobilis* (330). Potential antitermite compounds from *Juniperus procera* extracts were determined by TLC. The results were confirmed by GC (331). Measurement of lipo-philicity by RP-TLC of 19 N-(benzothiazol-2-yl)- α -amino alkyl phosphonic diesters with methanol–water mixtures was investigated. The concentrations of methanol were 75%, 80%, 85%, and 90%, respectively (332). Quantitative structure-retention relationships of O-alkyl, O-(1-methylthioethylideneamino) phosphoramidates were investigated by HPTLC on RP-18 layers with methanol–water solvent systems (333).

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Pharmaceuticals and Drugs**Szabolcs Nyiredy***Research Institute for Medicinal Plants, Budakalász, Hungary***Katalin Ferenczi-Fodor and Zoltán Végh***Chemical Works of Gedeon Richter Ltd., Budapest, Hungary***Gábor Szepesi***Qualintel Ltd., Budapest, Hungary***I. INTRODUCTION**

Chromatography is used extensively in the pharmaceutical industry as a separation tool for qualitative and quantitative analysis of various Pharmaceuticals and drugs. Although the popularity of capillary action planar chromatographic methods has decreased considerably during the past 10–15 years because of the replacement of several standard thin-layer chromatographic (TLC) separations with high-performance liquid chromatographic (HPLC) methods, they do claim a share of the market for chromatographic techniques, because many difficult analytical problems can be solved in small laboratories equipped with basic TLC equipment. Traditional TLC is inexpensive, and simple to use and requires minimal instrumentation, laboratory space, and maintenance. However, to achieve good precision, accuracy, and reproducibility, a certain degree of instrumentation is required; the use of densitometric evaluation is necessary at least for quantification.

A literature search of the last 20 years indicates that extensive reviews on TLC systems for Pharmaceuticals and drugs have been published (1–11). Several monographs, review papers, and book chapters are concerned with the detection and quantification of separated compounds (5, 6, 12–19).

Pharmacopoeias do not show the real importance of TLC in pharmaceutical analysis. Although the fourth edition of the European Pharmacopoeia (Ph. Eur. 4) (20) describes quantitative TLC in detail, its monographs include only qualitative identification or semiquantitative purity tests. The 25th edition of the United States Pharmacopoeia (USP 25) (21) contains a description of TLC in a general chapter, **621**, which has remained unchanged for about 20 years. However, recently Sherma gave a comprehensive outline of modern thin-layer chromatography in pharmaceutical and drug analysis in the

Pharmacopeial Forum (11). This article should be the basis for a future revision of the TLC chapter in Chromatography (621) for USP 26.

Governmental authorities require testing of pharmaceuticals for stability and impurity profiles before approval is given. Hence, the monitoring of the stability of drugs by TLC upon storage (22–26) and under stress (27, 28) is of concern. In addition, determination of bulk active ingredient purity and of the impurity profile using TLC has been reported (4, 5, 29–35).

To illustrate the applicability of planar chromatographic methods in pharmaceutical analysis, a brief outline is given below. Some aspects are well known and are treated only briefly, whereas others require a more detailed discussion. These aspects are listed in Table 1.

Table 1 Advantageous and Disadvantageous Properties of Capillary Action Planar Chromatographic Methods

Properties	Advantage	Disadvantage
	1. Ease of operation	
Solvent selection	Simple	
Detection	Off-line densitometry easy	
Automation		Difficult
Plate size	Easy to select	
	2. Sensitivity to experimental conditions	
Chromatographic parameters		
Eluent composition		Sensitive
Temperature		Sensitive
Chamber type		Sensitive
Chamber size		Sensitive
Relative humidity		Sensitive
Sample size	No problem	
	3. Selection of stationary phase	
Type of stationary phase	More uniform	
Nature of stationary phase		Limited
Applicability		Limited
Number of phases	Wide range	
Selectivity		Limited in general use
	4. Selection of mobile phase	

Separation selectivity	Wide range	
Variants in separation modes		Limited
5. Separation efficiency		
Plate efficiency	Good	
Peak symmetry		Problematic
Irreversible absorption		Frequently occurs
Solvent purity	No problem	
Spot shape		Dependent
Spot size		Dependent
Development modes		Highly dependent
Vapor phase		Highly dependent
6. Phase system optimization		
Number of variables		
Separation mode		Limited
Stationary phase		Limited
Mobile phase composition	Wide range	
Use of additives	No problem	
pH		Limited
Temperature		No
Relative humidity		No
Development mode	Wide range	
Chamber saturation		Limited
Time requirement	Fast	
Dynamic modification		
With organic solvent	Generally used	
With additives	Good alternative	
Properties	Advantage	Disadvantage
7. Detection possibilities		
In situ detection		
Visual	Qualitative good	Quantitative problematic
Densitometry without derivatization	Widely used	
Densitometry after derivatization	Widely used	

Spot elution		Problematic
8. Applicability (see Table 2 for data)		
9. Quantification-validation		
Precision		
Method		Problematic
System		Problematic
Accuracy	Good	
Selectivity	Good	
Limit of detection	Good	
Limit of quantitation	Good	
Linearity and range		Limited
Plate loadability	Good	
Sample stability		Problematic
Ruggedness		
Plate-to-plate variation		Problematic
Sample concentration	Good	
Sample size		Problematic
Spot type		Problematic
Chamber type		Problematic
R_f value		Problematic
Color reaction		Problematic
Detection	Good	
Mobile phase composition	Good	
Temperature		Problematic
Running distance	Good	

A. Ease of Operation

Conventional TLC is simple in instrumentation and in practice. The mobile phase can be easily prepared from organic solvents of conventional purity. Chromatograms can be visually evaluated after color reaction or under UV light. However, to obtain quantitative results densitometric evaluation is usually required. Although great efforts have been made to achieve complete automation, it is difficult and not widely used.

B. Sensitivity to Experimental Conditions

Planar chromatographic methods are sensitive to changes in environmental conditions. Small changes in the eluent composition and/or in temperature and relative humidity during development may cause dramatic changes in the retention characteristics of the compounds to be separated. This effect is more pronounced and more valid if unsaturated chambers are used for the development. The quality of any capillary action chromatographic separation is also a function of the type and size of the developing chambers.

C. Stationary Phase Selection

The most important stationary phases used in pharmaceutical analysis are described in textbooks (36–38). Modifications have been applied to improve the selectivity and efficiency of precoated layers in TLC. The following examples of modifications could be applied in pharmaceutical analysis:

- Bare silica and alumina

 - Precoated layers suitable for high-performance TLC (HPTLC)

 - Combinations of sorbents on a precoated layer

 - Hydrophobic and hydrophilic modification of bulk sorbents and precoated layers

More detailed treatment of this topic is given in Section III.A. 1.

D. Mobile Phase Selection

The correct choice of mobile phase composition for a given separation constitutes an important stage in achieving good separation in TLC. The basic strategy of mobile phase selection in TLC is similar to HPLC regarding solvent classification and eluotropic series (39–42). In general the selection of a particular solvent is simpler; it must be at least normally purified, have a relatively low boiling point and viscosity, and be inexpensive and compatible with the sorbent, support plate, and binder. Selection of appropriate mobile phase composition is more difficult. A great variety of factors can be adjusted, e.g., the solvent components, their relative concentrations, pH, and solvent strengths. A more detailed explanation is given in Section III.A.2.

E. Other Aspects

Successful planar chromatographic separation is dependent on several key factors. One factor relates to the quality of sample application.

The reproducibility of sample amount and spot size is quite important, but to achieve good chromatographic resolution and sensitivity of detection, the shape of the spots of the applied sample is also of importance. Techniques and apparatus for sample introduction are reviewed by Fennimore (43), Fennimore and Meyer (44), Jänchen (45), Kaiser (46),

and Omori (47). In summarizing the practical problems associated with various sampling procedures, the following principles should be considered:

1. There is a very narrow working range for the sample volume load if no focusing step is included in the sample application.
2. Sampling in the presence of solvents with a measurable elution strength can start the chromatographic process, resulting in significant loss in separation efficiency.
3. If a solvent used for sample preparation remains in the layer in or around the sampling area, the selectivity and the relative or absolute spot position in the chromatogram may be altered.
4. If part or all of the sample is solidified or adsorbed onto the stationary-phase surface, a slow dissolution effect can cause significant tailing of the spots.

The sample should be applied in the form of spots or streaks. The streak type of sample application, which forms very narrow lines, results in sharper spots and greater resolution, allowing for optimum separation efficiency. However, streak-type sample application is less precise than spot-type sample application.

The other aspects, i.e., phase system optimization, detection, quantification, and validation, are treated in detail in the following sections.

Sherma (11) summarized the advantages of TLC, comparing it to HPLC in tabulated form. The most important advantages of TLC are the reduced analysis time; the free solvent selection for the eluent, making a sample solution independent of the eluent used; there are no detection problems; chromatography of “dirty” samples is possible without precleaning.

II. ANALYTICAL AIMS IN PHARMACEUTICAL ANALYSIS

The most important tasks of capillary action planar chromatography in pharmaceutical analysis are summarized in Table 2.

Although HPLC has superseded TLC for many applications, TLC in both instrumental or noninstrumental forms has remained a standard method for solving many difficult analytical problems. With respect to the applicability and significance of TLC in pharmaceutical analysis, the methods used in pharmacopoeias can be strictly separated from the ones used in industrial pharmaceutical analysis.

Because of its useful role when cost effectiveness is essential, TLC is widely used as the standard technique for rapid and accurate identification of raw materials or finished products as

Table 2 Applicability of Capillary Action Planar Chromatography in Pharmaceutical Analysis (Data for Part 8 of Table 1)

Properties	Advantage	Disadvantage
Type of solute		
Very polar		Limited

Polar	Good	
Medium polar	Good	
Nonpolar	Good	
Structure of solute		
Structurally different compounds	Good	
Isomers	Good	
Homologous compounds		Limited
Size of solute		
Small	Good	
Medium large		Limited
Large		Limited
Analytical task		
Analysis of starting raw materials		
Plant extracts	Widely used	
Extracts of animal organs	Widely used	
Fermentation mixture	Widely used	
Analysis of intermediates		
Intermediates and crude products		Limited
Reaction mixtures	Applicable	
Mother liquors and secondary products	Applicable	
Analysis of pharmaceutical raw materials		
Identification	Generally used	
Purity testing	Alternative to HPLC	
Assay		Not applicable
Stability testing	Complementary to HPLC	
Analysis of formulated products		
Identification	Generally used	
Purity testing	Alternative to HPLC	
Assay	Complementary to HPLC	
Stability testing	Complementary to HPLC	
Content uniformity test		Limited

Dissolution test	Limited
Analysis of drugs and their metabolites in biological media	Limited applicability

well as for purity testing of raw materials and formulations in various pharmacopoeial prescriptions. The importance of TLC for assay methods, considering the difficulties experienced during its use, has decreased, and only a few official methods can be found in the pharmacopoeias, usually with a spot elution technique for quantification. In the field of industrial pharmaceutical analysis, the situation is different, because TLC instrumentation has reached a relatively high level of sophistication. In some special application areas, such as the analysis of extracts of medicinal plants and fermentation mixtures, modern TLC (precoated or HPTLC layers, densitometric evaluations) has a distinct role, because the interference of “unknown background materials” can be more easily eliminated than with other chromatographic techniques. Many chromatographers working in the pharmaceutical industry prefer to use reversed-phase HPLC in conjunction with normal-phase TLC or HPTLC to analyze raw materials for purity and impurities as well as for stability testing.

III. METHOD DEVELOPMENT

Method development (48), which results in sufficient separation, is one of the most critical steps in qualitative and quantitative analysis. Figure 1 summarizes the main steps in method development. These steps are described in the remainder of this section.

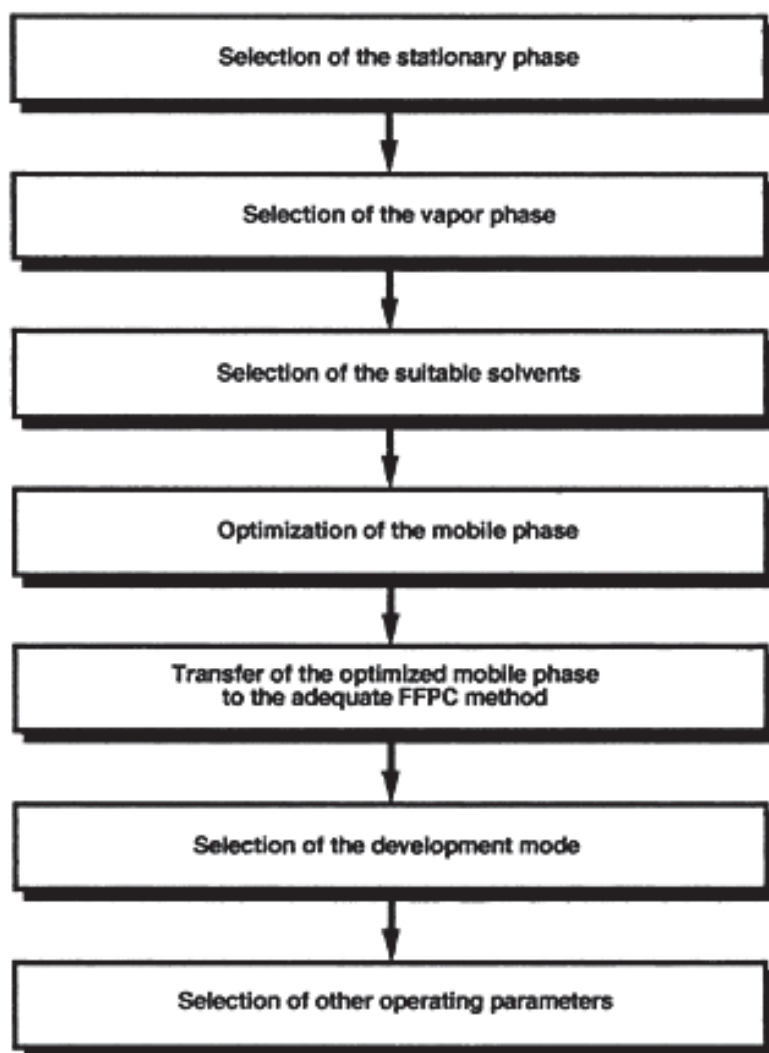


Figure 1 Schematic of method development.

A. Selection of the Stationary Phase

Separations for Pharmaceuticals and drugs can be performed using modified, nonmodified, and impregnated stationary phases that take advantage of the differences in chemical properties between the sorbent material and the sample compounds to be separated (49). Various types of chromatographic modes (straight-phase, reversed-phase, partition, and ion-exchange chromatography) are available based on the type of determining interactions involved. In TLC, silica is mainly (>90%) used for the separation of pharmaceuticals and drugs. However, of late, chemically bonded phases have become more popular to resolve certain separation problems (50, 51).

In straight-phase (normal-phase) chromatography, it is the hydroxyl groups on the surface of the silica that are the polar, active centers where most of the interactions take place in the retention of the compounds to be separated. These interactions are mainly hydrogen bonding and induced dipole–dipole interactions. Generally three characteristic

commonly used for precoated plates have an average particle size of about 11 μm , ranging from 3 to 18 μm ; for self-prepared analytical layers the average particle size is 15 μm , and the range of particle sizes is much greater. Precoated HPTLC plates have a small range of particle sizes, with an average particle size of 5–6 μm .

Precoated analytical layers with a preadsorbent zone are also commercially available for linear development. This zone serves to hold the sample until development begins. Compounds soluble in the solvent system pass through the preadsorbent zone and are concentrated in a narrow band before they enter the chromatographic layer, thus improving resolution.

B. Selection of the Vapor Phase

In planar chromatography, the separation process occurs in a three-phase system of stationary, mobile, and vapor phases, all of which interact with one another and with the operating parameters. Selection of the chamber type and vapor space is a variable that forms a third dimension of chromatographic parameters offered only by planar chromatography. The role of the vapor phase in TLC is well known, although little attention is given to it in practice (57).

1. Conventional Chromatographic Chambers

One can distinguish the normal (N) chamber and the sandwich (S) chamber (58). In the N-chamber there is a distance of more than 3 mm between the layer and the wall of the chromatographic tank (or between the layer and the lid of the chamber in horizontal development). If this distance is smaller, the chamber is said to have the S-configuration. Both types of chromatographic chambers can be used for unsaturated or saturated systems. The chambers used for forced-flow planar chromatography (FFPC) separation can also be assigned to these two categories.

2. Chambers for Forced Flow

The chambers used for overpressured layer chromatography (OPLC) (59–61) are unsaturated S-chambers devoid of any vapor space. This must be considered in the optimization of the solvent system, especially in connection with the disturbing zone and multifront effect (62), which are specific features of the absence of a vapor phase. The OPLC method is described in detail in Chapter 7.

The main difference between the chamber types used in rotation planar chromatography (RPC) (63–66) is the size of the vapor space, which is an essential criterion in RPC (66). Therefore, an additional symbol indicates the vapor space [normal chamber, microchamber, ultramicrochamber, and column RPC (N-RPC, M-RPC, U-RPC, and C-RPC, respectively)].

In N-RPC the layer rotates in a stationary N-chamber, where the vapor space is very large. Due to extensive evaporation, this chamber is practically unsaturated. The M- and U-chambers in RPC belong to the S-chamber type, the difference between these two chambers being that the former is saturated whereas the latter is unsaturated. Because in M-chamber RPC the chromatoplate rotates together with the small chromatographic

chamber, where the distance between the layer and the lid of the chamber is less than 2 mm, the vapor space is rapidly saturated. In the case of the ultramicrochamber, the lid of the rotating chamber is placed directly on the chromatoplate so that practically no vapor space exists.

3. Characterization of the Vapor Phase

For the characterization of the chamber saturation, a marker test dye mixture was proposed by Nyiredy et al. (58). The chromatograms of this dye mixture have to be developed with dichloromethane. The R_f range of the applied dyes depends on the chamber saturation. If the hR_f values of the marker dye mixture are the same in different chambers on a given stationary phase, the grade of the chamber saturation is identical. For comparison, the hR_f values obtained from different chambers can be depicted in a coordinate system. The hR_f values of any given system are plotted along the y axis, and those from the system being compared, along the x axis. If the chamber saturation values are identical, then a linear relationship is obtained, and $\tan \alpha$ for the line is 1.

Two basic possibilities arise during characterization of chamber saturation. If the hR_f values obtained in the second system are smaller, the vapor phase is more saturated and $\tan \alpha > 1$, or $\alpha > 45^\circ$. Conversely, if $\tan \alpha < 1$, or $\alpha < 45^\circ$, the vapor phase in the second system is less saturated. These possibilities are illustrated by results A and B in Figs. 3a and 3b, respectively.

With the help of this approach the chamber type can be characterized for a certain separation (X in Fig. 3) without specification of the vapor phase. Obviously, the separation can be influenced by changing the saturation of the vapor phase. The example depicted in Fig. 3a shows that increasing the degree of saturation of the chamber used for separation X would reduce the hR_f values of the compounds separated and reduce the resolution obtained. Figure 3b shows that use of a less saturated vapor phase increased the resolution obtained at lower R_f values.

These facts, together with the hR_f values of the test dye mixture, can be used to characterize the different types of chambers and simultaneously indirectly characterize the vapor-phase conditions used for a given separation (40). These results can be used for comparison of separations with given stationary and mobile phases, thus enabling prediction of the separation under other vapor-phase conditions, e.g., the different forced-flow planar chromatographic techniques. The technique also provides guidelines for the transfer of mobile phases between the different planar chromatographic methods.

As a rule of thumb, if the sample contains fewer than seven compounds to be quantitatively determined, then saturated chromatographic tanks have to be selected for the method development. If the sample contains more than seven substances for quantitative determination, or the separation is very difficult, then unsaturated chromatographic chambers have to be selected that enable the transfer of the optimized mobile phase for OPLC separation.

C. Selection of Suitable Solvents

The basis for the strategy of solvent selection is the solvent classification of Snyder (40), who classified more than 80 solvents into eight groups for normal-phase (NP)

chromatography according to their properties as proton acceptors (x_a), proton donors (x_d), and their dipole interactions (x_n) (see Fig. 4, part I). From these eight groups, 29 solvents were chosen that are commonly used in planar chromatography (48).

For the selection of suitable solvents, the first experiments can be carried out on TLC plates with the suggested neat solvents. After these first experiments the solvent strength must be either reduced or increased so that the substance zones are distributed between R_f 0.2 and 0.8. If the compounds to be separated migrate in the upper third of the plate, the solvent strength has to be reduced by dilution with hexane. If the substances do not migrate with the neat solvents, the solvent strength has to be increased by addition of water. In both cases the solvent strength can be varied so that a better distribution of the substance zones is obtained. Homologs of the solvents showing good separation or other solvents of the same group can also be tested. Consequently, the structures and properties of the compounds to be separated do not have to be known for the experiments. After these experiments the solvents giving adequate separations are chosen for further optimization of the mobile phase (48).

D. Mobile Phase Optimization

Mobile phase optimization is based both on the analyst's experience and intuition and on modifications of published data. However, as the sample composition becomes more complex, systematic solvent optimization becomes more important (56, 67). The methods used for optimizing isocratic mobile phases in HPLC are generally applicable, with some modifications, to TLC. Window diagrams have been successfully used (68–70) for the optimization of TLC mobile phases. Similarly, overlapping resolution maps were used as a criterion by Issaq et al. (71) and by Nurok et al. (72), who extended this approach to continuous development. Application of the sequential simplex method was also reported (73, 74).

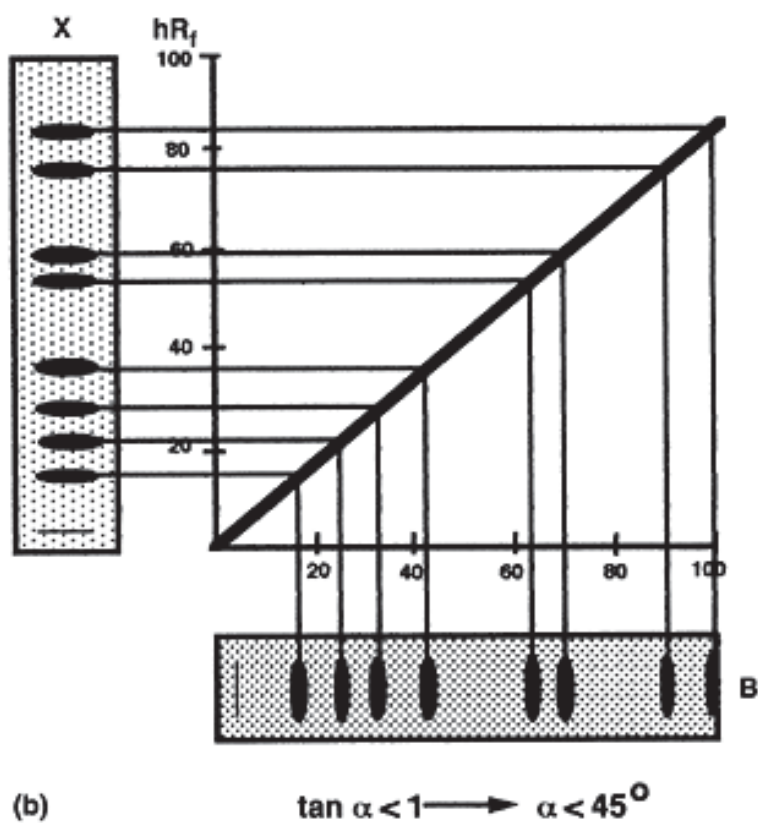
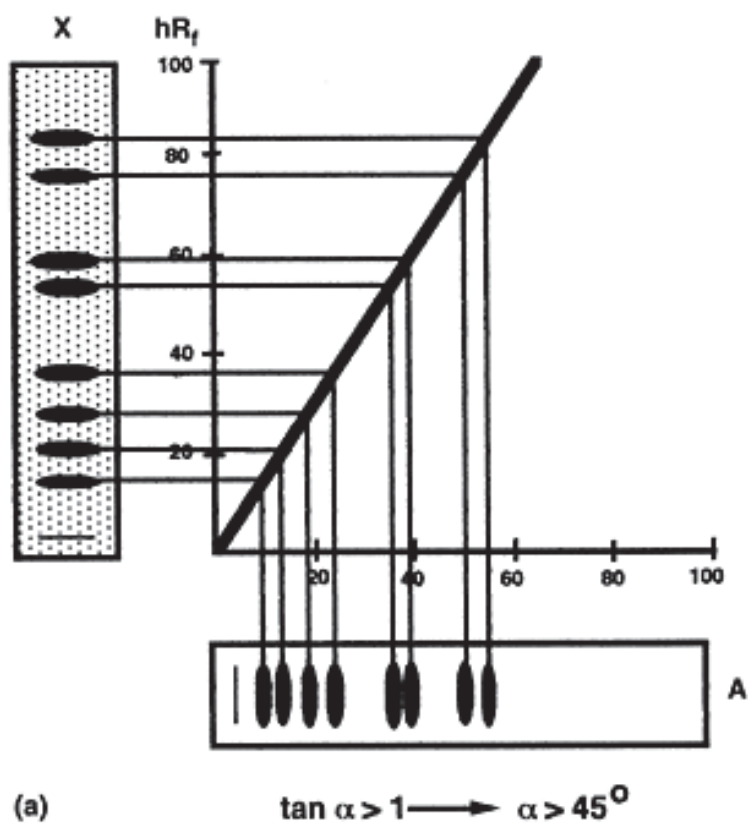


Figure 3 Calculation of the effect of the chamber saturation on the separation. (From Ref. 58.)

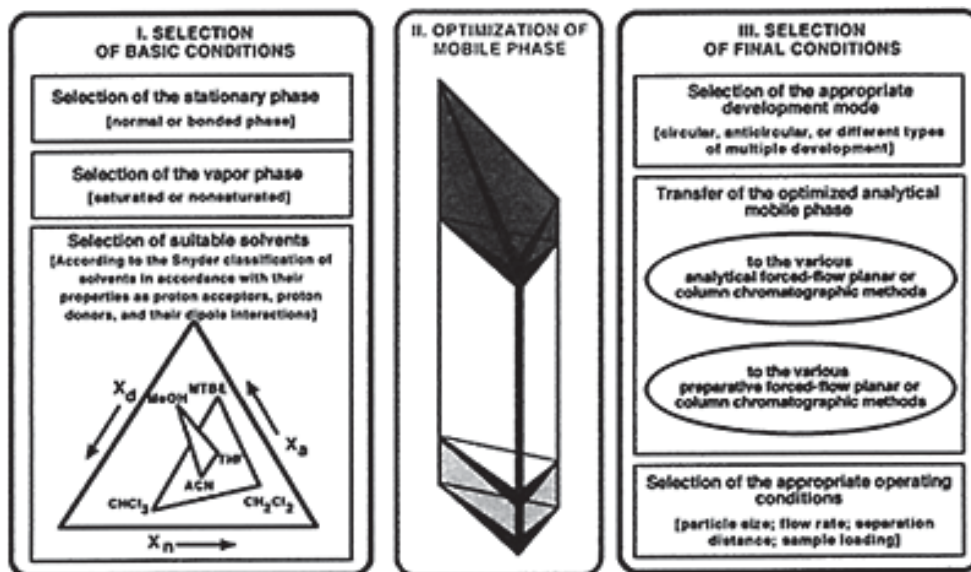


Figure 4 PRISMA optimization system for planar chromatographic separation. (From Ref. 79.)

A structural approach was suggested by Geiss (75) that assumes that selectivity and solvent strength are independent variables. For this optimization procedure a Vario KS chamber was used with three strong solvents. All three solvents (methyl *tert* -butyl ether, acetonitrile, and methanol) were diluted with a suitable amount of a weaker fourth solvent (e.g., 1,2-dichloroethane) to obtain a series of solutions spanning the solvent strength (ϵ^0) range 0.0–0.70 in increments of $0.05\epsilon^0$. In the next step the appropriate solvent strength must be determined. Once this has been identified, fine-tuning is accomplished by blending solvent mixtures of this strength but of different selectivities. Many elegant separations have been achieved in this manner; this method reduces the number of solvents available for optimization.

Based on Snyder's solvent characterization (39), a mobile phase optimization method, the PRISMA system (Fig. 4), was developed by Nyiredy et al. (76–82). The system consists of three parts. In the first part, the basic parameters such as the stationary phase, the vapor phase, and the individual solvents, are selected by TLC. In the second part, the optimal combination of the selected solvents is determined by means of the PRISMA model. The third part of the method includes selection of the appropriate FFPC technique (OPLC or RPC) and HPTLC plates, selection of the development mode, and finally application of the optimized mobile phase in the various analytical and preparative chromatographic techniques. This system provides guidelines for method development in planar chromatography.

The basis for an automatic mobile phase optimization procedure, the correlation between the selectivity points for saturated TLC systems at a constant solvent strength (horizontal function), was described (82) by the function $hR_f = a(P_s)^2 + P_s + c$, where P_s is the selectivity point, characterizing the composition of the mobile phase.

The vertical correlation at constant selectivity points between various solvent strengths was also described by $S_T = d \ln hR_f + e$. Because the vertical correlation can be linearized, measurements at three solvent strength levels are needed to calculate the hR_f values at all selectivity points in the spatial design. These correlations are also relevant when modifiers are used in constant amounts, using various substance classes of naturally occurring compounds. With these correlations of the hR_f values and the selectivity of the mobile phase, the chromatographic behavior of substances to be separated can be predicted at all selectivity points within the PRISMA model in saturated chromatographic chambers. The separation quality of predicted chromatograms is as assessed by the chromatographic response function (CRF). This optimal composition is found by a simple mathematical procedure that maximizes the CRF in dependence upon the mobile phase combination. Twelve measurements are necessary for a local optimum, and 15 for the global one. To increase accuracy, six measurements at three different solvent strength levels (18 experiments) are proposed (82).

Strategies for optimizing the solvent systems for planar chromatography, including two-dimensional TLC separations, were summarized by Geiss (75), Nurok (83, 84), and Härmälä et al. (85).

E. Mobile Phase Transfer

The possibilities of transferring mobile phases between the various chromatographic methods (48, 58) are summarized in Fig. 5. The thick lines indicate where direct transfers are possible; the thin lines indicate where transfers are possible but, in general, the selectivity will change. The dashed lines indicate the direct transfer possibilities for fully on-line separation processes. With the characterization of the different saturation grades of chromatographic chambers (58), excellent mobile phase transfer between analytical and preparative planar chromatographic methods and analytical HPLC can be achieved.

F. Selection of the Development Mode

The ascending linear development mode is most frequently used in TLC. Because ascending development has no theoretical advantage over horizontal development, the latter, being more adaptable, has become increasingly common in recent years.

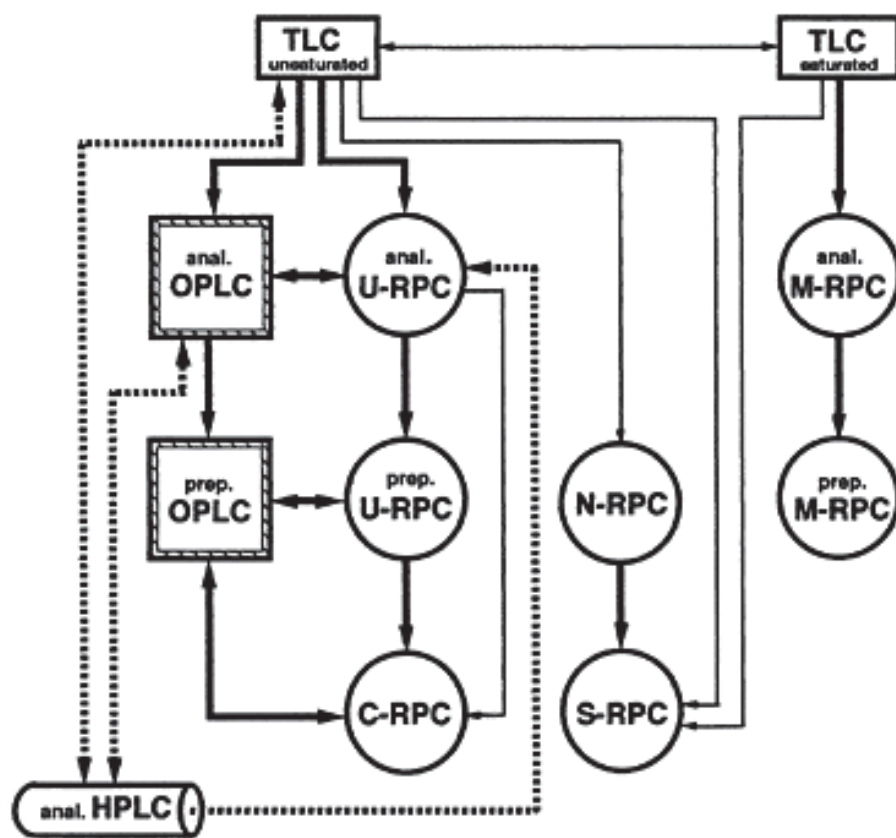


Figure 5 Possibilities of transferring the optimized mobile phase between different planar chromatographic methods, as well as HPLC and fully on-line FFPC techniques.

The advantage of circular development, where the solvent system migrates radially from the center of the plate to the periphery, is well known for the separation of compounds in lower R_f ranges (55, 75). Working with the same mobile phase, the resolution, particularly in lower R_f ranges, is about 4–5 times greater in the circular than in the linear development mode. The separation power of the circular development mode can be better exploited if samples are spotted near the center (62, 75).

In the anticircular development mode, the solvent system enters the layer at a circular line and flows toward the center. Because the solvent flow velocity decreases with the square of the distance but the area wetted also decreases with the square of the distance traveled, the speed of solvent system migration is practically constant. Therefore this developing mode is the fastest with respect to separation distance. Anticircular development is a widely accepted approach in analytical TLC if the resolution must be increased in the high R_f ranges (56).

From the point of view of development distance and mobile phase composition, multiple development (MD) techniques can be classified into four basic categories (86).

1. Unidimensional MD (UMD) means the repeated development of the chromatographic layer for the same development distance (D) in the same (S_{T1} , S_{V1}) solvent system (see Fig. 6). The mobile phase between development steps is removed by careful drying of the chromatoplate. The dried layer is returned to the development chamber for repeated development under the same chromatographic conditions as for the earlier development steps.
2. In the process of UMD called incremental MD (IMD) rechromatography is performed for increasing development distances (D_1-D_5) with the same (S_{T1} , S_{V1}) mobile phase (see Fig. 6). Using this multiple development technique, the first development length is the shortest, subsequent development steps are accomplished with a longer development distance each time, and the last front migration distance is the longest, corresponding to the useful development length of the chromatoplate and the mobile phase.
3. Gradient MD (GMD) is a multiple development technique where the successive chromatographic development steps are performed with a change in solvent strength and selectivity (S_{T1} , $S_{V1} \rightarrow S_{T5}$, S_{V5}) for the same chromatographic length ($D = \text{const.}$) (see Fig. 6).
4. Bivariate MD (BMD) is the most complex multiple development technique. The development distance and mobile phase composition vary simultaneously (D_1 , S_{T1} , $S_{V1} \rightarrow D_5$, S_{T5} , S_{V5}) during successive chromatographic runs (see Fig. 6).

The advantages of MD techniques are summarized in Ref. 86.

G. Selection of Other Operating Parameters

In TLC the solvent velocity is one parameter that, in principle, cannot be influenced by the chromatographer. Compared to TLC where the mobile phase migrates only by capillary action, the enhanced efficiency of FFPC techniques is due to the constant linear mobile phase velocity. FFPC techniques guarantee optimal H/u values. In OPLC the upper limit of velocity depends on the applied external pressure as well as on the viscosity. In RPC, the higher the rotational speed, the faster the migration of the mobile phase. The local mobile phase velocity can be influenced by the selection of the development mode.

In TLC the separation distance improves with the square root of the separation distance. However, the optimum depends on the quality of the plate (average particle size and size distribution of the stationary phase), the vapor space, the development mode, and the properties of the compounds to be separated.

In OPLC, because of the forced flow of eluent, the developing distance can be increased to about 18 cm by using a fine-particle sorbent layer by maintaining the high efficiency of separation.

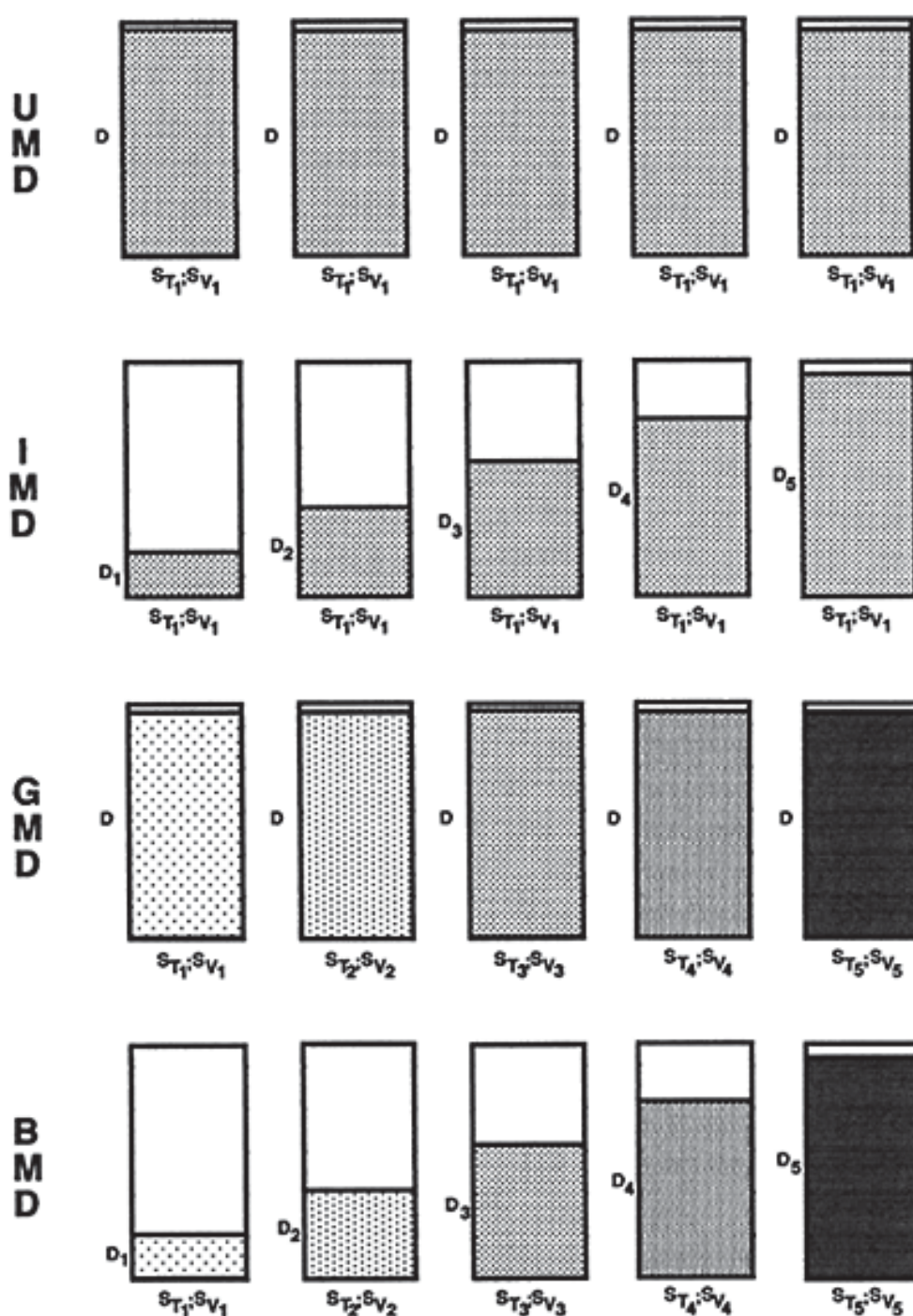


Figure 6 Schematic of multiple development techniques. See text for explanation.

A realistic possibility of increasing the efficiency and rapidity of planar chromatography is a novel aspect of multilayer OPLC; in long-distance OPLC (65, 66) the efficiency of the separation is increased significantly. With this technique the end of the first chromatoplate has a slitlike perforation to permit the mobile phase to migrate to a

second layer. A long separation distance can be achieved by adding one plate to another (87, 88).

Sample application is one of the most important steps for a successful planar chromatographic separation; this has been summarized by Kaiser (89).

During method development, the applied development mode, forced-flow technique, and development distance always depend on the separation distance. This is summarized in Fig. 7 in the form of a flow chart.

IV. DETECTION AND QUANTIFICATION

A. Conventional Detection Modes

For the visualization of compounds, one can use physical, chemical, or biological detection methods. Physical detection methods are based on substance-specific properties. The most commonly employed methods are the absorption or emission of electromagnetic radiation, which is measured by detectors. The β -radiation of radioactively labeled compounds can also be detected directly on the plate. These nondestructive detection methods allow subsequent isolation of compounds and can also be followed by microchemical and/or biological detection methods (90). Because physical detection methods are frequently not sufficient to establish the identity of compounds, they must be complemented by specific chemical reactions (derivatization). These reactions may be carried out either before or after chromatography.

Prechromatographic derivatization can be done either during sample preparation or on the chromatoplate. It is generally used to introduce a chromophore, leading to the formation of strongly absorbing or fluorescent derivatives; to increase the selectivity of the separation; to enhance the sensitivity of detection; and to improve the linearity. It includes oxidation, reduction,

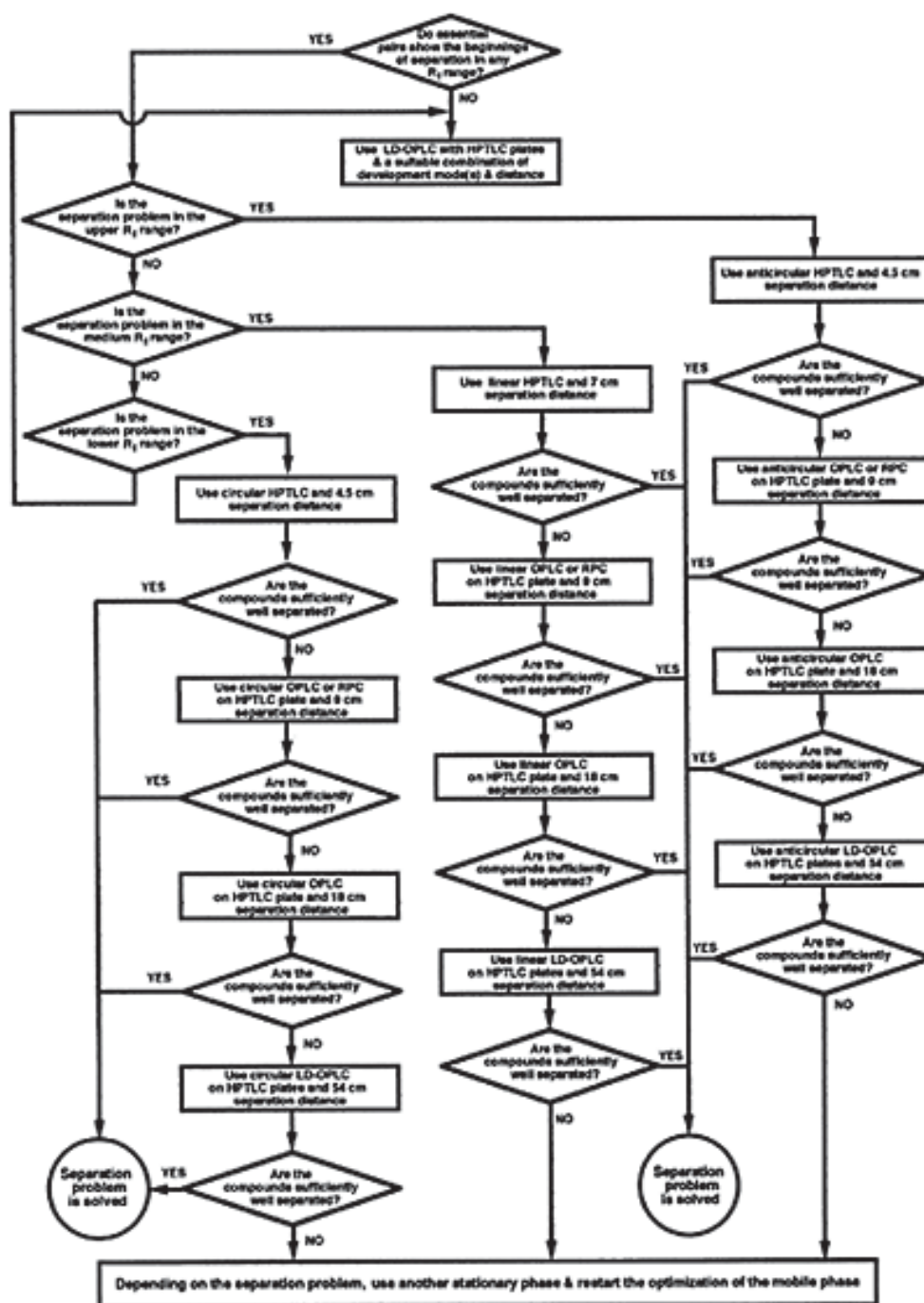


Figure 7 Flow chart for the selection of development mode, development distance, and forced-flow techniques.

hydrolysis, halogenation, nitration, diazotization, esterification, etherification, hydrazone formation, and dansylation (90).

The primary aim of postchromatographic derivatization is the detection of the chromatographically separated compounds for better visual evaluation of the

chromatogram. This step generally improves the selectivity and the detection sensitivity. Postchromatographic reactions can be carried out by spraying reagents onto the chromatoplate, by dipping the layer in reagent solutions, or by exposing the plate to vapors. The reagent can also be in the solvent system or in the adsorbent. In most instances, subsequent heating is necessary. Postchromatographic derivatizations have been extensively reviewed (90–92). In 1990, a new method, overpressured derivatization, was reported (93); this can be done by pressing an absorbent polymeric pad (prewetted with derivatization reagent) on the TLC plate.

For biological/physiological detection, the separated compounds can be transferred to the biological system (94). Alternatively, bioautographic analysis (95), reprint methods, and enzymatic tests may also be used.

Reagents and detection methods are summarized in a textbook by Jork et al. (54, 91). A summary of postchromatographic derivatization in quantitative TLC for pharmaceutical applications was reported by Duez et al. (96) and recently by Cimpan (97).

B. Instrumental Detection Modes

Absorption in the UV range can be detected by direct scanning at the wavelength of maximum absorption of the compound in the sample regardless of whether it is colored or colorless. Some types of compounds are naturally fluorescent, whereas others can be converted to fluorescent derivatives through pre- and/or postchromatographic reactions; this is a highly specific and sensitive method. Fluorescence quenching is limited to compounds that readily adsorb in the wavelength range of maximum excitation of a phosphor incorporated into the stationary phase (55).

Instruments to carry out scanning densitometry after the above treatments can be operated in the reflectance, transmission, or combined reflectance/transmission mode. Contemporary scanners work in the reflectance mode in most cases. Transmittance measurements are used predominantly for evaluation of gel electropherograms (98). Usually the sample beam is fixed and the plate is scanned by mounting it on a movable stage controlled by a stepper motor. The geometry of the light beam of scanners can be in the form of a slit or a spot (99). Various principal optical geometries are used in scanning densitometry: single-beam methods, which can be used in the reflectance, transmittance, or simultaneous mode, and double-beam methods. In the latter case the two beams can be either separated in time at the same point on the chromatoplate or separated in space and recorded simultaneously by two detectors (55).

Absorption spectra are rarely sufficient for identification, except when directly compared with the spectrum of a standard substance measured on the same chromatoplate. If possible, preference should be given to the fluorescence mode, which has a higher spectroscopic selectivity because two different wavelengths, an excitation wavelength and an emission wavelength, are used for each measurement. Fluorodensitometry has other benefits as well. By appropriate selection of these wavelengths the sensitivity can be increased by a factor of 10–1000 with respect to UV absorption, so the detection limit is decreased from, e.g., 10 ng to 500 pg (100). Linearity is better than in absorption densitometry; the calibration graph of the fluorescent signal is

linear over two or three orders of magnitude in sample amount and passes close to or through the origin (101, 102). The signal is independent of zone shape (103).

Diode array scanners (104, 105) as well as image analysis (106–109) are also used for evaluation of planar chromatograms. Planar chromatography coupled with spectroscopic techniques was reviewed by Somsen et al. (110). These techniques are frequently used for assays, and only a few publications describe their application for impurity control. A book edited by Görög is concerned with impurity profiling of drugs, and a chapter in it by Ferenczi-Fodor and Végh (33) deals with the application of planar chromatographic methods for impurity determinations.

Depending on the nature of the compound, the IR absorption of a TLC spot can be measured either after transfer to an IR-transparent substrate or in situ (111). By using Fourier transform infrared (FTIR) spectroscopy, almost all analytes can be identified if reference spectra are available (110). Moreover, the detection and quantification of non-UV-absorbing substances on TLC plates can also be done without postchromatographic derivatization (112). In the absence of reference spectra, relevant information about the molecular structure of the analyzed compound can be obtained by spectral interpretation (110).

The off-line method involves eluting about 5 μg of substance, evaporating the solvent, and pressing the substance into a micropellet suitable for recording spectra. Infrared spectra can also be recorded in situ by diffuse reflectance infrared Fourier transform (DRIFT) IR spectroscopy (113). To obtain suitable IR spectra, all solvents must be removed prior to measurement, and a background correction must be applied for absorption by the chromatoplate. Usually, more than 1 μg of compound is necessary for detection of individual functional groups and at least 10 μg for partial spectral recording. Kovar and coworkers (114, 115) reported a new method for on-line coupling of TLC and FTIR spectroscopy; a commercially available special HPTLC-DRIFT unit was constructed for this purpose. Achievements in this field are summarized by Rager and Kovar (116). Because of interactions between the analyte and the stationary phase (117), the HPTLC-DRIFT spectrum of a compound differs from the spectrum obtained by using the KBr technique (110). Thus, for reliable identification it is advisable to establish a special spectrum library containing spectra of adsorbed substances recorded from chromatographic plates (33). In a TLC-DRIFT system the detection limit is about 10 ng (118), which is approximately tenfold that of UV/Vis densitometry (110, 116). The identification limit is approximately 1 μg (111).

The moderate sensitivity of TLC/DRIFT can be improved by focusing the substance zones (spots) either by a second development perpendicularly to the first (119) or by using PMD (117), or AMD techniques (120, 121). Therefore, by zone-focusing and recording the substance-specific “window chromatograms,” identification limits can be improved by a factor of 3–6 (119), enabling stability tests of an active substance and its dosage form. On the other hand, the sensitivity of TLC-DRIFT can be enhanced by improving the IR reflectance of the stationary phase, e.g., by using a recently developed optimized sorbent for direct HPTLC-FTIR on-line coupling (122). This special adsorbent led to improvement of the detection limit for several pharmaceuticals by a factor of 2–3, and its evaluable IR range was extended by about 100 cm^{-1} into the “fingerprint” region. Determination and identification of five impurities in flurazepam bulk drug substance and capsules were performed (117) using this adsorbent.

Analysis of TLC spots by photoacoustic spectrometry (TLC/PAS) has been preferred over DRIFT analysis of strongly IR-absorbing samples (123). The spot containing 1–50 μg of the sample must be removed from the chromatoplate. After some preparation, it is placed in the photoacoustic cell for measurement. Recently, investigation of the in-depth distribution of the analytes in the sorbent layer was studied by photoacoustic spectroscopy (124). Vovk and Močnik gave a comprehensive review of photoacoustic and photothermal methods used in planar chromatography (124).

Reasonable spectra can be obtained by surface-enhanced Raman spectroscopy (SERS) (125, 126). A method was reported for preparing SERS-active surfaces in which colloidal silver spheres are deposited on HPTLC plates. The sensitivity of these activated HPTLC plates is so high that in situ vibrational investigations of spots are possible at the picogram level (127). Comparisons of the relative intensities of HPTLC-SERS spectra and normal Raman spectra were reported by Koglin (128). The results clearly demonstrate that varying electric charge density and hydrophilic character of the silver sol-activated nano-TLC plate strongly influence the HPTLC-SERS detection limit. The size and shape of the silver colloids are also important.

Various methods have been described for obtaining mass spectra of TLC spots (129–139). The zone of the compound to be identified can be scraped from the stationary phase, and after elution and solvent evaporation the residue is inserted into the mass spectrometer. Alternatively, the sample, together with the stationary phase, can be inserted directly into the spectrometer. In fast atom bombardment (FAB) and secondary-ion mass spectrometry (SIMS), a high-energy ion or atom beam is used to sputter molecules from the condensed phase into the gas phase for mass spectrometric analysis. However, most mass spectrometric measurements are destructive; FAB and SIMS spectrometry are surface-sensitive methods in which the material consumed in the analysis is sputtered only from the top layers of the sample spot. The sample required for FAB and SIMS is between 1 ng and 1 μg (130, 131). By using a special ion-source housing and an appropriate direct TLC-MS probe (132), one-dimensional mass spectra and mass chromatograms can be recorded from one track of the developed chromatographic plate (110).

Bush and coworkers (133, 134) reported an interface for the combination of TLC and MS that makes possible successful coupling between these techniques. By means of this device two-dimensional mass imaging was carried out. The FAB-SIMS liquid matrices (e.g., glycerol) that are used limit the time for imaging because of spot diffusion. To overcome this limitation, DiDonato and Busch used a solid matrix (135) (e.g., sorbitol or threitol, which is only melted by the primary ion beam) instead of a liquid, thus preserving the spatial resolution of the chromatogram for a longer time and making it possible to do the time-consuming 2-D MS mapping. Also, a charge-coupled device (CCD) for optical detection of sample bands was used (136). Crecelius et al. (137) described a matrix-assisted laser desorption time-of-flight mass spectrometric (MALDI-TOF-MS) system for the generation and characterization of an impurity profile of a pharmaceutical compound recorded directly from 65×2 mm strips of the developed aluminum-backed TLC plate. A mixture of a newly synthesized substance and three related impurities (25 μg of each) was separated by TLC on silica gel sheets over a 7 cm development distance, and mass chromatograms were recorded.

Wilson and coworkers (138–141) reported the application of TLC-MS-MS to drugs and their metabolites. They showed that FAB-MS alone gave spectra that were dominated by ions from the matrix. When MS/MS was used on the same samples, the resulting spectra were devoid of matrix interferences and contained only ions from the compounds of interest. Most recently, Busch (142) outlined the state-of-the-art results of TLC-MS and gave details of inherent problems of on-line TLC-MS coupling.

Wilson et al. (143) reported on solid-state NMR spectroscopy (high-resolution magic-angle spinning) for identification of Pharmaceuticals. The substances were separated on C₁₈-bonded sorbent, and the spots were detected by UV illumination. A usable spectrum was obtained for about 10 μ g of substance when the appropriate zone was scraped off the TLC plate, slurried with D₂O, and placed in the NMR tube without elution of the substance from the stationary phase.

For the quantification of separated radioactive substances, autoradiography, liquid scintillation counting, and direct scanning with radiation detectors can be used. Recently, a new detector for radiochromatography was reported (144) that measures the position and intensity of ionizing radiation on a two-dimensional TLC plate. Digital autoradiography (DAR) offers higher sensitivity than contact autoradiography, which makes possible significant reductions in the time needed to detect radiolabeled compounds and/or reduction in the quantity of analyte (145), as shown for metabolite products in dog urine samples. Implementation of OPLC separation improved the performance of the method (146–148). Klebovich (95) reviewed the applications of DAR in planar chromatography.

C. Quantification

A general approach to quantification in pharmaceutical analysis using TLC may be made on the basis of methods used for evaluation. Two basic approaches can be distinguished: direct methods, in which the separated spots are evaluated in situ on the plate, and indirect methods, in which quantitative measurements are carried out after elution of the spots on the chromatoplate. Although the importance of direct methods has increased considerably, the spot elution technique is also used (e.g., some assay methods in USP Pharmacopoeia). Direct methods can be divided into two major groups:

1. Visual comparison, where the spot intensities are established by visually comparing them with the intensities of simultaneously developed reference spots
2. In situ densitometry, with which the chromatogram is quantified directly on the plate by measurement of optical density by native or induced fluorescence of the separated spots

The semiquantitative estimation of impurities by visual comparison is used in some pharmacopoeias for purity testing of active raw materials as well as formulated products.

Four purity criteria can be defined:

1. The criterion does not allow the appearance of any spots other than the principal one on the chromatogram (single-spot criterion). In this case, a known quantity of the sample is transferred onto the plate. This procedure is simple, and does not require the simultaneous application of any reference materials; however, the result does not

- reflect the impurity profile of the material and is dependent on the experimental conditions (e.g., light intensity of the UV lamp, plate type and efficiency).
2. The criterion specifies the maximum allowable number of impurities and limits the quantity of each individual impurity. Only impurities that show R_f values identical to those of the simultaneously developed reference compounds can be present. Although this procedure is more accurate than the preceding one, it requires close conformity of the materials. (The impurity profile should be the same, which is a function of the manufacturing process.)
 3. The criterion limits the total intensity of the separated impurity spots. It does not limit the number and quantity of the individual impurities but does limit the total intensity of the separated impurity spots in comparison with the intensity of spots of simultaneously developed reference standards (principal components) applied in known quantities. Although this procedure is generally used for the evaluation of pharmacopoeial purity tests, it has two major limitations:
 - (a) Spot intensity may be a function of the chemical structure and R_f value of the impurities.
 - (b) The spot intensities must be summed visually.
 4. The criterion limits the quantity of each individual impurity and also the total amount of impurities. Standard dilutions of reference materials are simultaneously applied to the plate, and the purity of the material tested is evaluated by comparing the spot intensities of known impurities with those of standard dilutions of the same compounds; the quantity of other spots different from the reference spots is expressed in terms of the principal component. This evaluation procedure is the most accurate of the methods based on visual comparison.

Advantages and disadvantages of conventional and instrumental detection modes are discussed in Section IV. A brief summary of the most important advantages and limitations is presented in Table 3.

As with any quantitative method based on the interpretation of detector responses, several methods exist for evaluation of the results obtained. In the case of quantitative TLC a nonlinear relationship exists between the detector signal and the amount of substance to be tested. This nonlinearity is valid for both peak height and peak area measurements. A detailed interpretation of the problems is presented in the papers of Ebel and coworkers (149–156).

The next section is concerned with method validation of quantitative TLC techniques. Two questions must be answered prior to discussing the validation experiments: Should the statistically evaluated data elements such as precision, accuracy, and reproducibility be calculated on the basis of measured peak heights or peak areas? and Should internal or external standard methods or area normalization be used to yield quantitative results for the assay? The most important advantages and limitations of peak height and area measurements and those of the different methods of quantification are summarized in Table 4. As is apparent from Table 4, it is not easy to select the most appropriate method for quantification. The plot of peak height or peak area versus concentration is linear only within a narrow range of concentrations; therefore, the application of fivepoint calibration covering the total range of concentrations is recommended. Similarly, the usefulness of the internal standard method relates mainly to those analytical tasks that require complex

sample preparation procedures. Area normalization cannot be used for the evaluation of TLC results.

Table 3 Advantageous and Disadvantageous Properties on Spot Elution Technique and In Situ Densitometry

Advantage	Disadvantage
Spot elution technique	
Simple Identification of unknown components	Simultaneous use of appropriate blank is re-quired
By IR	Simultaneous use of appropriate reference material is required
By NMR	
By mass spectrometry	Losses caused by irreversible adsorption
Quantification can be performed by several methods, such as	Nearly quantitative extraction is required on accurate location of the spots
Colorimetry- spectrophotometry	Chemical reaction can be optimized
Spectrofluorimetry	The measured property of the derivatives can be stabilized
HPLC	
Gas chromatography	
In situ densitometry	
Chromatographic separation is independent of the detector in time and space.	Simultaneous running of appropriate refer-ence standard is required.
The chromatogram can be preserved after measurement.	Errors can be caused by incomplete distribu-tion of spraying.
The total amount of the sample can be spotted.	Layer structure varies along the plate.
The chromatogram is complete; every compo-nent can be detected.	Zones caused by solvent demixing can have a disturbing effect.
The sensitivity of the detection is higher by about two orders of magnitude.	Results vary from plate to plate. Derivatives formed on the plate are unstable.
Reflectance spectra of the spots can be taken, permitting certain identification of unknown components.	
Selectivity of the separation can be increased by changing the detection wavelength.	
Using transmittance and fluorescence modes, both the sensitivity and selectivity can be significantly increased.	

V. METHOD VALIDATION

The primary goal of every analytical investigation is to obtain qualitative and quantitative information about the sample being tested. Quantification includes the entire process of analysis, from sampling to interpretation of the final results. Because quantitative analytical results cannot be better than those of the weakest step in the entire process, each step should be considered separately either in method validation when developing methods or when interpreting results. Method validation is therefore a summary of several validation steps that provide exact proof and evaluation of the suitability, correctness, and precision of each chromatographic and nonchromatographic step and of the instrumental components.

This general approach is valid for analytical methodology using any type of chromatographic technique. Regulatory recommendations have been developed to promote correct method validation (157–160), and in the field of chromatography several reviews have been published that deal with the validation of analysis by TLC (161–168) and HPLC (169–173). A recent paper (168) gives acceptance criteria, based on practical experience, for individual validation parameters of

Table 4 Comparison of Peak Height vs. Peak Area Measurements and of Various Quantification Techniques

Peak height vs. peak area measurements			
Measurement	Advantages		Disadvantages
Peak height measurement	Simple Effective		Errors originating: Peak distortion Plate overloading Sensitivity to: R_f changes Baseline fluctuation
Peak area measurement	Higher precision, less sensitive to R_f changes and baseline fluctuation Ease in computerization		Linearity is valid in only a narrow concentration range.
Comparison of internal standard, external standard, and area normalization methods			
Property	Internal standard	External standard	Area normalization
Advantages	Not sensitive to loss during complex sample preparation More precise sample application	Simple Fast No additional peak	Simplest Fast No additional peak Easy to calculate No standard required
Disadvantages	Error addition Additional peak on the chromatogram Difficulties due to finding suitable internal standard	Insufficient accuracy and precision of sample application	Reliability of assay results originate from nonlinearity of concentration vs. detector response curve. Detector responses are function of chemical

planar chromatographic methods for various purposes and quantification levels. Examples of typical validation results for TLC methods are given by Sherma (11). We refer here to our own reviews and papers concerned with TLC methods used in pharmaceutical analysis (4, 5, 8, 15). The next section summarizes the parameters that must be taken into account in the course of a TLC validation process.

A. General Considerations

Prior to making any decision on validation experiments, several important factors should be considered:

Steps to be included in the analytical process

Selection of analytical performance parameters

Model solution (sample) to be used for validation experiments

Degree of instrumentation

Stability problems

1. Steps To Be Included in the Analytical Process

The TLC method intended for use may be divided into five distinct steps:

1. Sample preparation, including prechromatographic derivatization
2. Application of the sample to the plate
3. TLC separation
4. Detection, including postchromatographic derivatization
5. Quantification

Each step must be validated for error analysis and suitability in order to determine potential sources of error.

2. Selection of Analytical Performance Parameters

The aim of the analysis should be kept in mind when analytical performance parameters are selected for the validation experiments. Table 5 lists the most important analytical performance parameters used for validation of TLC and HPLC methods. As is apparent from the data presented in Table 5, the parameters used for the two chromatographic techniques are not vastly different. The importance of the parameters used in the experiments to validate TLC or HPLC methods varies, especially if the purpose of the analysis is also considered. This is demonstrated in Table 5, where the primary analytical parameters are indicated as a function of the analytical aims.

3. Model Solution (Sample) To Be Used for Validation Experiments

Prior to beginning the validation of a TLC method, a decision must be made as to which type of sample should be used for the experiments: a mixture of standards, a real sample, a spiked placebo, or a blank. It must be emphasized that without appropriate reference compounds, the identification of an unknown peak on the chromatogram is problematic, and the quantification will be questionable. A mixture of only standards provides better results than those that may be obtained from a real sample. Concentrations of the desired components in the sample may vary within a definite range, and this can influence the separation efficiency, resolution, detectability (lowest limit of detection and lowest detectable quantity), and quantification (accuracy, precision, and reproducibility).

The use of sample (either in a mixture or alone) that is in accordance with the analytical application is recommended. (Data for each analytical application are included in Table 5.) Samples used for validation experiments can be classified into four categories:

1. A mixture of a known number of the components of interest, the identities of which are known. The use of a mixture of reference compounds at concentrations similar to those likely to occur in the real sample is recommended (Model A in Table 5).
2. A mixture of a known number of the components of interest with a number of compounds that are not of interest but whose identities are known. The components of interest and other components should both be available in the pure form (i.e., the latter with the matrix absent). The use of a placebo spiked with the reference compounds in the concentrations desired is recommended (Model B in Table 5).
3. A mixture of a known number of the components of interest with a number of unknown (background) compounds. The components of interest should be available in pure form (i.e., with the matrix absent). A sample blank or a real sample spiked with the known components in the concentrations desired is recommended (Model C in Table 5).
4. A mixture of an unknown number of compounds. The use of real sample is recommended (Model D in Table 5).

4. Degree of Instrumentation

Unlike HPLC, TLC is an off-line technique: Sample application, chromatographic development, and detection are separated in time and space. The validation procedure and the significance of the various analytical performance parameters during validation research vary with the degree of instrumentation (see data in Table 5).

Table 5 Analytical Performance Parameters Used for Validation of HPLC and of Noninstrumentalized and Instrumentalized TLC Methods

Parameter	HPLC	TLC							
		No	A	D	A+B	A+R	D+R	A+R+D	
Precision									
Of method	++	—	—	++ ++	++	—	+++	++	
Of system	+	—	—	++ +	+	—	++	+	
Accuracy	++	—	—	++ +	++	—	++	++	
Reproducibility									
R, R_f	+	++ ++	+++	++ +	+++	++	++	+	
Within day	+	—	—	++ +	++	—	++	+	
Day to day	+	—	—	++ ++	+++	—	++	+	
Selectivity	++	+++ +	++++	++ +	+++	++	++	++	
LLD	++	+++	+++	++	++	++	++	+	
LDQ	++	+++ +	++++	++	++	+++	++	—	
LR	+	—	—	++ ++	+++	—	+++	++	
SL	+++	+++	++	++ +	++	++	++	+	
ST	+	++ ++	+++	++ ++	+++	++	+++	++	
Ruggedness	++++	+	+	++	+++	+	+++	++++	
			HPLC		TLC				
Analytical task			Primary parameters		Primary parameters I		Primary parameters II	Model mixtures	
			Analysis of starting raw materials						

Plant extracts	R_s , RE, LDQ	RE, LLD	R_s , RE, LDQ	Model D
Extracts of animal organs	R_s , RE, LDQ	RE, LLD	R_s , RE, LDQ	Model D
Fermentation mixture	R_s , RE, LDQ	RE, LLD	R_s , RE, LDQ	Model D
Analysis of intermediates				
Intermediates and crude products	R_s , P-S	R_s , LLD	R_s , P-S, LR	Model A
Reaction mixtures	R_s , P-M	R_s , LLD	R_s , P-M, RU	Model A
Mother liquors and secondary products	R_s	R_s , LLD	R_s , LR, RU	Model A
Analysis of pharmaceutical raw materials				
Identification	k	R_f	R_f	Model A
Purity testing	LDQ, RU, P-S	R_f , R_s , LLD	R_s , LR, P-S, RU	Model A
Assay	P-S, RU	—	RU, P-S, LR	Model A
Separation of closely related compounds	R_s , RU	R_f , R_s , LLD	R_s , RU, LR	Model A
Stability testing	RU, LDQ, R_s , P-S	R_s , LLD	RU, LR, R_s , P-S	Model A
Analysis of formulated products				
Identification	k	R_f	R_f	Model A
Purity testing	P-M, RU, RE	R_s , LLD	R_s , RU, LR, RE	Model B
Assay	P-M, RE, RU	—	P-M, RE, RU	Model B
Stability testing	R_s , RU, RE, LDQ	R_s , LLD	RU, R_s , LR, RE	Model B
Content uniformity test	P-M, RE, RU	—	RU, RE, P-M	Model A
Dissolution test	P-S, RU	—	P-S, LR, RU	Model A
HPLC		TLC		
Analytical task	Primary parameters	Primary parameters I	Primary parameters II	Model mixtures
Analysis of drugs and their metabolites in biological media				
Pharmacological and toxicological studies	RU, RE, LDQ	—	RU, LR, P-M	Model C
Pharmacokinetic study	RU, RE, LDQ	—	RU, LR, P-M	Model C

Metabolic study	R_s , LDQ, RE	R_s , LLD	R_s , LDQ, RU	Model D
Bioequivalence study	P-M, RE, RU	—	P-M, LR, RU	Model C
Compliance and pharmacodynamic studies	P-M, RE, RU	—	—	—

Abbreviations: A: automated sample application, D: in situ instrumentalized detection, R: automated chamber system, “—”: not applicable performance parameter, “+”: applicable performance parameter, number indicates the degree of importance or of difficulties.

LDQ, lowest limit of quantification; LLD, lowest limit of detection; LR, linearity and range; SL, sample loadability; ST, sample stability; k , capacity factor; P-M, method precision; P-S, system precision; RE, recovery; R_f , retention factor; R_s , resolution; RU, ruggedness.

Primary parameters I: conventional TLC system without any instrumentation. Primary parameters II: TLC system equipped at least with a densitometer.

Model mixtures A–D: see text.

5. Stability Problems

During the TLC process, several sources of error may originate from insufficient stability of the sample (sample components) and/or incomplete testing of the ruggedness of sample preparation, sample application, chromatography, or detection. The most important stability problems may appear in the following areas.

a. Handling of Samples (Stability of the Sample Prior to the Sample Preparation Procedure, ST-1). To minimize prelaboratory errors arising from inappropriate handling, when a laboratory performs an assay on samples derived mostly from biological media, medicinal plants, or animal organs, it should issue instructions for the collection, storage, and transport of the samples. One immediate problem that may lead to false results concerns the stability of compounds (drugs, metabolites and glycosides) in the sample. Instability may be associated with the presence of enzymes in the samples that can cause partial decomposition or transformation of sample components. The most useful procedure for ensuring the stability of sample components during storage is to freeze samples immediately after collection. Although several problems associated with frozen samples have been reported, freezing is useful for preventing alterations in concentration and composition of sensitive samples (i.e., those originating from biological fluids, medicinal plants, animal organs, and fermentation mixtures). For clarification, stability problems occurring during the storage of the sample are listed in Table 6.

b. Stability During Sample Preparation (ST-2). Besides establishing optimum conditions for extraction and/or partial purification of the samples, in order to exclude systematic and/or nonsystematic errors from these procedures it is necessary to consider two additional factors that occur as a result of the incomplete ruggedness of sample preparation and that have a strong influence on the validity of the assay results. The first relates to the chemical properties and purity of the extraction solvents: Chemical reaction of the solvents and their impurities can result in undesired by-products (artifacts) that produce one or more extra peaks on the chromatogram and

Table 6 Stability Problems and Control Methods

Description of the problem	Sample experiments	Evaluation
Sample storage (ST-1)		
Analyte concentration may decrease due to environmental conditions (temperature, light, moisture, etc.) or enzymatic degradation resulting in decreasing assay results.	Sample: Model C or D stored at -20°C , $+4^{\circ}\text{C}$, and room temperature in dark in tight containers. Analytical method: same as described in the analytical report.	Reanalyzing the same sample at zero time and after 1, 2, 4 weeks storage time and comparing the chromatograms for assay and extra peak(s)
Artifact formation during sample preparation (ST-2)		
Extra peaks(s) on the chromatogram, lower assay results	Sample: (a) Model A dissolved in methanol or chloroform; (b) Model B or C treated as described under sample preparation; (c) Model D (real sample), if necessary Analytical method: as described in the analytical report including sample preparation for cases (b) and (c)	Evaluating the chromatograms for assay and extra peak(s) obtained by using the prescribed analytical method, but for Model A no extra sample preparation step is included. Comparing the chromatogram of Model A with the one obtained for Model B (or C), and D (if necessary); further necessary experiments to clarify the problematic step(s) in sample preparation procedure must be decided.
Stability in sample solution (ST-3)		
Extra peak(s), decreasing assay results, and/or peak distortion or peak(s) disappearance on the chromatograms recorded after different time intervals of sample solution storage	Sample: (a) Model A dissolved in methanol or chloroform freshly prepared in each analysis time; (b) Model A, B, or C treated as described under sample preparation. Chromatography: same as described in the analytical report. Samples are applied in triplicate each time.	Evaluating the chromatograms at 0 time and after 1, 2, 3, 4, 5, and 6 h storage of sample solution stored at room temperature, and at $+4^{\circ}\text{C}$ if necessary, for assay and peak presence or disappearance.
Stability during spotting (ST-4)		
In the course of 2-D chromatography, one or more extra peak appear on the chromatogram in the first direction without additional tailing, and no further decomposition [new or the same extra peak(s)] is apparent on the chromatogram obtained in the second	Sample: Model A, dissolved in the same solvent as used for sample preparation, and Model B or C treated as described under sample preparation, if necessary.	(a) When the chromatograms obtained in both directions of flow are evaluated during sample application, no additional decomposition occurs if no extra peak(s) appear on any chromatogram.

direction developed with the same mobile phase.	Chromatography: Two-dimensional chromatography using the same eluent for both directions of flow.	(b) Additional decomposition during sample application occurs, if
Description of the problem	Sample experiments	Evaluation
Stability during spotting (ST-4)		
	Spotting conditions: (i) ST-4A: Spot and streak application of 2, 5, 10, 20, 50, and 100 μL sample solutions (each application with same amount of sample) (ii) ST-4B: 5 μL samples are spotted in triplicate and dried using cool and hot air without and with light protection (iii) ST-4C: 5 μL sample are spotted in triplicate and dried immediately. After 5 min new spottings are made under similar conditions. This procedure is repeated up to 30–60 min using 5 min time intervals between spotting periods.	(i) Extra peak(s) appear(s) on the chromatogram obtained in the first direction of flow but only peak A appears on the chromatogram obtained in the second direction of flow. (ii) Asymmetry factor for peak(s) D (decomposition product) obtained in first direction of flow is practically the same as obtained for peak D in the second direction of flow. $A_{\text{sfD1}} - A_{\text{sfD2}}$; see text for abbreviations.
Stability during chromatographic development (ST-5)		
In the course of two-dimensional chromatography, extra peak(s) appears on the chromatogram in both first and second directions of eluent flow with additional tailing.	Sample: Model A dissolved in the same solvent as used for sample preparation, and Model B or C treated as described under sample preparation, if necessary. Chromatography: One- or two-dimensional chromatography using the same eluent in both directions of flow. (a) Temperature and light effects. Develop the chromatogram in one dimension (i) at room temperature in reflected light; (ii) at room temperature in dark under cooling in reflected light and under cooling in dark. (b) Oxidative and hydrolytic decomposition, isomerization, and other decomposition reactions during the run. Two-dimensional chromatography using the same eluent in both directions. Only a	Evaluating the resulting chromatograms for assay and extra peak(s). (a) Decomposition occurs only during the run, if $A_{\text{sfD1}} > A_{\text{sfA1}}$ $A_{\text{sfD1}} - A_{\text{sfD2}}$ $A_{\text{sfD1}} > A_{\text{sfD2}} \times 2$ $PA_{\text{D1}} - PA_{\text{D2}}$ (b) Decomposition occurs during the spotting, spot drying and run, as well, if: $A_{\text{sfA2}} < A_{\text{sfD1}} < A_{\text{sfD2}}$ $A_{\text{sfD1}} > A_{\text{sfD2}}$ $PA_{\text{D1}} > PA_{\text{D2}}$ For abbreviations see the text.

gentle drying is performed after development in the first direction at room temperature.

Description of the problem	Sample experiments	Evaluation
Spot stability after chromatographic development (ST-6)		
Insufficient assay accuracy and precision results	Sample: Model A chromatography: as described in the analytical report recording the chromatogram immediately after running and 5, 10, 15, 20, and 30 min elapsed time. Five parallel runs constructing calibration curve are evaluated.	Peak area or peak height vs. recording time plot is constructed. Maximum acceptable time elapsed between running and recording is determined.

lead to false assay results. The second factor is concerned with the pH of the aqueous phase to be extracted. Several side reactions (hydrolysis, oxidation and isomerization) are catalyzed by the presence in the aqueous phase of hydrogen or hydroxide ions, which may be concentrated during the sample preparation, again leading to undesired artifact formation. Artifacts may also be formed when the aqueous or organic phase is evaporated to dryness. The procedure to be followed to clarify any possibility of artifact formation is given in Table 6.

c. Stability in Sample Solution (ST-3). Many solutes (analytes) readily decompose in sample solution prepared prior to TLC investigation. The stability of samples in a final sample solution may be determined from the “system stability” defined as a measure of bias in the assay results within a preselected time interval (in TLC, every hour up to 4–6 h) determined by replicate analysis of the same ready-made sample solution, each time using freshly prepared standard solution for comparison. The results are evaluated for major and/or minor components. System stability is considered appropriate if the relative standard deviation calculated for the assay results obtained after different time periods does not exceed more than twice the corresponding value of system precision. If the RSD value is higher, when the assay results are plotted as a function of time the maximum duration of the usability of a sample solution can be calculated. This requirement can be extended with two additional criteria: (a) The chromatogram of the final application should not contain any spot absent from the initial chromatogram and (b) any spot present in the initial chromatogram should be present in the final chromatogram. The recommended experiments are listed in Table 6.

d. Spot Stability (Stability of Sample Components After Spotting and Prior to Chromatography) (ST-4). A further possible source of error is the decomposition that can occur on the active sites of the stationary phase surface prior to chromatography. This stability term involves three considerations—(a) the quality of sample application (spotting technique and size and shape of the spots), (b) drying conditions (i.e., temperature of the air stream and chemical effect of light and air), and (c) the time the sample is allowed to stand between spotting and chromatography—all of which have a significant influence on the stability of the spots to be separated.

Spot stability should be investigated according to the procedure mentioned above. The effect of spot shape and size on spot stability is examined by applying a greater volume

of sample as a band or spots and comparing the resulting chromatograms. The experiments are followed by investigation of temperature variance and the effect of light during the drying process, using the spotting technique found to be optimum in previous experiments. Finally, the effect on spot stability of the amount of time elapsing between spotting and chromatography is clarified by applying the same volume of sample solution to the same plate as spots (or bands) of the same size and using the same drying conditions but with a preselected time period between consecutive applications of sample (details are given in Table 6). As discussed in the next subsection, by using two-dimensional chromatography with the same mobile phase it is relatively easy to distinguish between decomposition occurring during spotting and that occurring during chromatography.

e. Stability During Chromatography of the Compounds Being Separated (ST-5). Many compounds are very sensitive to the conditions used for chromatogram development. The most important factors contributing to oxidative or hydrolytic decomposition of the compounds during chromatography are (a) dissolved and/or atmospheric oxygen, (b) light and temperature effects, and (c) inadequate eluent composition. These effects, leading to apparent instability of the compounds, are typical methodological problems, although the third also indicates inadequate optimization of the TLC method.

To clarify the detrimental effects of temperature and exposure to light is relatively easy. The chromatograms are developed at room temperature in reflected light, at room temperature protected from light, in a cool place in reflected light, and in a cool place protected from light. Investigation of other factors such as the presence of dissolved and atmospheric oxygen and inadequate eluent composition is a more complicated task based on two principles: (a) How can the decomposition be minimized or excluded? and (b) How can decomposition during chromatography be differentiated from that arising from sample application?

Undesired oxidative decomposition can be reduced by use of a protective gas (nitrogen, carbon dioxide, or argon), both in the chamber and to remove the oxygen dissolved in the eluent. Use of a stabilizer in the eluent is also an effective way of protecting sample components from oxidative degradation. BP93 (174), and Ph. Eur. 2 (175) recommend the use of BHT (2,6-di-*tert*-butyl-4-aminophenol) as antioxidant in the eluent to protect ergocalciferol from oxidative decomposition during chromatography. A combination of the two procedures has been used by Szepesi et al. (176) with carbon dioxide as a protective gas in the chamber and BHT dissolved in the eluent as stabilizer for purity testing of sulfinpyrazone raw material.

Evidence of analyte decomposition during spotting and chromatography can be confirmed by two-dimensional chromatography using the same eluent in both directions. If decomposition takes place only during the spotting and spot drying procedures, then the chromatogram obtained in the second direction of eluent flow will be free from any impurity peak (single peak chromatogram). ($A_{sfD1} = A_{sf} * D2$; only peak D appears on the chromatogram, where A_{sf} is peak asymmetry, A and D refer to the peaks of decomposition product and analyte, respectively; 1 and 2 indicate the direction of flow, and the asterisk marks the chromatogram of the decomposition product separated in the first direction and measured in the second direction of flow.)

If decomposition occurs only during the run, the peak(s) of the decomposition product(s) formed can be observed on the chromatograms obtained for the analyte in both

the first and second directions of eluent flow. The shape of the peak of the decomposition product shows significant tailing compared with that of the analyte. The peak areas of the decomposition product observed in both directions are similar to each other. ($A_{sfD1} > A_{sfA1}$, $A_{sfD1} = A_{sfD2}$, $A_{sfD1} > A_{sfD2}$; $PA_{D1} = PA_{D2}$, where PA_{D1} and PA_{D2} refer to the areas of the peak of the decomposition product on the analyte chromatograms in the first and second directions of flow, respectively. If decomposition occurs during both spotting and chromatography, the situation is similar to that described above, but the peak of the decomposition product in the first direction exhibits less tailing than that obtained in the second direction, and the area of the peak obtained in the first direction of eluent flow is significantly higher than that obtained in the second direction ($A_{sfA2} < A_{sfD1} < A_{sfD2}$, $A_{sfD1} > A_{sfD2}$, $PA_{D1} > PA_{D2}$). This principle is also summarized in Table 6.

f. Spot Stability After Chromatographic Development (ST-6). There are two major sources of error originating from insufficient stability of the separated spots prior to densitometric evaluation. The first relates to the drying process after chromatogram development, and the second to the stability over a period of time of the derivatives formed in situ on the plate. The sensitivity of the separated compounds to heat can be controlled by systematically changing the drying conditions (temperature and time) before taking densitometric measurements of the spot intensities. The stability of the derivatives formed after elution must also be investigated as a function of time and concentration. With regard to quantification, the spot stability is sufficient if (a) the spot signal is constant (the derivative is stable) for at least 30 min or (b) a stable signal is available after a certain period of time within a suitable concentration range (the unstable derivative transforms into a stable one on the plate within a short period of time).

B. Validation Data Elements Specific to TLC

Table 4 summarizes the most important data used for validation of TLC experiments. The use of most of the parameters is similar to that in HPLC, and it is not necessary to redefine them.

Table 7 summarizes recommended procedures for the determination of various validation data specific to planar chromatography. Errors originating from sample preparation, sample application, and calibration are calculated, keeping in mind the most important factors that are influenced by chromatographic separation, detection, and quantification.

Another part of the validation process relates to chromatographic separation, including exact proof of separation selectivity and peak purity. If pure standards of each analyte are available, this information—determination of separation selectivity (specificity) and of the purity of each chromatographic peak—may be obtained by chromatographing each standard separately under the experimental conditions. (This process has several disadvantages that are not discussed here.) Assessment of peak purity is better performed by subjecting samples to appropriate stress conditions in order to produce decomposition products: Peak overlapping may then be more easily recognized. The chromatograms of treated and untreated samples, either unspiked or spiked with known concentrations of impurity standards, are recorded at two or three preselected wavelengths. Any change in peak shape, resolution, or peak asymmetry (determined by comparing the chromatograms obtained from treated and untreated samples) can indicate nonhomogeneity of a

chromatographic peak. The recommended procedure is summarized in Table 7 under "Peak purity." Peak homogeneity can also be investigated by two-dimensional chromatography, using a different mobile phase in the second direction than in the first direction of eluent flow. After trying various eluent compositions during the second run, when single-peak chromatograms are obtained, the peak can be considered a pure one.

C. Ruggedness Test

One of the most important experiments performed during the validation of an analytical method is testing of its ruggedness, defined as a measure of the reproducibility of the individual test results when the procedure is used repeatedly to analyze the same homogeneous sample under a variety of prespecified experimental conditions. This general term is valid for each analytical method, including every chromatographic and nonchromatographic technique. Although ruggedness testing, as a specified criterion of method validation experiments, is extensively used in this practice, the literature contains few data about how the experiments should be done and evaluated.

In general, for ruggedness testing of any TLC method the following experiments should be included:

1. Sample preparation should be checked for ruggedness by varying the shaking time, pH, temperature, composition of extraction solvents, phase ratio, and number of extractions.
2. Sample application should be checked for ruggedness by investigating the effect of spot shape and size and spot stability.
3. Separations should be performed on at least three plates (obtained from the same and/or different sources).
4. The separation is checked for ruggedness by varying the separation conditions (chamber system, eluent composition, temperature, and development distance).
5. The spot visualization technique (postchromatographic derivatization) is checked for ruggedness by changing the experimental conditions: spraying or dipping conditions, temperature, and reaction time.
6. The quantitative evaluation is checked for ruggedness by varying the conditions used for drying the plates and the detection wavelength.

Table 7 Validation Experiments Specific to Planar Chromatography

Validation data elements	Validation step	Sample	Experiments
I. Precision			
Method precision	Sample preparation Sample application Chromatographic run Detection-quantification	Model A, B, C, or D depending on the analytical aim	Five simultaneously prepared stock solution each with in-dividual weighings, one application from each solution onto the same plate. Experiments are performed on three

Precision of sam-ple application	Sample application	Model A-1: single stock solution prepared from standards Model A-2: standard solutions containing five different concentrations of the an-alytes in 2:4:10:14:16 concentration ra-tio, where 10 is equivalent to Model A-1	plates obtained from the same source, with same development, detection, and evaluation conditions.
			A-11: Five applications 50 μ L each from Model A-1 in spot form A-12: Five applications 50 μ L each from Model A-1 in streak form A-21: Five applications 10, 20, 50, 70, 80 μ L, each containing same concentrations of analytes as Model A-2 in spot form A-22: Five applications 10, 20, 50, 70, 80 μ L, each containing same concentrations of analytes as Model A-2 in streak form
II. Accuracy			
Recovery	Sample preparation	Model A and Model B or C	Sample stock solutions are prepared from Model B, C, or D in 10, 50, 75, 100, 125% concentrations of the claimed value using the sample preparation procedure as described. Each concentration of the stock solutions is prepared in parallel three times. Each individual sample stock solution is applied in triplicate. Simultaneously the standard solutions in the same concentrations and parallels are prepared from Model A and applied as described above. Sample (spiked, placebo, or blank) and standard solution (1:1) is spotted in triplicate onto the same plate using data pair technique [12 spots (3 $\times$ 3 from spiked standard and 1 $\times$ 3 from standard solutions) on one plate]. Chromatography, detection, and evaluation are made according to the description of the standard method.
Microsyringe	Sample application	Model A	Weight control of the injected amounts
Validation data elements	Validation step	Sample	Experiments
III. Reproducibility			
Within-day	Entire method	Model A and B, C, or D denending on the analytical	As described under I, the test is nerformed three times in

		task	same day.
Day-to-day	Entire method	Model A and B, C, or D depending on the analytical task	As described under I, the test is performed on three different days.

IV. Selectivity—Specificity—Peak purity

Selectivity—specificity	Chromatographic run Detection	Model A and B or C depending on the analytical task	Determination of $R_{s(min)}$, R_{sa} (Ref. 4), R_{sb} (Ref. 4) from the chromatograms
Peak purity	Chromatographic run Detection	Sample A: Model A prepared from standard(s) subjected to heat, light, and moisture treatment Storage conditions: +4°C, 40°C (1 week), 100°C (1 day), 40°C 80% RH (1 week), reflected light (1 week), UV, 254 nm (10 h), in 1% aqueous ethanolic solution at 100°C, pH=2/7/12 (3 h)	Appearance of new peaks, determination of $R_{s(min)}$, R_{sa} (Ref. 4), and R_{sb} (Ref. 4) from the chromatogram, dependence of R_s and A_{sf} on detection wavelength

V. Detectability

Detection limit	Detectability	Further dilution of lowest concentration of stock solutions prepared from Model A and Model B or C as described under II, Recovery	Lowest concentration of the analyte that can be clearly identified on the chromatogram at the preselected wavelength (3 times noise).
Quantification limit	Detectability, quantification	Same as above	Lowest concentration of the analyte that can be determined with the required precision (RSD: $\pm 25\%$, 10 times noise).
Linearity and range	Entire method	Combination of experiments described under II, Recovery. Same sample solutions are prepared as described under II, Recovery.	Calibration curve is produced by plotting concentration vs. peak height or peak area. Each point is determined from triplicate application of the same concentration of the analyte(s).

VI. Ruggedness Testing (see Tables 9–12)

7. The complete analytical method is performed by three different analysts, satisfactorily trained in TLC, who analyze the same sample under the same experimental conditions to determine any problems with the reproducibility of a TLC method.

The results of evaluation are reported as a percentage relative standard deviation (RSD), and a TLC method is judged to be rugged when, despite normal variation of the

parameters listed above, the system still yields acceptable RSD results in all variations and passes the system suitability test (see next subsection). Any parameter influencing the results unfavorably, i.e., a parameter to which the TLC method is sensitive, must be reported, and the degree of control must be specified.

1. Starting the Ruggedness Test

When a new TLC method is developed and validated, it is important to be aware of its sensitivity to normal variations in specific conditions. Each ruggedness testing experiment can be assigned to one of two major categories: (a) experiments that test the effects on the assay results of changes in the environmental conditions (Type A investigations) and (b) experiments that test the effects on the assay results of changes in the experimental conditions (Type B investigations).

a. Type A Investigations. Tests are based on variation of environmental conditions while on each occasion the TLC method is performed as specified in the method description. The following tests are involved in this study:

1. Analyses are performed in different laboratories. Interlaboratory examination of the reproducibility of a TLC method would be ideal for ruggedness testing. However, it is time-consuming and difficult to organize. The main aim of ruggedness testing is to exclude these difficulties and simulate the variances in the experimental conditions that occur when a TLC method is transferred from one laboratory to another.
2. Tests are performed in the same laboratory by different analysts under different instrumental conditions.

Table 8 lists the considerations important to Type A investigations.

b. Type B Investigations. Tests are based on different experimental conditions and are performed by the same analyst using the same instrument(s). The experimental conditions are varied within a range that may occur in normal practice.

For adequate performance of ruggedness testing, prior to the start of any investigation of ruggedness the following decisions must be made:

1. The factors (experimental variables) that are expected to have a significant influence on the assay results, i.e., plate type, temperature, and mobile phase composition, should be selected.
2. The maximum acceptable deviations from the specified experimental conditions should be defined [-1 (lowest value), 0 (nominal value), +1 (highest acceptable value)].
3. The analytical performance parameters (precision, R_f value, R_s , etc.) considered to be sensitive to changes in the experimental variables should be selected.
4. Acceptable deviation(s) from the value of the prespecified analytical performance parameter(s) can be defined, including maximum and/or minimum value.

Type B investigations can be divided into four major subgroups: (a) testing the ruggedness of sample preparation (Type B-1), (b) testing the ruggedness of sample preparation (Type B-2), (c) testing the ruggedness of parameters related to chromatographic separation (Type B-3), and (d) testing the ruggedness of spot visualization, detection, and quantification (Type B-4).

Testing of sample preparation for ruggedness. Four major techniques can be used for sample preparation: liquid-liquid extraction, solid-phase extraction, direct sample application including simple dissolution of the sample, and prechromatographic derivatization. Because there is no great difference between Type B-1 ruggedness testing for HPLC and for TLC, a summary of the experiments only (recommended performance parameters, variables, and limits) is given in Table 9.

Table 8 Type A Investigations

Environmental conditions	Experiments	Analytical performance parameters	Calculated data
Analyst-to-analyst variation	At least three analysts perform the full test from sample preparation to evaluation and quantification	Method precision System precision Reproducibility: within-day, day-to-day, each quarter Accuracy $R_{s(\min)}$ Linearity and range	RSD variation: for each analyst for three analysts
Sample application, manual or automated	One analyst performs the full test using manual and automated sample applications from the same sample solution with five parallel runs on the same plate.	System precision Resolution $R_{s(\min)}$	RSD variation
Densitometry (if more than one densitometer is available)	One analyst performs the full test in triplicate using two or more densitometers for evaluation and the same instrument settings.	Method precision Resolution $R_{s(\min)}$ Peak asymmetry Lowest detectable quantity	RSD variation Difference Difference RSD variation

Method: As specified in the Analytical Prescription applied. Sample: Real sample.

Testing of sample application for ruggedness (Type B-2). Successful quantitative TLC is strongly dependent on the quality of sample application. Reproducibility of sample amount and spot size is quite important, but to achieve good chromatographic resolution and sensitivity of detection, the shape of the spots of the applied sample is also of great importance. Accordingly, the method is tested for ruggedness to variations in:

- Spot shape and size (streak-type and spot-type application, ST-L1A stability problem; details given in Table 6)
- Sample volume
- Sample concentration
- Drying conditions (ST-4B stability problem; details given in Table 6)
- Standing time prior to run (ST-4C stability problem; details given in Table 6).

Recommended experiments are summarized in Table 10.

Testing the chromatographic separation for ruggedness (Type B-3). The recommended experiments are listed in Table 11. It is apparent from Table 11, that there are three major aspects to testing the ruggedness of the chromatographic separation: plate-to-plate variation, variations in the chamber systems and in the environmental conditions during the chromatography, and experimental variables.

Plate-to-Plate Variation. The complete ruggedness test includes investigation of the separation characteristics:

On at least three identically labeled plates of the same particle size and dimensions supplied by the same manufacturer

On at least two additional similarly labeled plates of the same particle size and dimensions supplied by different manufacturers

Table 9 Ruggedness Testing Plan for Sample Preparation (Type B1)

Variable	Liquid-liquid extraction limits	Solid-phase extraction limits	Direct sample application limits	Precolumn derivatization limits
Dissolution				
Solvent volume	±5% (rel)	±5% (rel)	±5% (rel)	±5% (rel)
pH/IF aqueous	±0.5 pH U	±0.5 pH U	±0.5 pH U	±0.5 pH
Temperature	±5°C	±5°C	±5°C	±5°C
Sample cleanup and enrichment				
Phase ratio	±20% (rel)			
Number of extractions	1 or 2			
Cartridge columns from the same source		Up to 3		
Cartridge columns from different sources		Up to 3		
Plates with concentrating zone from the same source			Up to 3	
Plates with concentrating zone from different sources			Up to 3	
Sample concentration (same volume)	−80% to +50%	−80% to +50%	−80% to +50%	
Sample volume (same concentration)	±80% (rel)	±80% (rel)	±80% (rel)	
Evaporation to dryness				

Temperature	±5°C	±5°C		±5°C
Time	±20% (rel)	±20% (rel)		±20% (rel)
Redissolution				
Temperature	±5°C	±5°C		±5°C
Solvent volume	±20% (rel)	±20% (rel)		±20% (rel)
Variable	Liquid-liquid extraction limits	Solid-phase extraction limits	Direct sample application limits	Precolumn derivatization limits
Washing/elution-preelution steps				
Temperature		±5°C		
Solvent volume		±20% (rel)	±20% (rel)	
Solvent composition		±10%	±20% (rel)	
Solvent pH		±1 pHU		
Flow rate		±20% (rel)		
Reaction conditions				
Reagent concentration				±30 (rel)
Reaction time				±50% (rel)
Solvent volume				±20% (rel)
pH/IF aqueous				±0.5 pH U
Temperature				±5°C
Excess of reagent				±20% (rel)
Elapsed time after completion of the reaction				1 to 5 hours
Sample and reagent stability				
ST-3 time	±50% (rel) if stated, zero time, 30 and 60 min, if not stated	±50% (rel) if stated, zero time, 30 and 60 min, if not stated	±50% (rel) if stated, zero time, 30 and 60 min, if not stated	±50% (rel) if stated, zero time, 30 and 60 min, if not stated
ST-3 temperature	±5°C	±5°C	±5°C	±5°C
ST-7		±50%, if stated, zero time, 15 and 30 min if		

	not stated	
ST-8 temperature		±5°C
ST-8 time		±50%, if stated, zero time 2 and 4 hours, if not stated
ST-8 pH (if aqueous)		±0.5 pH U

Analytical performance parameters

Method precision	Method precision	Method precision	Method precision
Recovery	Recovery	Recovery	Recovery
Quantification limit	Recovery	Lowest detectable quantity	Lowest detectable quantity
Sample stability	Lowest detectable quantity	Sample stability	Sample stability
	Sample stability		

Samples used for the experiments, Model A, B, or C

Sample stability	Experiments
ST-3: Stability in sample solution	
ST-3	
Temperature	
If the temperature is stated	Five parallel runs from sample solution stored at three different temperatures (stated, -5°C, +5°C)
If the temperature is not stated	Five parallel runs from sample solution stored at three different temperatures (20°C, +25°C, +30°C)
ST-3 time	
If the time is stated	Five parallel runs from sample solution allowed to stand at prescribed temperature for three different time intervals (stated, -50%, +50%)
If the time is not stated	Five parallel runs from sample solution allowed to stand at prescribed temperature for three different time intervals (zero time, 30, and 60 min) The method is performed according to the prescribed methodology
ST-7: Stability of the sample on cartridge column	Sample is transferred to the cartridge columns in triplicate, washed with the solvent indicated in the Method File, and allowed to stand on the columns for zero time, 15, and 30 min. The method is continued as described.
ST-8: Reagent stability in solution	Reagent solution is prepared as described and stored at three different temperatures (stated, -5°C, +5°C). Reaction conditions, sample preparation, chromatography, evaluation as described,
ST-8	
Temperature	
if stated	

if not stated	Reagent solution is prepared as described and stored at three different temperatures (stated, -5°C , $+5^{\circ}\text{C}$). Reagent solution is prepared as described and stored at three different temperatures ($+20^{\circ}\text{C}$, $+25^{\circ}\text{C}$, $+30^{\circ}\text{C}$). Reaction conditions, sample preparation, chromatography, evaluation as described.
ST-8 time if stated	Reaction conditions, sample preparation, chromatography, evaluation as described. Reagent solution is prepared as described and stored at the temperature prescribed. Reagent solution is used after allowing to stand for three different time intervals (stated, -50% , $+50\%$).
If not stated	Reaction conditions, sample preparation, chromatography, evaluation as described. Reaction conditions, sample preparation, chromatography, evaluation as described. Reagent solution is prepared as described and stored at the temperature prescribed. Reagent solution is used after allowing to stand for three different time intervals (zero time, 2, and 4 hours). Reaction conditions, sample preparation, chromatography, evaluation as described.
ST-8 pH	Reagent solution is prepared at three different pH (stated, -0.5 pH U, $+0.5$ pH U). Other conditions are the same as described. The method is performed according to the prescribed methodology.

Table 10 Checking Sample Application for Ruggedness (Type B-2 Investigations)

Variables	Limits	Experiments	Analytical performance parameter	Calculated data
Application in streaks	$\pm 5\%$ SLL	Three parallel applications on the same plate, changing length of line application and measuring length of line parallel to the mobile phase front (SLL) and width of line in separation distance (SLW) prior to run	Precision	RSD variation for A ($A = \text{SLL}/\text{SLW}$)
	$\pm 5\%$ SLL	The same measurements as mentioned above after running, calculated for least absorbing sample component	Precision resolution	RSD variation for B ($B = \text{SLL}^*/\text{SLW}^*$) and for $R_{s(\min)}$
Application in spots	$\pm 5\%$ SDL	Three parallel applications on the same plate, changing diameter of spots and measuring length of spot parallel to the mobile phase front (SDL) and width of spot in separation distance (SDW) prior to run	Precision	RSD variation for A ($A = \text{SDL}/\text{SDW}$)
	$\pm 5\%$ SDL	Same measurements as mentioned above after	Precision resolution	RSD variation for B ($B = \text{SDL}^*/\text{SDW}^*$)

		running, calculating the least absorbing sample component		and for $R_{s(\min)}$
Sample volume	$\pm 20\%$ (rel)	Different volumes are applied containing the same sample amount	Precision $R_{s(\min)}$, R_f values	RSD variations
Sample concentration	-80% to $+150\%$ (rel)	Same volumes are applied containing different sample amounts	Precision $R_{s(\min)}$, R_f values	RSD variations
Stability problems during sample applications (ST-4)				
Drying conditions Temperature	$\pm 5^\circ\text{C}$ (rel)	The temperature of air stream is changed. 3×3 parallel runs on the same plate. Drying time is constant.	Precision $R_{s(\min)}$	RSD variations
Time	± 10 min (rel)	Drying time is changed. 3×3 parallel runs on the same plate. Drying temperature is constant.	Precision $R_{s(\min)}$	RSD variations
Standing time on the plate prior to run after drying	$\pm 20\%$ (rel)	Three parallels are spotted and dried, next three are spotted and dried, followed by last three applications. The time intervals between two spottings are increased by 20% of the previous one.	Precision (peak area or peak height), $R_{s(\min)}$, A_{sf}	RSD variations

On one identically labeled plate of different particle size and/or dimensions obtained from the same manufacturer

On one identically labeled plate of the same particle size and dimensions with different support material (aluminum sheet vs. glass plate) and/or binder

Variation in Chamber Systems and in Environmental Conditions. One of the most important aspects of ruggedness testing is to determine the effects of variations in chamber systems and environmental conditions on the analytical performance parameters. The quality of the chromatograms obtained is influenced by the running conditions (type, shape, and size of chambers, presaturation conditions, temperature and humidity).

Experimental Variables. The mobile-phase composition (percent organic solvent in the eluent and eluent pH) is the most frequently used variable during ruggedness testing of a chromatographic method. Because in TLC the stationary and mobile phases are not in equilibrium prior to chromatography, an additional factor, possible pretreatment of the plate, may also have an important effect on the quality of the separation. Investigation of this variable should also be included in ruggedness testing.

Testing the visualization technique and in situ detection for ruggedness (Type B-4). In situ quantification of the separated compounds on a chromatoplate has several advantages and limitations. Two circumstances can be strictly distinguished:

Direct measurements, when the evaluation is based on the native UV absorbance or fluorescence emission of the separated compounds

Indirect measurements, when the separated compounds are converted into derivatives by introduction of chromophores or fluorophores into the molecules

Direct Methods for In Situ Quantification. Amounts of sample corresponding to the detection limit (DL) and quantification limit (QL) are the most important factors that can be tested with respect to ruggedness. Special attention is paid to determination of the signal-to-noise ratio, which depends partly on the plate structure (plate-to-plate variation) and partly on the inhomogeneity within one plate, i.e., variations in the layer thickness across the plate, variable packing density and microscopic changes in particle size and distribution (changes in precision and resolution), as well as on instrumental components (optical and electronic noise). The latter effect is examined under the term “instrumental conditions” in Table 12.

Indirect Method for In Situ Quantification. Because the derivatization procedure is usually an important part of quantitative or semiquantitative TLC and may exert a significant influence on the accuracy and precision of the entire method, the ruggedness testing of the derivatization reaction is important. Ruggedness testing of postchromatographic derivatization includes the following steps:

Investigation of conditions used for drying the plates after chromatography but prior to derivatization

Determination of the influence of spraying or dipping conditions on the accuracy and precision of the determination

Evaluation of the stability of the derivatives formed on the plate

The most important variables and the testing procedures recommended for use are listed in Table 12.

Planning and evaluation of ruggedness testing. The most important experiments required to test the ruggedness of TLC methods are listed in Table 12. To perform these experiments the basic problems to be considered are Should all the experiments be performed or only a selected part? and Having acquired a set of experimental results, how should the ruggedness of the entire procedure be evaluated?

Planning Ruggedness Testing. It should be noted that not all of the experiments can be performed in each circumstance. It is recommended that only those critical variables that can

Table 11 Checking Chromatographic Separation for Ruggedness (Type B-3 Investigations)

Plate-to-plate variation	Chamber system environmental conditions, variable limits	Experimental variables, variable limits
Plates from the same source: The same experiments are performed on three plates supplied by the same manufacturer. Analytical performance parameters: R_f , R_s , precision, lowest detectable quantity.	Conventional chamber: The same experiments are performed on plates with same particle size and dimension changing the environmental conditions. Analytical performance parameters: R_f , R_s , precision, quantification limit	Mobile phase composition: The same experiments are performed on the same plates using the same chamber system and environmental conditions but changing mobile phase composition. Analytical performance parameters: R_f , R_s , precision, lowest detectable quantity.
Sample: Model A and B or C.	Sample: Model A and B or C.	Sample: Model A and B or C.
Same dimension (particle size, length, etc.)	Saturated chamber	Percent nonpolar $\pm 5\%$ (rel) (normal phase) or polar (reversed phase) organic solvent in binary eluent system
Different dimension: The same experiments as above performed on HPTLC plate using three different running distances.	Saturation $\pm 5\%$ (rel) time	Percent polar $\pm 5\%$ (rel) solvent (ternary system)
Plates from different sources: The same experiments are performed on two additional plates supplied by different manufacturers. Analytical performance parameters: R_f , R_s , precision, lowest detectable quantity	Temperature $\pm 5^\circ\text{C}$	pH ± 0.5 (if aqueous)
Sample: Model A and B or C. Same dimension (particle size, length, etc.) Different dimension	Humidity $\pm 10\%$ Eluent $\pm 10\%$ (rel) amount prior to run Unsaturated Temperature $\pm 5^\circ\text{C}$ Humidity $\pm 10\%$ Eluent $\pm 10\%$ (rel) amount prior to run Duration $\pm 5\%$ (rel) prior to run	Buffer $\pm 5\%$ (rel) concentration (if aqueous) Additive $\pm 10\%$ (rel) concentration Plate pretreatment: The same experiments are performed on the same plates using the same chamber system, environmental conditions, and mobile phase composition but changing pretreatment conditions of the plates. Analytical performance parameters: R_f , R_s , precision, lowest

detectable quantity.

Sample: Model A.
 Preactivation of the plate
 Time $\pm 5\%$ (rel)
 Temperature $\pm 5^\circ\text{C}$ (rel)
 Impregnation of the plate
 Prerunning $\pm 5\%$ (rel) solvent composition

UM chamber: The same experiments are performed on plates with same particle size and dimension as in conventional chamber. Analytical performance parameters: R_f , R_s , precision, quantification limit. Sample: Model A and B or C.

Temperature $\pm 5^\circ\text{C}$.
 Eluent $\pm 10\%$ (rel) amount prior to run.
 Forced-flow chamber: Instrumental conditions are changed. Analytical performance parameters: R_s , R_f , precision, lowest detectable quantity.
 Sample: Model A and B or C.
 Temperature $\pm 5^\circ\text{C}$
 Flow rate $\pm 10\%$ (rel)

Drying time $\pm 10\%$ (rel)
 Drying temperature $\pm 5^\circ\text{C}$
 Running distance: The same experiments are performed, but the running distance differs by $\pm 10\%$ (rel). Analytical performance parameters: R_s , precision
 Sample: Model A.
 Sample stability (ST-5, Table 6)

Table 12 Checking Visualization Technique and Detection for Ruggedness (Type B-4 Investigations)

Spot visualization, drying conditions	Detection, visual evaluation
Temperature $\pm 5^\circ\text{C}$	Sample amounts corresponding to limit of detection: 3 parallel runs from each sample solution containing sample amounts corresponding to the detection limit, to -20% , to $+20\%$ respectively, using daylight and/or UV lamp of 254 and 366 nm for evaluation.
Time $\pm 20\%$ (rel)	Sample amounts corresponding to quantification limit; three parallel runs from each sample solution containing sample amount corresponding to the limit of quantification, -20% , and $+20\%$, respectively, using daylight and/or UV lamp of 254 and 366 nm for evaluation.
Spraying or dipping reagent	Instrumental conditions
Reagent concentration $\pm 10\%$ (rel)	Wavelength precision: The same wavelength is selected three times and the same chromatogram is scanned in triplicate. RSD of peak height or peak area is calculated.
Reagent-to-reagent variation I: Three solutions prepared from the same batch	Wavelength accuracy: The chromatogram is recorded at three wavelengths (Prescribed, and ± 1 nm).
Reagent-to-reagent variation II: Three	Sample amounts corresponding to limit of detection

solutions prepared from different batches	three parallel runs from each sample solution containing sample amounts corresponding to the detection limit, -20%, +20%, respectively, under prescribed instrumental conditions.
Reagent stability: The same reagent solution is used at zero time and after 2 and 4 hours.	Sample amounts corresponding to limit of quantification: three parallel runs from each sample solution containing sample amount corresponding to the limit of quantification, -20%, and +20%, respectively, under prescribed instrumental conditions.
Spraying technique	Stability of the formed derivatives on the plate (ST-6, Table 6)
Analyst to analyst variation: RSD variation of peak heights or peak areas from 5 parallel runs of the same sample solution. Dipping technique	
Analyst to analyst variation: RSD variation of peak heights or peak areas from 5 parallel runs of the same sample solution.	
Sample amounts corresponding to limit of detection: 3 parallel runs from each sample solution containing sample amounts corresponding to the detection limit, to -20%, to +20% respectively.	
Time $\pm 10\%$ (rel): 3 parallel runs from sample solution on three different plates and the plates are dipped into the dipping solution for three different time intervals (prescribed, +10% more, -10% less).	

significantly influence the results of an analysis be investigated in detail. Another principle, which can determine the depth of each ruggedness test, relates to the aim of analysis.

Evaluation of Ruggedness Testing. In general, a TLC method is considered to be

- Rugged, if the same or very close values of the analytical performance parameters [relative deviation range (RDR) is higher than 0.9 (for definition see text below)] have been obtained for each variable within the maximum acceptable deviation range (MDR).
- Semirugged either if the same values can be obtained for the tested performance parameters only by significant change of the experimental conditions or plate-to-plate variation, or if for not more than two variables, one or more analytical performance parameters fail to meet criteria but the analytical performance parameters meet criteria within 75% of the prespecified deviation ranges (RDR is higher than 0.75). Variables that are problematic should be mentioned in the analytical report.

- (c) Sensitive, if one or more performance parameters fail to meet criteria on one of the plates tested, or if for more than two variables, one or more analytical performance parameters fail to meet criteria but the analytical performance parameters meet criteria within 75% of the prespecified deviation ranges (RDR is higher than 0.75), or if for not more than two variables, one or more analytical performance parameters fail to meet criteria within 50% of the prespecified deviation ranges (RDR is higher than 0.5). The problem caused by the higher deviation range should be indicated in the analytical report.
- (d) Not rugged in all other circumstances. The exact description of the analytical method, with special emphasis of the variables leading to the failure of the performance parameters to meet criteria, is absolutely necessary in the analytical report.

If one or more analytical performance parameters fail to meet criteria, the simplest way to estimate the deviation range to be used in daily practice is to construct a response window for the limit values of a criterion in a given variable range, as illustrated in Fig. 8.

As revealed in Fig. 8, the distances A_1 and A_2 , represent the maximum acceptable deviation range (MDR) within which the criterion must be met. M_1 and M_2 represent measured points that lie outside the limits set by the criterion. A_3 and A_4 are the values of a variable obtained by measuring the distances between the nominal value and the interpolated value of this variable; they indicate the lowest and highest acceptable values of this performance parameter. If a criterion is met within the acceptable limit in the prespecified deviation range, then $A_3=A_2$ and $A_4=A_1$. The percentage of the useful variable space (relative deviation range) is given by the formulas

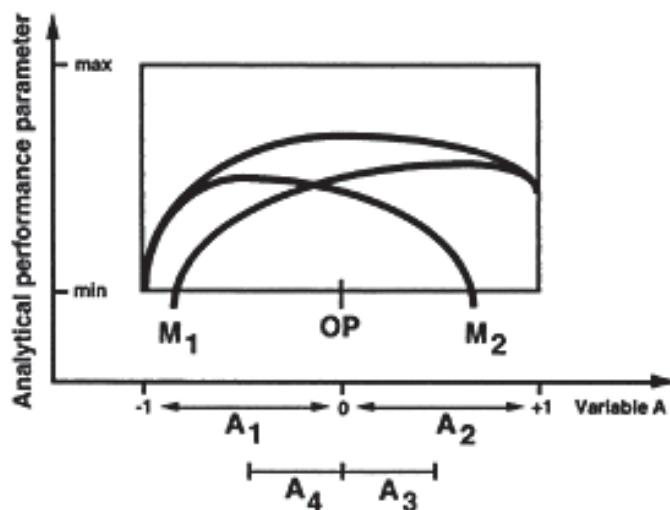


Figure 8 Schematic of response window for the limit value or values of a criterion in a given variable range.

$$\text{MDE (maximum acceptable deviation range)} = A_1 + A_4$$

$$\text{RDR (relative deviation range)} = \frac{A_3 + A_4}{A_1 + A_4} = \frac{A_3 + A_4}{\text{MDE}}$$

Should the method fail the ruggedness test and thus not be validated, evaluation and interpretation of the results obtained from the ruggedness test should provide a diagnosis of the problems leading to the failure. It might then be possible to propose the necessary changes.

D. System Suitability Test

The system suitability data serve to demonstrate chromatographic parameters that are satisfied by successful reproduction of a TLC method in daily practice. System suitability data can be divided into three groups;

1. Instrument qualification including control of sampling. If an automated sample system is used (RSD is calculated for five replicate samplings), control of detector sensitivity (peak height is measured for the peaks obtained from the above experiment), a similar procedure can be followed if manual sample application is used. Instrument qualification can be performed for a preselected period of time, e.g., each quarter of a year.
2. Separation system qualification including data related to the chromatographic separation (R_f , R_s , peak asymmetry).
3. Qualification of sample preparation procedure (if needed), including production of a three-point calibration curve on each day from a placebo or blank spiked with the lowest, median, and highest concentration estimated to be present in the test sample.

The system suitability test should be performed each time the system is assembled for the assay. Naturally, not all tests mentioned above are performed in every instance. The extent of any system suitability test may be a function of the frequency with which the investigation is performed and the constancy of the experimental conditions; it will also depend on the analytical problem to be solved as indicated in Table 13.

VI. QUALITY ASSURANCE OF EXPERIMENTAL DATA AND DOCUMENTATION

An important topic in method validation is the quality assurance of the experimental data. Quality assurance (QA) fills multiple functions. One is that if the results of validation indicate that something is wrong (validation fails), then QA rejects the analytical results, giving instructions to the analyst to repeat method development. The second function relates to the documentation activity of method validation. Four important activities carried out by QA are included in this topic:

1. Collect the validation package.
2. Generate the analytical report.
3. Create standard operating procedures for the satisfactorily validated TLC methods according to GLP requirements.
4. Perform statistical treatment of the experimental data based on well-established mathematical equations.

Of these activities, the collection and content of the method validation package is mentioned herein.

To demonstrate adequate performance of the TLC system, all results must be well documented and criteria must be formulated (limits and minimum requirements) that should be controlled prior to the use of the TLC system (system suitability test). The items necessary for documentation (method validation package) include the following:

1. A listing of

All samples used for the experiments, including their sources, lot numbers, analytical

Table 13 System Suitability Data Specific to Planar Chromatography

I. System qualification and analytical tasks												
Data to be checked	Identification			Purity testing			Assay			Stability test		
	IQ	SSQ	QSP	IQ	SSQ	QSP	IQ	SSQ	QSP	IQ	SSQ	QSP
R_f or R_M values from five replicate applications (injections) of standard solution	–	+	–	–	(+)	–	–	(+)	–	–	(+)	–
Precision calculated from five replicate applications (injections) of standard solution	–	–	–	–	(+)	–	(+)	–	–	(+)	–	–
Precision calculated from three parallel runs of spiked placebo or blank solutions with different concentrations of analyte(s), each applied in triplicate	–	–	–	–	(+)	–	–	(+)	–	–	(+)	–
Peak asymmetry	–	–	+	–	–	+	–	–	+	–	–	+
Peak height for one or more peaks	–	–	–	+	–	–	+	–	–	+	–	–
II. System suitability tests												
Data	Data to be checked											
	A			C			B					
Informatory R_f value	+			+			+					
Minimum value of desired R_s	+			+			+					
Maximum value of peak asymmetry	+			–			+					

Desired peak height	+	+	+
Detection limit	4	—	—
Limit of quantification	+	—	—
Precision (for IQ)	+	(+)	(+)
Precision (for QSP)	+	(+)	(+)

IQ=instrument qualification; SSQ=separation system qualification; QSP=qualification of sample preparation; —check is not necessary; +to be checked; (+) to be checked if necessary.

A: Full test. The method is used after a long period of time or an attempt is made to use the method for a different analytical purpose. B: Basic test. The method is in continuous use on the same analysis, but something, e.g. chromatoplate, has been replaced. C: Abbreviated check. The system is in continuous use under the same experimental conditions.

results (if available), and storage conditions

All required instrumentation

Reagents necessary for sample preparation, their required dilutions or mixtures, and storage conditions, indicating shelf life

Materials and reagents used for mobile phase preparation, including their required dilution and mixing (in detail)

Chromatoplate: type, particle size, dimensions, and other important parameters

Chamber used for the experiments (type, size)

Experimental conditions (sample application, temperature, saturation, developing)

Visualization technique including detailed description of preparation of spraying reagent, spraying conditions

Detection parameters

2. Detailed description of the entire analytical process including sample preparation, preequilibration, weights, volumes, extraction times, solvent range adjustments
3. Summary flow chart of synthesis (if available)
4. Summary of developmental data for formulated products
5. Degradation scheme for the active ingredients and for formulations
6. All validation data, including definitions, determinations, and calculations of the analytical performance parameters, together with densitograms (if available)
7. Proposed criteria for system suitability test
8. Methods reported as properly written standard operating procedures, in detail
9. Detailed evaluation of test method validation procedure, containing all of the recommendations and precautions to be taken into consideration when the TLC method is being routinely used

Some recommendations should be given as to when and why revalidation might be necessary.

VII. ROLE OF TLC IN PHARMACEUTICAL ANALYSIS

In the previous sections the importance of TLC methods in the field of pharmaceutical analysis was emphasized. In this section a brief summary of current practices and

procedures when TLC is used to analyze bulk active raw materials and formulations is given. It is especially appropriate to consider this topic, because analytical methodology is in a period of transition, owing to technological advances and modified policies of governmental regulatory agencies. The regulations in many countries and the practices of drug manufacturers have changed because of the ability of analysts equipped with modern instruments to determine low concentrations of analytes and the structures of impurities.

A. Identification of Known and Unknown Compounds

The basic test in each Pharmacopoeia is the identification of the compounds by TLC. In Ph. Eur. 4 (20), 90 monographs contain TLC methods; most of them are identity tests. The TLC is carried out by using standardized experimental conditions, and the spot of the substance being examined with or without derivatization is compared to the spot of similarly developed reference material applied in the same concentration. The sizes and positions of the spots of the principal component and the reference standard must be similar.

In most cases, different TLC systems are used for identification of substances rather than for purity testing. The reason is simple; in the first case the main aim of the analysis is to differentiate compounds belonging to the same type (group-type separation, separating compounds with similar but not the same structure), whereas in the second instance (purity testing), structurally related compounds can be separated.

When synthesizing a new compound, various chromatographic techniques are used to discover the impurity profile of the new substance. In this case, in addition to HPLC, GC, CC, TLC have important roles (177).

The identification of unknown compounds is a difficult analytical task. The use of retention data alone is not sufficient for this purpose, because of the high risk of coelution of the compound in question with many other structurally similar or different compounds in the same chromatographic system. Off-line combined (or coupled) techniques such as TLC-HPLC, TLC-MS, and TLC-IR are frequently applied for structure elucidation and identification of an unknown compound in the chromatogram.

A method for identification of an unknown compound after TLC separations was published by Nyiredy et al. (178). It is based on the retention data measured, and for the declaration that two components are identical if the identification probability value is higher than 0.5. Identification probability is defined as the area of a triangle formed by three mobile phases characterized by different values for total solvent strength (S_r), and total selectivity value (S_v), where the hR_f values of the unknown compounds are identical with those of the standard substances.

B. Purity Testing

Many countries require impurities in bulk raw material should be below 1%, and any individual impurity present at concentrations above 0.1% must be identified. The official methods (pharmacopoeial methods) in this regard are different from the methods used in industrial analysis: HPLC is the premier method, and TLC is the second choice. Pharmacopoeias are in agreement that conventional TLC is used without instrumentation.

In industrial pharmaceutical analysis, HPLC and instrumental TLC are more or less equally used, in many cases in conjunction. It should be noted that TLC has been revitalized by the recent availability of new bonded phases and by better instruments for development and quantification.

TLC is usually involved in the estimation of an impurity profile. The reason for this, in addition to the well-known advantages of this technique, is that the first indication of the presence of certain impurities may originate from recent TLC investigations, which are usually part of the routine analytical procedures in pharmacopoeias. Several methods have been published that use instrumental TLC for stability testing of active ingredients and formulated products (e.g., Refs. 25 and 26). However, if the impurities cannot be identified with the aid of authentic reference standards of the supposed impurities, then spectroscopic investigation of the separated impurities is necessary. Another problematic feature is that artifacts may be formed in the course of the separation and isolation and can be confused with real impurities.

A special area of purity testing is the cleaning validation, where the cleanliness of the manufacturing equipment is checked to avoid cross-contamination of the pharmaceutical products manufactured in the same equipment at different times. Because in this case numerous samples of swabs or rinse solvents have to be tested at the same time, thin-layer chromatography is advantageously used for this purpose. Ranitidine and ursodeoxycholic acid residuals from equipment were tested by a quantitative HPTLC method (179) using toluene–methanol–diethylamine (9:1:1, v/v) and *n*-heptane–ethyl acetate–glacial acetic acid (5:5:1 v/v) eluents on silica gel sorbents. The comprehensive validation of the method is also described in Ref. 179.

Using a bidirectional OPLC method, one can double the number of tested substances. Katona et al. (180) used this technique for cleaning validation of equipment in which five different steroid drug substances were produced consecutively. This method was used to test 27 samples on the same chromatographic plate in a short time and at low cost.

C. Assay Methods

Assays are expected to be accurate (giving the true contents of analytes in the mixture), selective (being able to distinguish between analytes and related compounds), and relatively simple. These guidelines explain the general preference for chromatographic methods. As was mentioned, HPLC superseded TLC methods in the case of APIs from this analytical field owing to the problems and difficulties related to quantification in TLC.

In the case of formulated products, the densitometric TLC/HPTLC assay method is frequently used. Nearly 200 examples of this type of analysis are reported in the book by Sethi (10). These methods provide fast and precise results.

Using a 50 mm developing distance and a 15 min migration time, Jamshidi et al. (181) determined the ibuprofen content in a licorice-containing tablet. On an HPTLC silica sorbent layer, a toluene–acetone–formic acid (70:28:2 v/v) mixture was used as mobile phase, and the chromatograms were evaluated by densitometry at 230 nm. The RSD of the repeatability test was 0.13%, and the accuracy of the method was 99.9–100.5%. Similar precision data can be found in other papers (e.g., 182).

The same chromatographic system was used by Jamshidi et al. (183) to assay acetylsalicylic acid and its free salicylic acid impurity on an HPTLC silica gel sorbent layer with ethyl acetate–methanol–acetic acid (80:19:1 v/v) as eluent. Densitometric evaluation was done at 300 nm. Validation data were also described.

Active substances of multicomponent dosage forms were determined by TLC densitometry (184). Naproxen with diflunisal and paracetamol with chlorzoxazone were separated with an ethyl acetate–methanol–aqueous ammonia solution (85:15:5 v/v) solvent mixture, and chlorphenoxamine with 8-chlorotheophylline and caffeine were separated with ethyl acetate eluent. The method was used for sugar-coated tablets, capsules, and suppositories.

TLC densitometric methods are also suitable for testing the content uniformity of tablets. For H_2 receptor antagonist Pharmaceuticals containing ranitidine and famotidine, an HPTLC densitometric method was developed using the eluent toluene–methanol–diethylamine (9:1:1, v/v) (185).

D. Standardization Possibilities

Official methods described in various pharmacopoeias can be considered standardized methods. Among these methods, some general ones are worthy of mention and are listed in Table 14.

VIII. ROLE OF TLC IN DRUG ANALYSIS

With increasing interest in medicinal plants and plant-derived substances, the use of planar chromatography is important for the identification, quantitative determination, and isolation of naturally occurring compounds. The identification of compounds in complex samples, e.g., medicinal plant extracts, is difficult, because of the similarity of chemical structures. An identification schematic is given in Fig. 9.

Because in routine laboratory work, the identification process is usually carried out without isolation, the following strategy is demonstrated using chromatographic and spectroscopic data. Therefore, in addition to the retention data the similarity of the UV/Vis spectra before and after postchromatographic derivatization are required (186).

A. Chromatographic Identification

Snyder (40) characterized the solvents used in liquid chromatography on the basis of their proton acceptor (X_e), proton donor (X_d), and dipole interaction (X_n) and characterized their selectivity in a triangle diagram, where the solvents were divided into eight groups according to their physicochemical properties. Solvents belonging to the same group have similar chromatographic behavior (55, 79). For the characterization of chromatographic behavior of the solvents a new definition was created and the individual selectivity value (S_v), was defined as a proportion of X_e and X_d (178).

For the characterization of a multicomponent mobile phase the total solvent strength (S_T) can be defined as the sum of S_i components, weighted by multiplication with their

volume fraction (76). The total selectivity value (S_v) can be calculated similarly to the S_T value.

If the hR_f value of the compound to be identified has about the same value (± 3) as the reference substance in three mobile phases with different solvent strengths and selectivity factors, these values can be depicted as a triangle in a coordinate system. The area of the triangle, the

Table 14 Methods Used for Group-Type Separation in USP and Ph. Eur.

Analytical aim	Stationary phase	Mobile phase	Detection	Literature source
Identification of fatty oils	HPTLC octadecylsilyl silica gel	Methylene chloride–glacial acetic acid–acetone (20:40:50)	Spray with 10% phosphomolybdic acid in alcohol	Ph. Eur. 4, 2.3.2
Identification of phenothiazines	Kieselguhr G impregnated with a mixture of 10% phenoxyethanol +50 g/L of Macrogol 300 in acetone	50 mL light petroleum, 1 mL diethylamine saturated with phenoxyethanol	In UV (366 nm) after spraying with 10% ethanolic sulfuric acid	Ph. Eur. 4, 2.3.3
Test for foreign oils	Kieselguhr G, impregnated with a mixture of 10 vol liquid paraffin and 90 vol petroleum spirit	Water–glacial acetic acid (10:90)		Ph. Eur. 4, 2.4.22
Identification of tetracyclines	Octylsilanized silica gel	0.5 M oxalic acid (previously adjusted with ammonium hydroxide to pH 2.0)–acetonitrile–methanol (80:20:20)	Ammonia vapor, then UV, 366 nm	USP 25 (193)
Test for aflatoxins in materials of plant origin	Silica gel	Chloroform–acetone–isopropyl alcohol (85:10:5)	UV, 366 nm	USP 25 (561)
Identification by TLC, general	Silica gel	Chloroform–methanol–water (180:15:1)	UV, 254 nm	USP 25 (201)

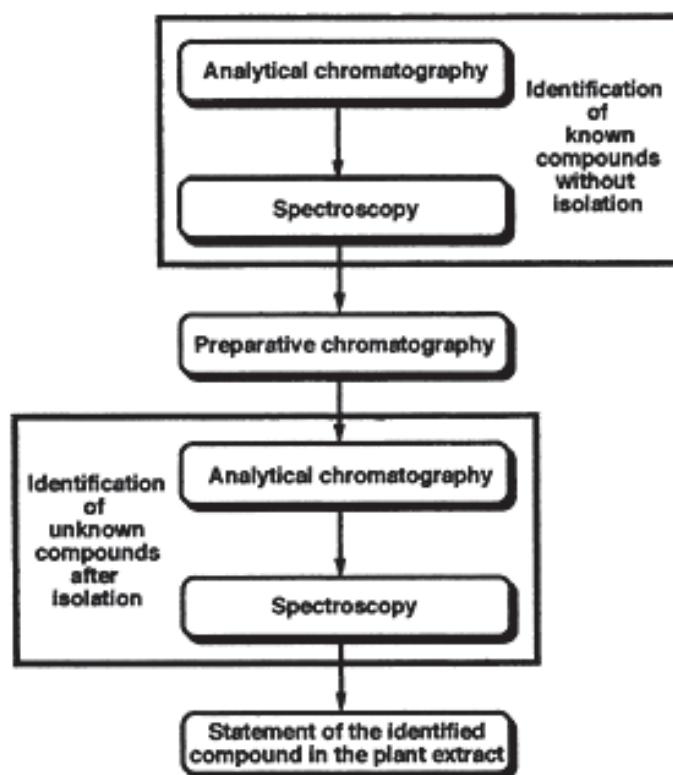


Figure 9 Schematic of identification procedure.

$I_{P(\text{chr})}$ value characterized by the goodness of the chromatographic identification (see Fig. 10). This value can be calculated according to the rules of coordinate geometry.

For chromatographic identification, the sample and the reference substances have to be spotted on the same chromatoplate (55). The hR_f values have to be calculated from the densitograms. Two compounds can be considered identical if the variance of the ΔhR_f values of the compounds to be identified and the standard substances are less than 3. The higher the value of $I_{P(\text{Chr})}$, the greater the probability that the two compounds are identical. If the $I_{P(\text{Chr})}$ value is less than 0.1, the identification is inadequate. For routine laboratory work, the $I_{P(\text{Chr})}$ value has to be between 0.1 and 0.5. If the value of $I_{P(\text{Chr})}$ is higher than 0.5, the substances are chromatographically identical with a high degree of probability. If the identification is of a new naturally occurring compound, the value of $I_{P(\text{Chr})}$ has to be higher than 0.5 (178).

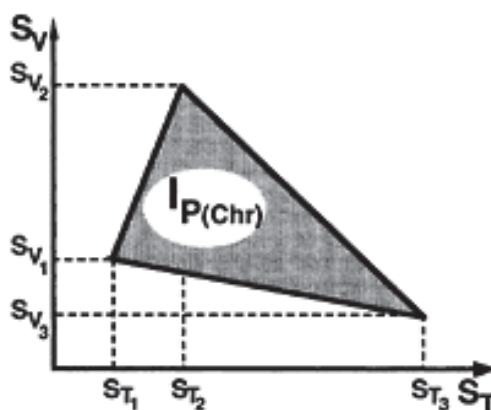


Figure 10 Representation of chromatographic identification probability, $I_{P(Chr)}$.

B. Spectroscopic Identification

For identification of certain compounds, it may be possible to compare the spectra of a sample and the reference compound to provide not only chromatographic evidence of identity but also spectroscopic confirmation. UV/Vis spectra have been recorded directly on the plate for many years (54) to characterize the compound and at the same time obtain information about the optimal wavelength for quantitative determination. This process is simple when the resolution is adequate and the concentrations are the same. Then the spectra of the reference compound and the substance in the sample to be identified have to be identical. If two spectra cover each other, they can be considered spectroscopically identical. Slight variations in amounts of substances do not alter the positions of the adsorption maxima and minima to any extent. Unfortunately, in working with drugs the coincidence of the spectra is often not accomplished. For identification of similar spectra, the $\lambda_{\min}$ and $\lambda_{\max}$ values of the reference substances and the compounds in the sample to be identified can be compared. However, theoretically it could occur that the minimal and maximal values of the spectra are not identical. Therefore, to impose a certain distance (X) between two adequate adsorbance values (between $\lambda_{\min}$ and $\lambda_{\max}$ in Fig. 11) was suggested (187).

If possible, $\lambda_{\min}$ should be higher than 220 nm. The distance between this linear (X) and the $\lambda_{\min}$ and $\lambda_{\max}$ values ($d_1, d_2, \dots, d_n$) can be determined, as demonstrated in Fig. 11. By definition the distance under the linea (X) has a negative value. From this value all possible ratios can be derived. From the data in Fig. 11 there are 15 possible combinations. Based on the rule of combination without repetition $n(n-1)/2$ combinations can be created. Clearly, as mentioned before, the values of all ratios in which d_4 or d_6 occurs, are negative, except for the ratio d_4/d_6 . Representation of the corresponding ratios ($D=d_1/d_2, d_1/d_3$, etc.) in a coordinate system indicates that a linear relationship has to be obtained if the reference and the compound to be identified are depicted on the x and y axes, respectively. The higher the value of r^2 , the better is the probability that two compounds can be spectroscopically identical.

For the spectroscopic identification probability [$I_{P(Sp)}$], regression coefficients [calculated from the λ_{min} and λ_{max} values, as well as the ratios of relative adsorbance (D)] have to be high. Based on experimental results for $I_{P(Chr)}$ and both $I_{P(Sp)}$ value empirical categories, high, medium, and low identification assurance levels can be established. In planar chromatography, two compounds

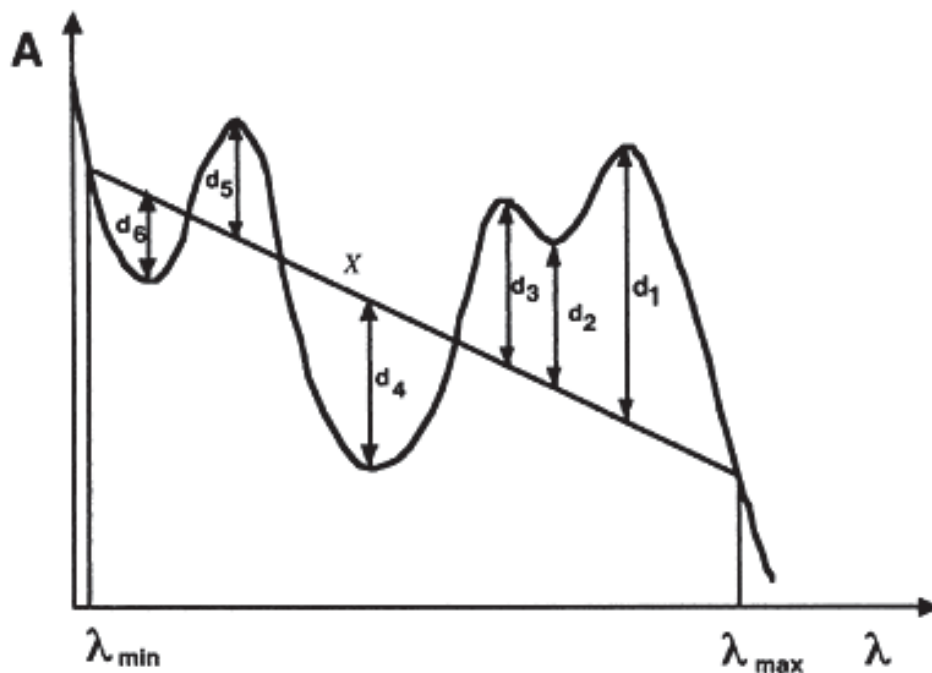


Figure 11 Schematic of method for the comparison of spectroscopic identification probabilities $I_{P(Sp)}$ using UV and/or visible spectra.

(the reference and the substance to be identified) have to be identical with adequate assurance if the chromatographic and spectroscopic identification probabilities have minimum medium levels (187), as is demonstrated in Fig. 12.

Based on the planar chromatographic data, two compounds have to be identical if the following chromatographic and spectroscopic conditions obtain (178, 188):

1. The identities of the retention data have to be ascertained with at least three mobile phases with different solvent strengths and selectivities using the same type of stationary phase.
2. The identities of the spectroscopic data have to be ascertained with the correlation of all local λ minima and maxima values, with a high regression coefficient value.
3. The identities of the spectroscopic data have to be ascertained with the correlation of the ratio of the adsorbance minima and maxima, compared to an optional baseline.

This can be expressed with the value of a high regression coefficient.

From the retention data the calculated chromatographic identification probability [$I_{P(Chr)}$], and from the UV/Vis spectra the calculated spectroscopic identification probabilities

$[I_{P(Sp)}]$ are adequate values to characterize and increase the probability of the identification of drugs.

The most widely prescribed formulated drugs from the American Druggist, animal-derived drugs from the Animal Health and Nutrition Service, as well as the common illicit drugs were complied by Ng (7).

IX. GENERAL CONCLUSIONS

This overview of the present status of TLC as an analytical technique for Pharmaceuticals and drugs suggests that TLC covers a special range of analytical methods, especially with newly introduced plates and identification techniques as well as modern forced-flow methods. Planar chromatography (189) does not compete with column liquid chromatography or gas chromatography. Instead, the three approaches are complementary, and together provide for successful and rapid separations. In our opinion, TLC has and will probably always have a role in the analysis of Pharmaceuticals and drugs.

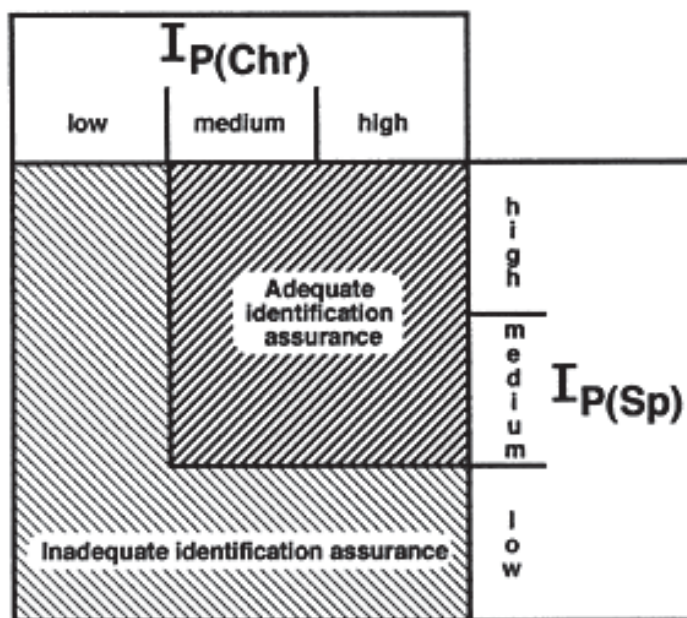


Figure 12 Representation of adequate identification assurance based on the chromatographic and spectroscopic identification probabilities.

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29

Phenols, Aromatic Carboxylic Acids, and Indoles

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I. PHENOLS

This chapter reviews developments in the thin-layer chromatography (TLC) of phenols, aromatic carboxylic acids, and indoles that have taken place since publication of the previous definitive text (1). Both natural and technical phenolic compounds are discussed, and the approach adopted is similar to that in other chapters. The phenolic group occurs in a vast array of natural products, extending from the alkaloids (e.g., morphine), through the lipids (e.g., polyunsaturated alkenylphenols), steroids (e.g., estrone), glycosides (arbutin, carminic acid), and cannabinoids to numerous other groups. At the risk of some duplication, this review therefore includes not only monocyclic phenols and phenolic acids but also some references to more complex systems. The majority of the investigations reviewed have been fundamental, and applied studies concerned with environmental, pollution, and toxicity problems have been relatively limited. With the increasing number of separatory processes available, their speed of application, and new commercial equipment, there has been a significant increase in quantitative studies aimed at improving detection limits. Impregnated layers have been more widely used in recent work, and with the availability of good chemically bonded commercial plates, the application of partition methods has increased. In general, standard methodologies have been used by workers, and the TLC of phenolic compounds has not been at the threshold of a novel "breakthrough" in technique. The development of high-performance thin-layer chromatography (HPTLC) and the potential for other innovations such as overpressured thin-layer chromatography (OPTLC) with this group of compounds is of considerable interest and counteracts an earlier feeling that high-performance liquid chromatography (HPLC) might displace planar techniques. To the contrary, in advanced work both approaches have often been used together. The art of chromatography is demonstrated in TLC, and the science in HPLC would have been true a decade ago. Now they are more indistinguishable. It is perhaps significant that one of the major scientific advances, "genetic fingerprinting," is a planar technique.

A. Preparation of Samples

The isolation of a concentrate of a natural or technical product is a vital operation requiring skilled application, and its importance cannot be overemphasized. At all stages of isolation processes, thin-layer chromatography can be used as a monitoring aid to follow the active material. At the point of sample application to the plate, the sample solvent can be of great importance. In TLC with liquid stationary phases on the support medium, the sample solvent should be immiscible to avoid streaking (2).

The unit operations of representative sampling, solvent extraction, steam distillation, column chromatography (cleanup), vapor absorption, freeze-drying, and chemical reaction followed by solvent extraction are but a few of the procedures discussed in the following examples.

1. Solvent Extraction

Technical products such as medical and pharmaceutical rubber articles (3) and industrial vulcanizates (4) have been extracted with acetone. Natural cashew phenols of *Anacardium occidentale* were extracted from the crushed shell by a variety of solvents (5). In the determination of phenol additives in various edible oils by initial solvent extraction, the coextracted oil components were eliminated by the use of precoated silica plates (6).

Cannabinoids were extracted from plant material (Indian hemp) with light petroleum (<50°C), the extracts were treated with active carbon, and after evaporation at ambient temperature the residue was examined by TLC in methanol solution (7). Phenolic acids solvent-extracted from *Saxifraga vayredana*, *Centranthus ruber*, and *Lythrum salicaria* have been examined (8). Phenolic metabolites in microbial extracts have been isolated by solvent extraction (9). This technique has invariably been only one step in more complex recoveries.

2. Column Chromatography

Phenol materials in apple juice have been recovered with methanol from poly amide columns (at 40°C) after elution of sugars and acids with water (10). Column chromatography on alumina has been used as a first step in the identification of phenolic material from *Colchicum lusitanum* (11). Extrelut 20 columns have been employed in the enrichment of phenolic acids in urine from the metabolism of catecholamines (12). And in the determination of phenol and *o*-cresol in fresh urine, solvent extraction with diethyl ether or dipropyl ether has been used following a preliminary hydrolysis with 60% perchloric acid (13).

3. Chemical Reaction and Extraction

Alkaline hydrolysis of forest soils followed by recovery of phenolic acids has been used (14). Leaf extracts of 33 varieties of *Ribes nigrum* were hydrolyzed to liberate the phenolic acids (15). Other plant extracts yielding phenolic acids have been treated similarly (16). Rather more elaborate procedures have involved treatment of plant material with hot water to denature enzymes, adjustment of the pH of the homogenized

(blender) material to 4.6, nonspecific glycosidase treatment, and extraction of the phenolic material with hot water followed by centrifugation. The filtrate was treated to hydrolyze phenolic acid esters, and it was found essential to have a reducing atmosphere (with sodium borohydride) at the hydrolysis stage (17). Nordihydroguaiaretic acid and quercetin in edible fats were extracted with methanol, reacted with isoniazid, and the colored products isolated by solvent extraction (18). An extraction procedure of labile phenols in plant material has been based on the use of radioisotope dilution and a subsequent hydrolysis stage (19). Derivatization has been used to locate phenolic material. Mono-, di-, and trihydric phenolic inhibitors have been acetylated with acetic anhydride/pyridine (20), with which only the unhindered phenols reacted. Azo dye formation from phenols with diazotized 4-nitroaniline (21, 22) and with Fast Blue Salt BB (23, 24) has been employed, followed in each case by chloroform extraction. 4-Acetyl-2-nitrophenyl derivatives of phenolic compounds have been used (25).

4. Miscellaneous Methods

The TLC of phenolic compounds in beer was effected after preliminary freeze-drying followed by solvent extraction (26). Monohydric phenols were separated from polyhydric members by steam distillation and then solvent-extracted (27). Phenols in air were absorbed in water, coupled with diazotized *p*-nitroaniline, and the azo dye extracted with diethyl ether (28).

In the determination of carbamate insecticide residues, the parent phenols were coupled with diazotized *p*-nitroaniline and the azo dye isolated (29). In a combined TLC/GLC procedure for quantitative determination of the polyunsaturated constituents of the cashew phenols following an initial TLC separation, the eluted phenols cardanol, 2-methylcardol, and a small proportion of anacardic acid each containing mono-, di-, and triene constituents were examined by GLC (30).

B. Chromatographic Properties

In this section the thin-layer chromatographic properties of phenolic compounds as revealed by their R_f values are given. The first subsection covers essentially the substituted derivatives of monocyclic phenols, the second is concerned with phenolic acids (with their derivatives), and the third, with more complex phenolic compounds such as phenolic glycosides, flavonoids, anthocyanins, lignan derivatives, phenolic steroids, cannabinoids, and other types. Some overlap between subsections is inevitable, but this feature serves incidentally to interrelate the thin-layer chromatographic properties of phenolic compounds as a whole. The material in the last section has been dealt with in other monographs (1), and more emphasis has been placed in this review on substituted phenols and phenolic acids and their simple derivatives.

1. Substituted Phenols

The term "substituted" refers to alkyl, alkenyl, halogen, nitro, hydroxy, and methoxy groups, either singly or collectively in di-, tri-, and penta-substituted compounds. Other groups are occasionally mentioned in tables and form a means for interrelating

compounds. Adsorption, partition, and reversed-phase partition methods have all been used, with increasing popularity of the latter technique and silica gel as the generally preferred support. Other major stationary phases have been alumina, cellulose, poly amide, and a number of specialized impregnated layers. In certain investigations in the above areas, which have been very comprehensive in including an extensive range of monocyclic derivatives, it is of interest that self-prepared layers have frequently been used. For each layer system there have been a large number of investigations in the past three decades, and for this reason it is possible to give only a selective description of the properties in terms of R_f values.

a. Types of Thin Layers

Silica gel. Silica gel and other layers have been used for halogenated phenols and nitrophenols (31) and for long-chain alkylphenols and derivatives (32–34). A comprehensive range of substituted phenols has been examined (35) quantitatively on silica gel, Si 5000, and amino and cyano-type plates (35a) and on silica gel plates impregnated with Cu^{2+} and Al^{3+} ions (35b). Silica gel foils and various spray reagents were employed for isomeric alkylphenols (36–38). Aniline-impregnated silica gel was used for the separation of phenol and derivatives followed by spraying with sodium nitrite solution (39). Phenols were separated with chloroform-methanol (98:2) and detected with diazotized orthonilic acid or with diazotized dianisidine (39a). A comparison of five different layers with 11 solvent systems indicated that silica gel with dichloromethane as solvent is best for separating chlorinated cresols (40). A related study was made with chlorophenols (41) and on chloroanisoles and veratroles with silica gel type 60 and RP-18 plates (41a). Separations of alkylphenols were improved on silica gel impregnated with diazotized 4,4'-diaminostilbene-2,2'-disulfonic acid (42). Related systems with Fast Blue Salt BB were described for isomeric alkylphenols and naphthols (43) and calcium carbonate-impregnated silica gel plates for phenols (43a). Naphthols were also separated on silica gel 60 F₂₅₄ and alumina 60 F₂₅₄ (43b). Reaction of phenols with 4-aminoantipyrene followed by oxidation was used prior to separation on a thin layer (44). An alkaline chloramine-T reagent was used to detect separated phenolic compounds on silica gel G (45). Chlorinated guaiacols were studied on silica gel G with a comprehensive range of solvents (46). Antioxidants were analyzed on a 1:1 layer of silica gel G with alumina (47), and hindered phenols and their methyl ethers on silica gel G (48). Impregnation of silica gel G with aliphatic amines (49), with ammonium molybdate (49a) for the separation of a wide range of monohydric phenols, and with silver nitrate for polyhydric phenols (49b) were studied. The structural isomers of cyclopentylphenol were separated on silica gel G (50). Reversed-phase and soap thin-layer separations were achieved with a wide range of alkyl and halogenated phenols on silanized (C_2) silica gel impregnated either with triethanolamine dodecylbenzene sulfonate or *N*-dodecylpyridinium chloride (51, 52). Chlorinated phenols were also examined in the presence of soluble β -cyclodextrin polymers (52a). Silica gel with a chemically bonded C_2 , C_8 , or C_{18} layer was examined for a range of substituted phenols by the reversed-phase method (53). Binary and ternary mobile phases were studied for the separation of phenol derivatives (54). A “new” solvent system for phenols was described, namely formic acid–diethyl ether–acetonitrile saturated with pentane (54a). The lipophilic properties of a wide range of substituted phenols were studied on C_{18} -bonded silica gel by the reversed-phase procedure (55). The effect of surfactants in the mobile phase on the

separation of phenols with C₁₈ silica gel was examined (56). Silufol plates were used to study polychlorophenol separations from urine samples with three different solvent systems and detection by ammoniacal silver nitrate followed by exposure to ultraviolet (UV) light (57). Pentachlorophenol in the wood of containers and in urine was determined by HPTLC on silica gel treated with dipotassium hydrogen phosphate (57a) and in tallow by separation on silica gel, detection with ammoniacal silver nitrate, and densitometric quantification (57b). Aminophenols and related compounds were detected with a modified ferric ferricyanide spray reagent (58). Developments in the use of diazido-4,4'-stilbene-2,2'-disulfonic acid for the identification of phenols were described (59). A potassium persulfate-silver reagent was used for the colorimetric identification of amines, phenols, and their derivatives on silica gel G (60). A combined flash chromatographic/TLC procedure on silica gel was valuable for resolving minor components of cashew phenols (61).

Alumina. The migration properties on alumina of a larger range of phenols and their derivatives are detailed in Ref 35. Some of the most comprehensive work on the relationship between molecular structure and thin-layer chromatographic properties was carried out on various alumina systems (62, 63). Alumina-calcium hydroxide layers (2:1) were used for phenol separations (64). Thin-layer procedures were studied generally by statistical methods (65).

Cellulose. Cellulose has been much favored after silica gel G as a layer for phenolic separations. A wide range of alkylphenols were studied on cellulose impregnated with ethyl oleate by re versed-phase methods with aqueous ethanol as solvent (66). Halogenated phenols were also comprehensively examined (67). Cellulose impregnated with formamide, *N*-methylformamide, or *N,N*-dimethylformamide was used with hexane as solvent for the separation of alkylphenols and partially hindered and hindered phenols (68). Two-dimensional thin-layer separations of 40 phenols with detection by UV and with a 4-nitroaniline reagent have been described (69).

Polyamide. Monohydric and polyhydric phenol were separated on 20% polyamide-impregnated silica gel (70). Micropolyamide plates were used for the separation of phenolic substances, particularly catechol, from cigarette smoke condensate with benzene-methanol-acetic acid (45:8:4) and detection with diazotized benzidine (71). α -Cyclodextrin was employed in the mobile phase for the separation of substituted phenolic and naphtholic compounds (72). Phenols from urine were separated on a polyamide-11 layer (73).

Miscellaneous layers. Resin and cellulose ion exchangers were used for separating phenols (74, 75), and aqueous and mixed solvents were used for phenol separations on AG 1-X4 and BDcellulose anion exchangers (76). Amine and phenolic antioxidants were separated on resin ion exchangers and cellulose layers with methanol-water (3:1) or 1 M aqueous acetic acid (77). Semicrystalline stannic tungstate layers were used for the separation of a variety of phenols with butanol-formic acid as solvent (78). Phenolic compounds including chlorogenic acid were separated on poly-*N*-vinylpyrrolidone-calcium sulfate (5:1) (79), and 30 phenols were examined on calcium sulfate alone, which was found useful for the separation of resorcinol and phloroglucinol from mixtures (79a). Titanium dioxide and stannic oxide layers were studied for the separation of a variety of solutes including structural isomers of substituted phenols, and the former for the unsaturated constituents of cashew phenols (80). Stannic arsenate-silica gel layers

were employed for the separation of nitro- and aminophenols occurring as pesticide residues (80a). A kieselguhr G-silica gel G (1:1) layer was employed for phenolic aldehyde separations with identification by 2,4-dinitrophenylhydrazone formation (81). A mixed layer of silica gel and one impregnated with an aqueous heteropoly compound followed by detection with Kodak CD-3 reagent were used (81a). Synachrom (an organic porous polymer similar to Poropak Q) was used for the separation of phenol and quinolines from hydrocarbons by pH variation (82). Related work was done on Poropak Q (a porous ethylvinylbenzene polymer) (83). The preparation, characterization, and chromatographic properties of chitin as a TLC support modified with ferric chloride were studied in the separation of phenols (83a), and a range of mobile phases were examined for such compounds and their derivatives (83b). Eggshell powder either impregnated or unimpregnated was employed for the separation of phenols and amines (83c).

b. Selected Examples of Chromatographic Properties of Substituted Phenols on Various Layers. In the early literature, no comprehensive data on R_f values were listed. In this section detailed tables are given on a wide range of substituted phenols with a variety of layer systems and solvents.

Silica gel. The separation and identification of individual members from a range of 126 phenolic compounds were described by means of three different one-dimensional developments in benzene, diisopropyl ether, and ethanol, respectively, on both silica gel 60₂₅₄ and alumina F₂₅₄ (35). A two-dimensional method routinely used for water samples containing phenolic traces was also employed with two different layers on one plate and two different solvents. Table 1 lists the R_f values for some of the substituted phenols examined on silica gel in benzene and diisopropyl ether (with values obtained on alumina plates in the same solvents). The comparison between the two layers is predictable. In many cases the presence of a 2-substituent results in an increase in the R_f value attributable to hydrogen bonding or a steric influence. Practically all the well-known functional groups are present in the compounds studied (with the exception of the cyano group), and the extensive examples give a pattern in which polarity, hydrogen bonding, and steric effects are involved. Halogenated phenols produced by the metabolism of chloropesticides were examined (57) on Silufol in the solvents shown in columns I-III of Table 2, which lists the R_f values for the dichloro, trichloro, and tetrachloro isomers and of pentachlorophenol. Sensitive detection was achieved with an ammoniacal solution of silver nitrate followed by irradiation with UV light for 5 min. It can be seen that some isomers have almost identical R_f values, and it should be emphasized that these compounds were separable by gas-liquid chromatography (GLC). The R_f values of an almost identical group of compounds were examined (31) on silica gel G containing 5% amylopectin in the three solvents shown in columns IV-VI of Table 2. Detection was by means of bromine vapor at ambient temperature, excess halogen was removed in a stream of cold air during 2 min, and the plates were finally sprayed with 2% aqueous potassium iodide. Most of the results from the two layers show a striking similarity, with the remarkable exception of pentachlorophenol, and it would seem to be arguable whether the impregnant amylopectin has any effect. The close similarity of R_f values for certain of the isomers implies that further experimentation might enable a specific separation to be achieved without the need to resort to GLC.

Reversed-phase thin-layer chromatography on silanized silica gel and soap thin-layer chromatography have enabled certain difficult separations to be effected. Soap TLC is

effectively the use of silanized silica gel G with an impregnated anionic or cationic detergent. These techniques have been comprehensively investigated for a wide variety of compounds, and their applications to alkyl, halogenated nitro, and polyhydroxy phenols are shown in Table 3 (51), which gives the R_f values for silanized (C_2) silica gel G (A) alone and (B) impregnated with 4% dodecylbenzenesulfonate (DBS) as the triethanolamine salt. The layer was prepared by mixing silanized silica gel 60 HF (C_2) in 95% ethanol with a known concentration of the detergent. The phenols were detected by exposing the wet layer successively to nitrogen dioxide and ammonia (84). With the exception of the isomeric alkylphenols, the separations of isomeric halogenated phenols are somewhat better than by the adsorption method. In the case of alkylphenols the use of 1 M, 4 M, 6 M, 8 M, and 10 M solutions of ammonia in 20% methanol as eluent enabled the structural isomers to be distinguished. With increase in the apparent pH of the solvents, deprotonation of the phenols occurs, resulting in an increase in polarity and a predictable increase in the R_f values for all the compounds. Strongly acidic phenols likewise show higher R_f values than the weaker ones, and a correlation between the R_f value and the pK_a value can be observed. At the lowest pH (with solvent 1), the effect of the substituent group on the R_f value can be seen irrespective of the deprotonation effect. A characteristic feature of the impregnated (B) series was the compactness of the spots resulting from the generally lower R_f values and the shorter development time required. This has preparative separately value in the case of polyhydroxyphenols. Relatively few separations have been described on silanized C_{18} silica gel layers. In Table 4 the R_f values are listed for such systems with solvents that are either neutral or weakly acidic and correspond approximately to solvent 1 in Table 3. It is of interest that aminophenols were included in this work, and their R_f values are in the expected region. A range of phenolic mixtures on RP-2, RP-8, and RP-18 type silica gel were separated with a variety of mixed eluents, and the relationship between R_m values and mobile phase composition was studied. The effect of hydrophobicity of the phenol on retention times was demonstrated (53b).

Table 1 R_f Values of Substituted Pehnols on Silica Gel 60F₂₅₄ and on Alumina F₂₅₄

Compound	Silica gel		Alumina	
	C_6H_6	Iso- $(C_3H_7)_2O$	C_6H_6	Iso- $(C_3H_7)_2O$
Phenol	0.16	0.74	0	0.13
2-Methylphenol	0.24	0.78	0.02	0.32
3 -Methylphenol	0.16	0.75	0	0.20
4-Methylphenol	0.15	0.74	0	0.20
2,3-Diniethylphenol	0.25	0.80	0.02	0.39
2,4-Dimethylphenol	0.28	0.83	0.06	0.28
2,5-Dimethylphenol	0.25	0.77	0.03	0.44
2,6-Dimethylphenol	0.40	0.76	0.16	0.52

3,4-Dimethylphenol	0.15	0.75	0	0.22
3,5-Dimethylphenol	0.15	0.73	0	0.26
2,3,5-Trimethylphenol	0.25	0.80	0.04	0.46
2,4,5-Trimethylphenol	0.19	0.71	0	0.23
2,4,6-Trimethylphenol	0.38	0.79	0.17	0.51
3,4,5-Trimethylphenol	0.18	0.80	0.03	0.16
2-Ethylphenol	0.27	0.77	0.06	0.32
3-Ethylphenol	0.17	0.72	0	0.14
4-Ethylphenol	0.15	0.69	0	0.24
2-Isopropylphenol	0.31	0.77	0.07	0.50
4-Propylphenol	0.17	0.74	0	0.19
2- <i>tert</i> -Butylphenol	0.54	0.87	0.17	0.79
3- <i>tert</i> -Butylphenol	0.22	0.79	0	0.30
4- <i>tert</i> -Butylphenol	0.16	0.75	0	0.26
4- <i>tert</i> -Pentylphenol	0.18	0.76	0	0.26
4- <i>tert</i> -Nonylphenol	0.24	0.79	0	0.35
3-Methyl-5-ethylphenol	0.16	0.69	0	0.16
2-Isopropyl-6-methylphenol	0.51	0.78	0.31	0.44
4-(1,1,3,3-Tetramethylbutylphenol)	0.21	0.77	0	0.32
2-Phenylphenol	0.41	0.76	0.04	0.24
4-Phenylphenol	0.20	0.76	0	0.06
2-Chlorophenol	0.42	0.75	0	0.04
3-Chlorophenol	0.20	0.74	0	0.04
4-Chlorophenol	0.16	0.69	0	0.04
2,3-Dichlorophenol	0.38	0.63	0	0
2,4-Dichlorophenol	0.38	0.65	0	0
2,5-Dichlorophenol	0.41	0.77	0	0
2,6-Dichlorophenol	0.56	0.83	0	0
3,4-Dichlorophenol	0.15	0.63	0	0
2,3,6-Trichlorophenol	0.49	0.76	0	0
2,4,5-Trichlorophenol	0.32	0.67	0	0
2,4,6-Trichlorophenol	0.49	0.76	0	0

2,3,4,6-Tetrachlorophenol	0.36	0.50	0	0
Pentachlorophenol	0.20	0.21	0	0
2-Methyl-4-chlorophenol	0.24	0.73	0.02	0.13
3-Methyl-4-chlorophenol	0.18	0.69	0	0.06
3-Methyl-6-chlorophenol	0.41	0.68	0	0.05
2-Chlorothymol	0.38	0.81	0.07	0.33
2-Nitrophenol	0.69	0.79	0	0
3-Nitrophenol	0.07	0.62	0	0
4-Nitrophenol	0.04	0.46	0	0

Compound	Silica gel		Alumina	
	C ₆ H ₆	Iso-(C ₃ H ₇) ₂ O	C ₆ H ₆	Iso-(C ₃ H ₇) ₂ O
2,4-Dinitrophenol	0.07	0.03	0	0
2,5-Dinitrophenol	0.32	0.18	0	0
2,6-Dinitrophenol	0.04	0.03	0	0
2,4,6-Trinitrophenol	0	0	0	0
2-Methyl-5-nitrophenol	0.11	0.78	0	0.03
3-Methyl-4-nitrophenol	0.05	0.54	0	0
4-Methyl-2-nitrophenol	0.70	0.79	0	0
4-Methyl-3-nitrophenol	0.08	0.65	0	0
2-Methyl-4,6-dinitrophenol	0.33	0.07	0	0
2-Chloro-4-nitrophenol	0.13	0.28	0	0
3-Methyl-6-nitrophenol	0.69	0.81	0	0
2,6-Dimethyl-4-nitrophenol	0.19	0.51	0	0
2- <i>tert</i> -Butyl-4,6-dinitrophenol	0.73	0.32	0	0
4-Chloro-2-nitrophenol	0.73	0.65	0	0
2-Fluorophenol	0.25	0.80	0	0.03
3-Fluorophenol	0.18	0.73	0	0.06
4-Fluorophenol	0.15	0.68	0	0.06
Catechol (1, 2-dihydroxy)	0.02	0.49	0	0
Resorcinol (1, 3-dihydroxy)	0	0.39	0	0
Hydroquinone (1, 4-dihydroxy)	0	0.43	0	0.02
4-Hexylresorcinol	0	0.39	0	0.02

Pyrogallol (1, 2, 3-trihydroxy)	0	0.17	0	0
Phloroglucinol (1, 3, 5-trihydroxy)	0	0.11	0	0
1,2,4-Trihydroxybenzene	0	0.14	0	0
2-Methoxyphenol	0.30	0.66	0	0.04
2-Methoxy-4-methylphenol	0.26	0.60	0	0.07
2-Methoxy-4-propenylphenol (eugenol)	0.31	0.70	0	0.04
Isoeugenol	0.32	0.68	0	0.03
2-Hydroxybenzaldehyde (salicylaldehyde)	0.53	0.79	0	0
4-Hydroxybenzoic acid	0	0.22	0	0
4-Hydroxy-3-methoxybenzaldehyde (vanillin)	0.05	0.30	0	0
4-Hydroxycarbonylpropoxybenzene	0.03	0.64	0	0
4-Hydroxythioanisole	0.11	0.63	0	0.08
3-Ethylamino-4-methylphenol	0.03	0.56	0	0.08
1-Naphthol	0.29	0.81	0	0.11
2-Naphthol	0.16	0.74	0	0.06
2,4-Dinitro-1-naphthol	0.04	0	0	0

Source: Adapted from Ref. 35.

Alumina and cellulose. Although neither alumina nor cellulose was favored in early work compared with silica gel B, classic studies of these two substances were made with a very wide range of alkyl-, nitro-, and other substituted phenols (62, 63, 67). Table 5 lists the chromatographic properties of a number of halogenated phenols on alumina and on cellulose, the former obtained by the normal adsorption method and the latter by a reversed-phase procedure. Alumina layers were prepared from column-grade neutral alumina (Brockmann activity I and II, 100–200 mesh) by crushing and use of the 200–230 mesh fraction. Cellulose plates were prepared by slurring

Table 2 R_f Values of Hologenated Phenols and Nitrophenols on Silica Gel G^a

	Silufol			Silica gel G (+5% amylopectin)		
	I	II	III	IV	V	VI
Phenol		—	—	0.39	0.17	0.47
2-Chlorophenol	—	—	—	0.52	0.21	0.50
3-Chlorophenol	—	—	—	0.40	0.16	0.47
4-Chlorophenol	—	—	—	0.38	0.16	0.43
2,3-Dichlorophenol	0.51	0.26	0.29	0.44	0.19	0.41

2,4-Dichlorophenol	0.49	0.24	0.33	0.44	0.19	0.42
2,5-Dichlorophenol	0.52	0.32	0.38	0.48	0.20	0.51
2,6-Dichlorophenol	0.70	0.43	0.45	0.59	0.30	0.60
3,4-Dichlorophenol	0.32	0.10	0.37	0.38	0.17	0.41
3,5-Dichlorophenol	0.40	0.12	0.43	—	—	—
2,3,4-Trichlorophenol	0.45	0.21	0.38	—	—	—
2,3,5-Trichlorophenol	0.51	0.27	0.40	—	—	—
2,3,6-Trichlorophenol	0.72	0.37	0.47	—	—	—
2,4,5-Trichlorophenol	0.49	0.22	0.44	0.45	0.17	0.43
2,4,6-Trichlorophenol	0.68	0.40	0.52	0.54	0.20	0.50
3,4,5-Trichlorophenol	0.38	0.10	0.44	—	—	—
2,3,4,5-Tetrachlorophenol	0.49	0.19	0.44	—	—	—
2,3,4,6-Tetrachlorophenol	0.72	0.41	0.43	—	—	—
Pentachlorophenol	0.69	0.34	0.50	0.23	0.09	0.09
4-Fluorophenol	—	—	—	0.36	0.09	0.41
4-Bromophenol	—	—	—	0.37	0.10	0.42
2-Nitrophenol	—	—	—	0.71	0.54	0.59
3-Nitrophenol	—	—	—	0.35	0.12	0.33
4-Nitrophenol	—	—	—	0.30	0.09	0.34

Solvents: I, benzene–methanol (95:5); II, light petroleum–benzene (1:1); III, *n*-hexane–acetone (3:1); IV benzene–ethanol (95:5); V, *n*-hexane–ethanol (95:5); VI, diethyl ether–*n*-hexane (1:1).
Source: Silufol data adapted from Ref. 57; silica gel data from Ref 31.

cellulose with a 0.75 v/v solution of ethyl oleate in diethyl ether. In both cases phenols were detected by spraying the dried plates with alkaline potassium permanganate solution, which resulted in yellow spots against a purple background. The R_f values on alumina layers are mostly complementary to those obtained on cellulose. The solvents for the alumina layer are more appropriate than those used in later work (35), and generally the separations appear to be better. On the cellulose layer, as on alumina, increase in solvent polarity increases the R_f values. Although by the adsorption method isomeric compounds show similar R_f values, on cellulose by the reversed-phase technique separations are frequently more favorable. Hydrogen bonding, either intermolecular between the substrate alumina and groups in the aromatic ring or intramolecular in the case of 2-substituted phenols, was considered to be a significant factor in determining the observed R_f values. In the case of cellulose it was generally thought that electronic effects were of secondary importance to the influence of the molar volume of the halogen atoms, although positional effects were significant. Generally this whole study represents a major contribution to the relationship between molecular structure and chromatographic

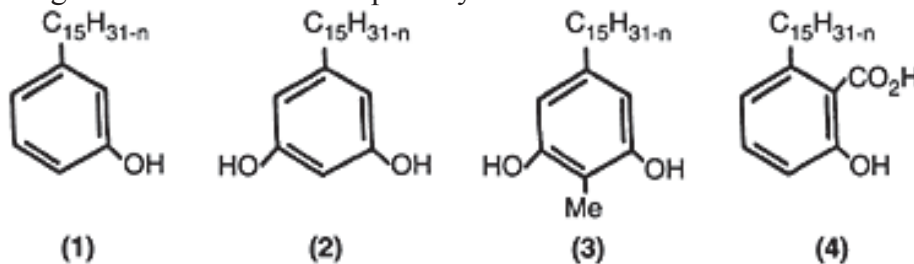
behavior. Tables 6 and 7 summarize the R_f values of halogen-substituted alkylphenols and the role of the relative position of halogen and alkyl groups on both alumina and cellulose layers.

Alkyl-substituted phenols have been studied extensively by the same authors, and R_f values are summarized in Table 8 for similar solvent systems for alumina and cellulose. At the end of Table 8 the properties of some alkoxyphenols are given.

For the alumina layer it was concluded that the hydrogen-bonding associative effect is predominant and tends to mask the influence of the substituent effect except in the case of 2-substituted compounds, where a steric factor impedes the association. With cellulose, the separation of the phenols is governed by their relative partition. The phenols, initially dissolved in the stationary phase, are removed by solvation of the phenolic hydroxy group by the mobile phase. In the case of 2-substituted compounds, solvation of the phenolic group is hindered and lower R_f values result. For cellulose systems the Martin additivity principle (85), relating to the contribution of substituent groups, was broadly valid, whereas with alumina it was inapplicable.

2-Substituted phenols including cryptophenols (partially hindered phenols) and hindered phenols were studied in detail on cellulose impregnated with *N*-methylformamide or *N,N*-dimethylformamide by the reversed-phase method (68). Table 9 summarizes the R_f values for a variety of essentially 2-substituted compounds on cellulose impregnated with various concentrations of *N*-methylformamide as the stationary phase and *n*-hexane as the mobile phase. Cellulose MN 300 HR was homogenized with the respective amide, and the plates were developed in a double-saturation chamber with a polythene bag technique (86); the phenols were visualized with alkaline potassium permanganate solution. The R_f values obtained with formamide as stationary phase were considerably higher than with either *N*-methyl- or *N,N*-dimethylformamide, which were similar in their effect. As a general rule, plots of R_m [$=\log(1/R-1)$] versus the logarithm of the concentration of amide in the solvent for layer preparation were linear. It was considered that interaction between the π electrons of the aromatic ring and the substituents on the nitrogen atom of the amide were significant in influencing R_f values in those cases where the primary hydrogen bonding mechanism was negligible.

By contrast with the reversed-phase technique, hindered phenols and their methyl ethers were examined on silica gel G (type 60) in weakly polar solvents as shown in Table 10. The methyl ethers have lower R_f values than the corresponding phenols, indicating an unusual reversal of polarity.



Long-chain alkylphenols have been investigated extensively (33) by both adsorption and reversed-phase partition methods (34). The cashew phenols (from *Anacardium occidentale*) comprise cardanol (1), cardol (2), 2-methylcardol (3), and anacardic acid

(4), each existing as a mixture of the saturated, 8'-monoene, 8',11'-diene, and 8',11',14'-triene constituents. In ammoniated solvent (light petroleum–diethyl ether–ammonia, 60:40:2) the acidic compound anacardic acid remained practically on the baseline, whereas in an acidic solvent (light petroleum–diethyl ether–formic acid, 70:30:1) it migrated toward the solvent front as an intramolecularly bonded substance. In natural cashew nut shell liquid, the phenols and their unsaturated constituents have the R_f values of 0.20 (cardol), 0.36 (2-methylcardol), 0.58 (cardanol), and 0.76 (anacardic acid). On argentated silica gel G (10% w/w silver nitrate) the unsaturated constituents separate as shown in Fig. 1 (30, 87). This illustrates the CNSL phenols before and after hydrogenation (solvent, diethyl ether–light petroleum–formic acid, (30:70:1). The spots were visualized by H_2SO_4 charring. Samples 2, 3, 5, and 7 were cardol, 2-methylcardol, cardanol, and anacardic acid, respectively, each showing (from the bottom) triene, diene, and monoene constituents with 5 and 7 also showing trace amounts of the saturated compound. Samples 2, 4, 6, and 8 represent the hydrogenated samples.

Table 3 R_f Values of Phenols on Thin Layers of Silanized Silica Gel (A) Alone and (B) Impregnated with 4% DBS Solution^a

Phenol	1		2		3		4		5		pK_a (25°C)
	A	B	A	B	A	B	A	B	A	B	
Phenol	0.36	0.35	0.36	0.35	0.37	0.35	0.51	0.47	0.73	0.62	10.02
<i>m</i> -Cresol	0.21	0.19	0.22	0.19	0.24	0.20	0.38	0.31	0.53	0.40	10.09
<i>p</i> -Cresol	0.21	0.19	0.22	0.19	0.23	0.19	0.35	0.29	0.48	0.36	10.17
<i>o</i> -Cresol	0.22	0.20	0.22	0.20	0.23	0.20	0.35	0.29	0.48	0.35	10.32
3,5-Dimethylphenol	0.13	0.12	0.13	0.12	0.13	0.12	0.24	0.21	0.33	0.25	10.19
3,4-Dimethylphenol	0.14	0.12	0.14	0.12	0.14	0.12	0.22	0.19	0.29	0.24	10.36
2,5-Dimethylphenol	0.13	0.11	0.13	0.11	0.13	0.11	0.22	0.19	0.26	0.21	10.41
2,3-Dimethylphenol	0.13	0.11	0.13	0.11	0.13	0.11	0.22	0.19	0.26	0.20	10.54
2,4-Dimethylphenol	0.12	0.11	0.12	0.11	0.13	0.11	0.20	0.18	0.26	0.19	10.60
2,6-Dimethylphenol	0.13	0.12	0.13	0.12	0.13	0.12	0.20	0.18	0.25	0.20	10.63
2,3,5-Trimethylphenol	0.07	0.06	0.07	0.06	0.07	0.06	0.13	0.10	0.14	0.12	—
2,4,6-Trimethylphenol	0.07	0.06	0.07	0.06	0.07	0.06	0.13	0.10	0.13	0.10	10.88
2,3,6-Trimethylphenol	0.07	0.06	0.07	0.06	0.07	0.06	0.13	0.10	0.14	0.11	—
<i>m</i> -Ethylphenol	0.11	0.11	0.11	0.11	0.12	0.11	0.25	0.21	0.32	0.28	9.9 ^b
<i>p</i> -Ethylphenol	0.12	0.11	0.12	0.11	0.12	0.11	0.23	0.20	0.28	0.24	10.0 ^b
<i>o</i> -Ethylphenol	0.11	0.10	0.11	0.10	0.11	0.10	0.20	0.18	0.25	0.21	10.2 ^b
<i>o</i> -Chlorophenol	0.20	0.19	0.21	0.19	0.32	0.24	0.69	0.68	0.93	0.89	8.48

<i>m</i> -Chlorophenol	0.16	0.12	0.16	0.12	0.20	0.14	0.50	0.42	0.82	0.64	9.02
<i>p</i> -Chlorophenol	0.16	0.12	0.16	0.12	0.20	0.14	0.43	0.33	0.67	0.50	9.38
2,6-Dichlorophenol	0.12	0.10	0.16	0.13	0.56	0.55	0.72	0.73	0.95	0.88	6.79, 6.79 ^c
2,5-Dichlorophenol	0.09	0.06	0.11	0.06	0.34	0.28	0.62	0.63	0.93	0.85	7.50, 7.35 ^c
2,3-Dichlorophenol	0.08	0.06	0.09	0.06	0.28	0.22	0.59	0.58	0.93	0.85	—, 7.45 ^c
2,4-Dichlorophenol	0.08	0.05	0.08	0.05	0.22	0.16	0.55	0.52	0.81	0.78	7.89, 7.75 ^c
3,5-Dichlorophenol	0.07	0.04	0.07	0.04	0.14	0.09	0.43	0.42	0.79	0.64	8.18, 7.93 ^c
3,4-Dichlorophenol	0.07	0.04	0.07	0.04	0.12	0.07	0.39	0.35	0.76	0.57	—, 8.39 ^c
2,3,6-Trichlorophenol	0.05	0.05	0.13	0.12	e.s.	0.45	e.s.	0.49	0.83	0.82	6.12
2,4,6-Trichlorophenol	0.04	0.04	0.09	0.08	0.37	0.37	0.49	0.47	0.81	0.73	6.42
2,3,5-Trichlorophenol	0.03	0.03	0.05	0.04	0.26	0.25	0.43	0.43	0.76	0.69	7.23
2,4,5-Trichlorophenol	0.03	0.03	0.05	0.04	0.25	0.22	0.43	0.43	0.78	0.70	7.33
2,3,4-Trichlorophenol	0.03	0.03	0.05	0.03	0.23	0.18	0.43	0.43	0.77	0.68	7.59
3,4,5-Trichlorophenol	0.02	0.02	0.03	0.02	0.12	0.09	0.33	0.34	0.64	0.52	7.74
2,3,5,6-Tetrachlorophenol	0.02	0.02	0.12	0.12	0.24	0.25	0.33	0.33	0.64	0.50	5.44
2,3,4,5-Tetrachlorophenol	0.01	0.01	0.02	0.02	0.14	0.14	0.29	0.30	0.61	0.46	6.96
Pentachlorophenol	0.01	0.01	0.09	0.09	0.14	0.13	0.24	0.21	0.46	0.33	5.26
<i>o</i> -Bromophenol	0.18	0.15	0.18	0.15	0.26	0.20	0.68	0.59	0.93	0.88	8.44
<i>m</i> -Bromophenol	0.13	0.10	0.13	0.10	0.19	0.12	0.49	0.38	0.78	0.62	9.03
<i>p</i> -Bromophenol	0.13	0.10	0.13	0.10	0.18	0.11	0.42	0.30	0.65	0.50	9.36
2,6-Dibromophenol	0.08	0.07	0.11	0.09	0.49	0.46	0.64	0.64	0.93	0.89	6.6
2,4-Dibromophenol	0.05	0.03	0.06	0.04	0.11	0.10	0.51	0.43	0.85	0.75	7.8
<i>p</i> -Nitrophenol	0.26	0.23	0.31	0.26	0.67	0.66	0.83	0.89	0.96	0.92	7.15
<i>o</i> -Nitrophenol	0.20	0.17	0.23	0.19	0.62	0.61	0.79	0.83	0.96	0.92	7.23
<i>m</i> -Nitrophenol	0.25	0.22	0.25	0.22	0.36	0.29	0.69	0.68	0.96	0.92	8.40
2,6-Dinitrophenol	0.53	0.68	0.54	0.76	0.66	0.77	0.71	0.78	0.96	0.92	3.71
2,4-Dinitrophenol	0.48	0.58	0.51	0.74	0.63	0.77	0.71	0.78	0.96	0.92	4.09
2,5-Dinitrophenol	0.32	0.31	0.49	0.65	0.63	0.73	0.70	0.77	0.96	0.92	5.22
3,4-Dinitrophenol	0.21	0.18	0.40	0.51	0.58	0.68	0.70	0.78	0.96	0.92	5.43
2-Chloro-5-methylphenol	0.11	0.09	0.11	0.09	0.13	0.12	0.55	0.45	0.89	0.77	—
4-Chloro-3-	0.08	0.06	0.08	0.06	0.09	0.06	0.28	0.20	0.51	0.30	—

methylphenol

4-Chloro-2-methylphenol	0.08	0.06	0.08	0.06	0.09	0.06	0.25	0.16	0.44	0.24	—
Gallic acid	0.92	0.94	0.91	0.94	e.s.	e.s.	e.s.	e.s.	e.s.	e.s.	4.41
Pyrocatechuic acid	e.s.	0.93	0.85	0.95	e.s.	e.s.	e.s.	0.95	e.s.	0.95	—
Phloroglucinol	0.76	0.73	0.74	0.72	e.s.	e.s.	0.95	0.95	0.95	0.95	8.45
Pyrogallol	0.74	0.68	0.74	0.67	e.s.	e.s.	e.s.	e.s.	e.s.	e.s.	9.01
Resorcinol	0.58	0.58	0.56	0.55	0.56	0.55	0.74	0.70	0.95	0.87	9.81
Hydroquinone	0.67	0.66	0.66	0.66	0.67	0.68	e.s.	e.s.	e.s.	e.s.	10.35
Orcinol	0.47	0.48	0.46	0.46	0.47	0.46	0.62	0.57	0.89	0.80	—
BHA ^b	0.02	0.02	0.02	0.02	0.02	0.02	0.04	0.03	0.05	0.03	—
<i>n</i> -Propyl gallate	0.19	0.24	0.21	0.23	e.s.	e.s.	e.s.	e.s.	e.s.	e.s.	—
Octyl gallate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—
Dodecyl gallate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—

^aEluents: (1) 0.1 M CH₂COOH+0.1 M CH₃COONa in 30% CH₃OH (pH 5.00); (2) 0.1 M KH₂PO₄ in 30% CH₃OH (pH 7.00); (3) 0.1 M NH₃+0.1 M NH₄Cl in 30% CH₃OH (pH 9.02); (4) 1 M NH₃+0.1 M NaCl in 30% CH₃OH (pH 11.30); (5) 1 M NH₃ in 30% CH₃OH (pH 11.30).

^b*pK_a* values at 28°C.

^c*pK_a* values at 29°C.

^d2,(3)-*t*-Butyl-4-hydroxyanisole (mixed isomers).

Source: Ref. 31.

Table 4 *R_f* Values of Substituted Phenols on Silica Gel C₁₈ by Reversed-Phase TLC

Compound	Solvent ^a			
	I	II	III	IV
Phenol	0.52	0.55	0.58	0.54
4-Methylphenol	0.40	0.48	0.48	0.44
3-Methylphenol	0.42	0.49	0.50	0.46
3-Aminophenol	0.76	0.72	0.80	0.72
4-Aminophenol	0.80	0.76	0.86	0.78
4-Chlorophenol	0.34	0.44	0.43	0.41
2-Chlorophenol	0.40	0.46	0.47	0.44
2-Methoxy-4-propen-2-ylphenol	0.26	0.36	0.36	0.32
2-Methoxy-4-propen-1-ylphenol	0.24	0.34	0.34	0.30
4-Hydroxy-4-propen-1-ylphenol	0.78	0.76	0.86	0.78

2-Methoxy-4-propen-1-ylphenol	0.49	0.52	0.55	0.50

^aI, CH₃CO₂H–HCONMe₂–0.21 M NaCl–dioxane–CH₃OH (1:5:39:8: 47); II, tetrahydrofuran–0.21 M NaCl–CH₃CH (5:42:53); III, 0.21 M NaCl–HCONMe₂–CH₃CN (41:15:44); IV, 0.21 M NaCl–CH₃SOCH₃–CH₃CN (41:15:44).

Source; Adapted from Ref. 53.

The homologous unsaturated cardol constituents present in cereal grains such as rye and wheat were separated by the reversed-phase partition method through the use of a two-way development procedure involving preliminary separation of the homologous saturated, monoenes, and dienes on argentated silica gel G with benzene–ethyl acetate (85:15). Separation of the C₁₃–C₂₅ members of each unsaturated group was achieved after removal of the excess silver nitrate, drying, impregnation of the plate with paraffin oil, and development in acetone–methanol–water (60:15:25). The two-dimensional separations of 5-*n*-alk(enyl)resorcinol homologs are shown in Fig. 2. Detection was with Fast Blue B. Columns 1–7 of Fig. 2 represent C₁₃, C₁₅, C₁₇, C₂₁, C₂₃, and C₂₅ homologs, and rows A, B, and C, the saturated, monoene, and diene constituents of each.

2. Phenolic Acids and Their Derivatives

As with phenols, adsorption, partition, and reversed-phase partition methods have all been applied to phenolic acids and their derivatives with a variety of stationary phases. This review is selective in describing the major work. Silica gel has been the layer favored by most investigators.

a. Types of Thin Layers. The phenolic acids arising from clinical studies (88) were examined by TLC on cellulose (88–90) and with cellulose on Silufol (91). TLC was considered superior to paper chromatography. The identification of phenolic acids in varieties of *Ribes nigrum* has been described (15) as well as separation of phenolic acids from plant material or silica gel G and quantitative analysis with spectrophotometry (92). Phenolic acids extracted from plants were best separated on silica gel G with chloroform–ethyl acetate–formic acid (5:4:1) (8). TLC on silica gel combined with scanning spectrophotometry was used to separate nine phenolic acids in wine, with hexane–ethyl acetate–formic acid (15:9:2) (93) the preferred solvent of the 10 examined. Twenty naturally occurring phenolic acids were separated by a combination of one- and two-dimensional TLC and development with three solvents (94), and phenolic acids (and some phenols) related to humic acid were examined on alumina with water as solvent (94a). Silica gel F₂₅₄ (Silica Rapid Platten Woelm in preference) was used with dichloromethane–toluene–formic acid (50:40:10) or dichloromethane–water–acetic acid (100:50:50, lower phase) followed by spraying with ferric chloride or titanium tetrachloride (17). The TLC of 37 phenolic acids was

Table 5 R_f Values of Substituted Phenols on Alumina (by Normal TLC) and on Cellulose (by Reversed-Phase TLC)

Compound	Alumina		Cellulose	
	$\text{C}_6\text{H}_6\text{--C}_2\text{H}_5\text{OH}$ (95:5)	$\text{C}_6\text{H}_6\text{--C}_2\text{H}_5\text{OAc}$ (3:7)	$\text{H}_2\text{O--C}_2\text{H}_5\text{OH}$ (25:75)	$\text{H}_2\text{O--C}_2\text{H}_5\text{OH}$ (37.5:62.5)
Phenol	0.29	0.50	0.795	0.90
2-Chlorophenol	0.24	0.16	0.535	0.79
3-Chlorophenol	0.22	0.10	0.43	0.67
4-Chlorophenol	0.22	0.10	0.46	0.68
2,3-Dichlorophenol	0.10	0.06	0.28	0.53
2,4-Dichlorophenol	0.11	0.05	0.22	0.48
2,5-Dichlorophenol	0.10	0.06	0.20	0.46
2,6-Dichlorophenol	0.10	0.06	0.31	0.55
3,4-Dichlorophenol	0.13	0.05	0.215	0.46
3,5-Dichlorophenol	0.13	0.05	0.14	0.355
2,4,5-Trichlorophenol	0.04	0.02	0.075	0.235
2,4,6-Trichlorophenol	0.03	0	0.15	0.275
2,3,4,6-Trichlorophenol	0.03	0	0.045	0.135
Pentachlorophenol	0	0	0.02	0.02
2-Fluorophenol	0.18	0.13	0.755	0.86
3-Fluorophenol	0.21	0.18	0.69	0.795
4-Fluorophenol	0.25	0.24	0.70	0.83
2-Bromophenol	0.26	0.16	0.48	0.73
3-Bromophenol	0.22	0.14	0.37	0.595
4-Bromophenol	0.22	0.14	0.39	0.60
2-Iodophenol	0.28	0.16	0.34	0.56
3-Iodophenol	0.20	0.14	0.28	0.49
4-Iodophenol	0.20	0.14	0.29	0.51
2,4-Dibromophenol	0.10	0.05	0.13	0.35
3,5-Dibromophenol	0.10	0.06	0.10	0.22
2,4,6-Tribromophenol	0.03	0	0.07	0.21
2,4,6-Triiodophenol	0.03	0	0.07	0.07

Source: Adapted from Refs. 63 and 67.

done in chloroform–acetic acid–water (2:1:1) and 1% aqueous acetic acid (95). The identification of phenolic acids in Tunisian medicinal plants (*Globularia alypum*) was carried out by two-dimensional TLC (96). Trichloroacetate-impregnated silica gel was used with dichloromethane–toluene–acetic acid (12:2:0.5) or toluene–acetic acid (8.5:1.5), and *E* and *Z* isomers were separated (97). Since the earlier separations obtained on cellulose (98), silica gel has been more widely used. Phenolic acids, polyhydric phenols and catechins were separated on silica gel G with detection by iodine vapor, ferric chloride, ferric chloride–ferricyanide, and vanillin–sulfuric acid reagent (98a). Reversed-phase TLC of hydroxycinnamic acids with silanized silica gel 60 H F₂₅₄ or silica gel H impregnated with liquid paraffin was carried out in sandwich tanks with 2% acetic acid and aprotic solvents (99). Ion-pair TLC with silica gel impregnated with Aliquat 336 in an Aliquat-saturated buffer (pH 7) with methanol was used for the separation of phenolic acids (100). The TLC of phenolic acids (and aldehydes) from the degradation of lignin was investigated on silica gel 60 F₂₅₄ impregnated with ferric chloride or nitrate (101) and reversed-phase separation of phenolic acids on silanized silica gel (sil C₁₈-50 and RP-18) together with 4% detergent (either *N*-dodecylpyridinium chloride or dodecylbenzenesulfonic acid) (102). The TLC separations of

Table 6 *R_f* Values of Chloro- and Bromo-Substituted Alkylphenols on Alumina (by Normal TLC) and on Cellulose (by Reversed-Phase TLC)

Phenol substituent	Alumina		Cellulose	
	C ₆ H ₆ – C ₂ H ₅ OH (95:5)	C ₆ H ₆ – C ₂ H ₅ OAc (3:7)	H ₂ O– C ₂ H ₅ OH (25:75)	H ₂ O– C ₂ H ₅ OH (37.5:62.5)
4-Chloro-3-methyl	0.24	0.37	0.31	0.56
4-Chloro-2,3-dimethyl	0.36	0.74	0.115	0.43
4-Chloro-2,5-dimethyl	0.42	0.73	0.115	0.45
4-Chloro-2,6-dimethyl	0.44	0.85	0.10	0.41
4-Chloro-3,5-dimethyl	0.26	0.38	0.125	0.50
4-Chloro-2,3,5-trimethyl	0.34	0.75	0.05	0.29
4-Chloro-3-methyl-5-ethyl	0.28	0.42	0.09	0.34
2-Chloro-4,5-dimethyl	0.52	0.31	0.21	0.57
2,4-Dichloro-6-methyl	0.39	0.43	0.05	0.235
2,4-Dichloro-3,5-dimethyl	0.21	0.20	0.055	0.24
2,4-Dichloro-3,6-dimethyl	0.40	0.46	0.035	0.15
2,6-Dichloro-4-methyl	0.11	0.08	0.16	0.37

2,6-Dichloro-3,4-dimethyl	0.22	0.20	0.09	0.37
2,4,6-Trichloro-3-methyl	0.10	0.02	0.07	0.16
2,4,6-Trichloro-3,5-dimethyl	0.14	0.03	0.02	0.09
2,4,6-Trichloro-3-methyl-5-ethyl	0.14	0.06	0	0.05
2-Bromo-4-methyl	0.29	0.21	0.30	0.66
2-Bromo-4-cyclohexyl	0.03	0.34	0	0.10
2-Bromo-4-phenyl	0.28	0.34	0	0.19
2-Bromo-3,4,6-trimethyl	0.54	0.39	0.05	0.195
2-Bromo-3-methyl-4,6-di- <i>t</i> -butyl	1.00	1.00	0	0.03
2,4-Dibromo-5-methyl	0.40	0.22	0.04	0.19
2,4-Dibromo-6-methyl	0.37	0.28	0.05	0.155
2,4-Dibromo-6- <i>t</i> -butyl	0.65	0.94	0	0.06
2,4-Dibromo-6-cyclohexyl	0.62	0.78	0	0.03
2,4-Dibromo-6-phenyl	0.53	0.74	0	0.06
2,4-Dibromo-3,6-dimethyl	0.53	0.45	0.03	0.12
2,4-Dibromo-5,6-dimethyl	0.53	0.41	0.04	0.115
2,4-Dibromo-3,5,6-trimethyl	0.64	0.63	0	0.06
2,6-Dibromo-4-methyl	0.32	0.25	0.07	0.255
2,6-Dibromo-4- <i>t</i> -butyl	0.35	0.30	0	0.120

Source: Adapted from Refs. 63 and 67.

phenolic acids on silica gel, silica gel RP-8, and polyamide layers have been compared (103). The separation of phenolic acids was studied on diol-bonded silica gel with binary mobile phases from heptane with ethyl acetate, isopropanol, tetrahydrofuran, or dioxan, which was found to give the greatest selectivity (103a). On silanized silica gel, the effect of β -cyclodextrin on the reversed-phase performance of a wide range of phenolic acids was examined (103b). Other approaches to the separation of phenolic acids were achieved by two-dimensional TLC on cellulose (104). This methodology was later extended and a new system optimized for a mixture of 14 solutes (104a).

The possible separation of phenolic acids of the triphenylmethane series by using micellar mobile phases comprising surfactants was studied (104b). A large group of phenolic acids and related structures were examined in four solvents on cellulose. Their R_f values and colors with

Table 7 R_f Values Showing the Effect of Relative Position of Halogen and Alkyl Groups in Phenols on Alumina (Normal TLC) and Cellulose (Reverse Partition)

Phenol	Alumina			Cellulose	
	C ₆ H ₆ – MeOH (95:5)	C ₆ H ₆ – EtOH (95:5)	C ₆ H ₆ – EtOH (3:7)	H ₂ O– EtOAc (25:75)	H ₂ O– EtOH (37.5:62.5)
2,4,5-Trimethylphenol	0.39	0.44	0.72	0.30	0.57
4-Chloro-2,5-dimethylphenol	0.37	0.42	0.73	0.115	0.45
2,4,5-Trichlorophenol	0.07	0.04	0.02	0.075	0.235
2,4,6-Trimethylphenol	0.50	0.55	0.83	0.280	0.540
4-Chloro-2,6-dimethylphenol	0.43	0.44	0.85	0.100	0.410
2,4-Dichloro-6-methylphenol	0.39	0.39	0.43	0.05	0.235
2,6-Dichloro-4-methylphenol	0.18	0.11	0.08	0.16	0.37
2,4,6-Trichlorophenol	0.06	0.03	—	0.15	0.275
2,3,4,6-Tetramethylphenol	0.52	0.54	0.86	0.18	0.37
2,6-Dichloro-3,4-dimethylphenol	0.21	0.22	0.20	0.09	0.27
2,4,6-Trichloro-3-methylphenol	0.12	0.10	0.02	0.07	0.16
2,3,4,6-Tetramethylphenol	0.03	0.03	0	0.045	0.135

Source: Adapted from Ref. 67.

diazotized 4-nitroaniline and with sulfanilic acid have been listed (105). Cellulose layers were employed for phenolic acids and their derivatives with an extensive range of chromogenic sprays (106), and phenolic acids in *Ginkgo biloba* were separated by TLC and HPTLC on similar layers (106a). Traces of ginkgolic acids in *Ginkgo biloba* leaves were also examined (106b). Phenoxyacetic acid and its herbicidal 2,4-dichloro derivative were examined by TLC (106c).

b. Selected Examples of Chromatographic Properties of Phenolic Acids and Their Derivatives

Phenolic acids. Table 11 lists the R_f values for a number of phenolic acids and certain of their relatives on silica gel, silanized silica gel, cellulose, and poly amide layers. Silica gel (Silica Rapid Platten Woelm F₂₅₄) was used with solvents I (dichloromethane–toluene–formic acid, 50:40:10) and II (dichloromethane–acetic acid–water, 100:50:50, lower phase), followed by UV examination of the plates and subsequent spraying with 1% methanolic ferric chloride. HPTLC precoated plates with silica gel 60 F₂₅₄, RP-8, and

Wang polyamide were used with solvents III (benzene–ethyl acetate–formic acid, 40:10:5), IV (ethanol–water, 55:45), and IX (benzene–ethyl methyl ketone–methanol, 60:26:14), respectively. The upper part of Table 11 lists the R_f values of hydroxybenzoic acids, and the lower part those for hydroxycinnamic acids. For columns V– VIII, the solvents used were 2% formic acid (V), 20% potassium chloride solution (VI), isopropanol–ammonium hydroxide–water (8:1:1 (VII), and 10% acetic acid (VIII). For each layer the relative trends of the R_f values follow the polarity of the compounds listed.

Table 12 gives the R_f values for certain phenolic acids on silver oxide–impregnated silica gel G by two-dimensional development on cellulose (107). The retention behavior of phenolic acids in normal-phase TLC on silica, alumina, and polyamide has been reported (107a). The efficiency of 10 TLC systems for the separation of the phenolic acids and flavones from *Sambuci flos* was investigated by three different mathematical techniques (107b). TLC and HPLC have been used for the free phenolic acids and those obtained upon hydrolysis in *Asclepias syriaca*, milkweed (107c). Caffeic and protocatechuic acids in *Rhizoma cibotii* and *Rhizoma pteris*, used in Chinese medicine, have been determined by TLC scanning (107d). Retention data for a series of phenolic acids on polar-bonded cyanopropyl and diol stationary phases have been obtained by HPTLC (107e).

Table 8 R_f Values of Alkylphenols and Alkoxyphenols on Alumina (by Normal TLC) and on Cellulose (by Reversed-Phase TLC)

Phenol	Alumina			Cellulose	
	C ₆ H ₆ – MeOH (95:5)	C ₆ H ₆ – C ₂ H ₅ OH (95:5)	C ₆ H ₆ – C ₂ H ₅ OAc (3:7)	H ₂ O– C ₂ H ₅ OH (25:75)	H ₂ O– C ₂ H ₅ OH (37.5:62.5)
Phenol	0.26	0.29	0.50	0.795	0.90
2-Methylphenol	0.40	0.38	0.67	0.025	0.785
3-Methylphenol	0.34	0.32	0.56	0.075	0.83
4-Methylphenol	0.33	0.32	0.55	0.66	0.83
2,3-Dimethylphenol	0.43	0.41	0.70	0.455	0.68
2,4-Dimethylphenol	0.41	0.40	0.69	0.425	0.69
2,5-Dimethylphenol	0.43	0.41	0.70	0.475	0.68
2,6-Dimethylphenol	0.52	0.54	0.80	0.40	0.635
3,4-Dimethylphenol	0.32	0.33	0.58	0.53	0.765
3,5-Dimethylphenol	0.32	0.32	0.60	0.515	0.74
2,3,4-Trimethylphenol	0.39	0.42	0.70	0.29	0.575
2,3,5-Trimethylphenol	0.39	0.44	0.72	0.29	0.55
2,3,6-Trimethylphenol	0.52	0.56	0.83	0.28	0.54

2,4,5-Trimethylphenol	0.39	0.44	0.72	0.30	0.57
2,4,6-Trimethylphenol	0.50	0.55	0.83	0.28	0.54
3,4,5-Trimethylphenol	0.30	0.33	0.62	0.43	0.68
2,3,4,5-Tetramethylphenol	0.39	0.44	0.70	0.24	0.50
2,3,4,6-Tetramethylphenol	0.52	0.54	0.86	0.18	0.37
2,3,5,6-Tetramethylphenol	0.54	0.55	0.86	0.15	0.36
2-Ethylphenol	0.45	0.46	0.75	0.40	0.61
3-Ethylphenol	0.36	0.36	0.59	0.485	0.70
4-Ethylphenol	0.35	0.36	0.58	0.455	0.69
2- <i>n</i> -Propylphenol	0.49	0.49	0.84	0.20	0.46
4- <i>n</i> -Propylphenol	0.35	0.35	0.62	0.24	0.55
2-Isopropylphenol	0.41	0.38	0.62	—	—
4-Isopropylphenol	0.35	0.35	0.62	0.25	0.585
4- <i>t</i> -Butylphenol	0.35	0.35	0.63	0.11	0.375
2- <i>sec</i> -Butylphenol	0.56	0.51	0.85	0.115	0.34
4- <i>sec</i> -Butylphenol	0.38	0.40	0.65	0.14	0.44
2- <i>t</i> -Butylphenol	0.61	0.59	0.92	0.08	0.24
3- <i>t</i> -Butylphenol	0.45	0.40	0.65	0.215	0.48
4- <i>t</i> -Butylphenol	0.45	0.43	0.65	0.23	0.50
4- <i>n</i> -Amylphenol	0.35	0.35	0.65	0.06	0.21
4- <i>sec</i> -Amylphenol	0.38	0.39	0.67	0.055	0.18
4- <i>t</i> -Amylphenol	0.44	0.43	0.67	0.15	0.355
4-(3-Methylbutyl)phenol	0.35	0.35	0.64	0.075	0.390
4- <i>n</i> -Octylphenol	0.44	0.45	0.78	0.04	0.06
2- <i>n</i> -Octylphenol	0.63	0.62	0.92	0	0.04
4- <i>n</i> -Nonylphenol	0.42	0.42	0.74	0	0.06
2-Allylphenol	0.47	0.48	0.70	0.28	0.575
4-Allylphenol	0.35	0.35	0.53	0.35	0.67
4-(But-2-enyl)phenol	0.36	0.36	0.56	0.17	0.50
2-Phenylphenol	0.51	0.53	0.76	0.13	0.415

4-Phenylphenol	0.30	0.30	0.42	0.110	0.445
4-Benzylphenol	0.40	0.42	0.69	0.105	0.42
4-Cumylphenol	0.42	0.44	0.71	0.08	0.22
4-Cyclopentylphenol	0.36	0.37	0.66	0.13	0.37
4-Cyclopent-2-enylphenol	0.37	0.37	0.64	0.19	0.47
2-Cyclohexylphenol	0.54	0.54	0.81	0.06	0.195
4-Cyclohexylphenol	0.38	0.39	0.69	0.07	0.22
2-Methyl-4- <i>t</i> -butylphenol	0.42	0.44	0.81	0.085	0.32
3-Methyl-5-ethylphenol	0.35	0.35	0.62	0.30	0.61

Phenol	Alumina			Cellulose	
	C ₆ H ₆ – MeOH (95:5)	C ₆ H ₆ – C ₂ H ₅ OH (95:5)	C ₆ H ₆ – C ₂ H ₅ OAc (3:7)	H ₂ O– C ₂ H ₅ OH (25:75)	H ₂ O– C ₂ H ₅ OH (37.5:62.5)
3-Methyl-4-isopropylphenol	0.35	0.35	0.68	0.245	0.55
3-Methyl-5-isopropylphenol	0.35	0.35	0.66	0.175	0.485
3-Methyl-5- <i>sec</i> -butylphenol	0.40	0.42	0.69	0.07	0.31
3,5-Di- <i>t</i> -butylphenol	0.48	0.49	0.86	0	0.11
2-Methyl-4-octylphenol	0.46	0.46	0.84	0.02	0.06
2- <i>t</i> -Butyl-3-methylphenol	0.63	0.64	1.00	0.04	0.12
2- <i>t</i> -Butyl-4-methylphenol	0.63	0.64	1.00	0.05	0.15
2-Octyl-4-methylphenol	0.63	0.63	1.00	0	0.02
2,6-Dimethyl-4- <i>n</i> -propylphenol	0.60	0.56	0.89	0.08	0.24
2,6-Dimethyl-4-allylphenol	0.60	0.58	0.89	0.095	0.31
2,6-Di- <i>t</i> -butylphenol	1.00	0.97	1.00	0.03	0.05
2-Methyl-4,6-di- <i>t</i> -butylphenol	0.77	0.77	1.00	0	0.05
2,6-Di- <i>t</i> -butyl-4-methylphenol	1.00	0.97	1.00	0	0.02
2-Methoxyphenol	0.30	0.41	0.68	0.81	0.855
3-Methoxyphenol	0.20	0.39	0.65	0.685	0.835
4-Methoxyphenol	0.20	0.38	0.66	0.68	0.835

3,5-Dimethoxyphenol	0.15	0.28	0.39	0.17	0.87
4-Ethoxyphenol	0.22	0.37	0.65	0.66	0.825
4-Cyclopentyloxyphenol	0.22	0.37	0.67	0.315	0.62
4- <i>n</i> -Heptyloxyphenol	0.25	0.44	0.70	0	0.12
4- <i>n</i> -Dodecyloxyphenol	0.26	0.43	0.70	0	0.02
4- <i>n</i> -Tetradecyloxyphenol	0.32	0.43	0.70	0	0.02
4- <i>n</i> -Hexadecyloxyphenol	0.32	0.43	0.70	0	0.02
4-Phenoxyphenol	0.21	0.39	0.58	0.30	0.515
3,5-Dicarbonylmethoxyphenol	0.12	0.11	0.22	0	0

Source: Adapted from Refs. 62 and 66.

Phenolic aldehydes and ketones. The R_f values of phenolic aldehydes as their phenylhydrazones formed in situ were determined on silica gel G and kieselguhr G (in equal parts) in four solvents (108) and on cellulose in four solvents. The collected results for a number of hydroxy and methoxy compounds are shown in Table 13. The R_f values for various hydroxybenzophenones and hydroxydiphenylmethanes on silica gel G F_{254} were determined in the five solvents shown in Table 14 (109). The values show the effect of both the presence of hydrogen bonding and its suppression either by the solvent or by the intervention of another polar group.

Long-chain alkylphenolic acids and their derivatives. Methylol derivatives of 3-pentadecylphenol have been separated (32) on silica gel G in the solvent light petroleum (60–80°C)–diethyl ether–dimethylformamide–glacial acetic acid (75:85:5:1) as shown in Fig. 3, in which A, B, and C are the 2-, 6-, and 4-monomethyl derivatives, respectively; D is a mixture of 2-, 2,6-di-, and 2,4-dimethylols; E and F are both the 4,6-dimethylol; G is the 2,4,6-trimethylol; H is a mixture of all seven, and I is the 6-methylol from a synthesis.

Simpler solvent systems have been used to separate related homologous materials as shown in Table 15.

Phenolic wood extractives, flavones, and catechol derivatives. A large number of phenolic substances comprising wood extractives, lignin precursors, and other guaiacyl propane structures were investigated by TLC on cellulose and on silica gel (110), which were also used for the polyphenols associated with the aqueous processing of olive oil (110a), the catechins from green tea (110b), and those from fresh cassava root (110c). The R_f values of a series of structures with a guaiacyl nucleus are shown in Table 16. They were detected by their specific colors with

Table 9 R_f Values of Phenols and Hindered Phenols on Cellulose (Normal TLC in Hexane)

Phenol	Concentration of <i>N</i> -methylformamide in the slurrying solvent, acetone (mol/L)					
	0.5	1.0	2.0	3.0	4.0	5.0
Phenol	0.16	0.09	0.05	0.035	0.02	0
3-Methylphenol	0.26	0.16	0.085	0.05	0.04	0.03
3,4-Dimethylphenol	0.36	0.21	0.12	0.08	0.06	0.05
3,5-Diethylphenol	0.50	0.31	0.17	0.11	0.08	0.06
3-Methyl-5-isopropylphenol	0.55	0.35	0.20	0.14	0.10	0.08
2-Methylphenol	0.38	0.23	0.12	0.095	0.075	0.05
2-Methyl-3-ethylphenol	0.51	0.34	0.20	0.13	0.09	0.07
2-Methyl-5-isopropylphenol	0.64	0.44	0.27	0.20	0.14	0.11
2-Ethyl-5-methylphenol	0.56	0.34	0.19	0.13	0.09	0.07
2- <i>n</i> -Propyl-4-methylphenol	0.62	0.43	0.26	0.18	0.13	0.11
2-Hexylphenol	0.83	0.69	0.51	0.40	0.43	0.27
2-Cyclohexylphenol	0.80	0.65	0.50	0.40	0.33	0.27
2,6-Dimethylphenol	0.67	0.43	0.24	0.18	0.14	0.11
2,6-Diethylphenol	0.78	0.63	0.43	0.31	0.25	0.22
2,6-Diisopropylphenol	0.92	0.86	0.74	0.65	0.57	0.51
2,6-Diallylphenol	0.71	0.54	0.36	0.26	0.20	0.16
2,6-Di- <i>sec</i> -butylphenol	1.00	0.94	0.88	0.82	0.76	0.72
2,6-Di- <i>t</i> -butylphenol	1.00	0.94	0.88	0.82	0.76	0.72
2,6-Di- <i>t</i> -butyl-4-methylphenol	1.00	1.00	1.00	0.94	0.91	0.89

Source: Adapted from Ref. 68.

Table 10 R_f Values of Hindered Phenols and Methyl Ethers on Silica Gel G

Phenolic compound	Solvent ^a				
	LP 100	LP-C (80:20)	LP-C (60:40)	LP-C (40:60)	C 100
2-Methoxy-6- <i>n</i> -pentadecylphenol	0.11	0.29	0.38	0.60	0.70
2-Methoxy-6- <i>n</i> -pentadecylanisole	0.65	0.17	0.24	0.48	0.65
2-Methoxy-4,6-di- <i>t</i> -butylphenol	0.14	0.41	0.52	0.68	0.73
2-Methoxy-4,6-di- <i>t</i> -butylanisol	0.08	0.29	0.38	0.57	0.64
2,6-Di- <i>t</i> -butyl-4-methylphenol	0.36	0.64	0.75	0.83	0.84
2,6-Di- <i>t</i> -butyl-4-methylanisole	0.27	0.57	0.75	0.83	—
2- <i>t</i> -Butyl-6-methoxy-4-methylphenol	0.12	0.31	0.44	0.66	0.76
2- <i>t</i> -Butyl-6-methoxy-4-methylanisole	0.045	0.20	0.32	0.55	0.66
2- <i>t</i> -Butyl-6-methoxy-5-methylphenol	—	—	—	—	—
2- <i>t</i> -Butyl-6-methoxy-5-methylanisole	0.10	0.33	0.38	0.59	0.67
2-Carbonylmethoxy-3- <i>n</i> -pentadecylphenol	0.17	0.34	0.52	0.58	—
2-Carbonylmethoxy-3- <i>n</i> -pentadecylanisole	0.04	0.21	0.45	0.52	—

^aLP, light petroleum; C, chloroform. Visualization with ethanolic Rhodamine 6G.

Source: Adapted for Ref. 48.

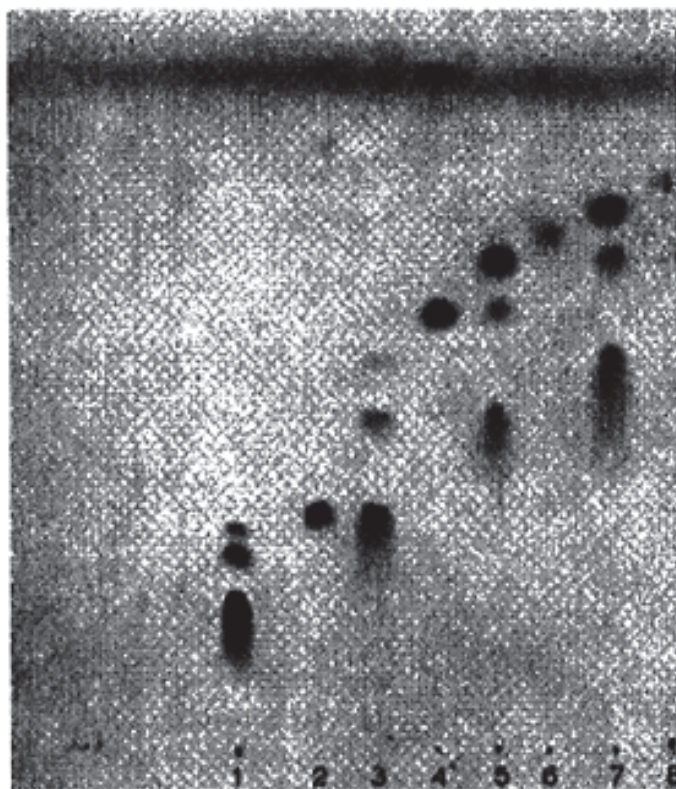


Figure 1 TLC of CNSL phenols in silica gel G (+10% AgNO₃). (From Ref. 30.)

diazotized sulfanilic acid. Flavones have been widely examined in classic work by paper chromatography (111, 112), although relatively less work has been carried out in specific areas by TLC such as with 6- and 8-methoxyflavones from several species of the Labiatae family (113). Table 17 lists for a number of polyhydroxy and polymethoxy flavones the R_f values obtained on silica gel G with (I) benzene–methanol–acetic acid (45:3:2) and (II) chloroform–*n*-hexane–methanol (40:40:3). Detection was by examination under UV light. The data illustrate the role of intramolecular hydrogen bonding. Flavones were investigated by automated multiple development (AMD) on silica gel by HPTLC with the solvent system methanol–dichloromethane–water–

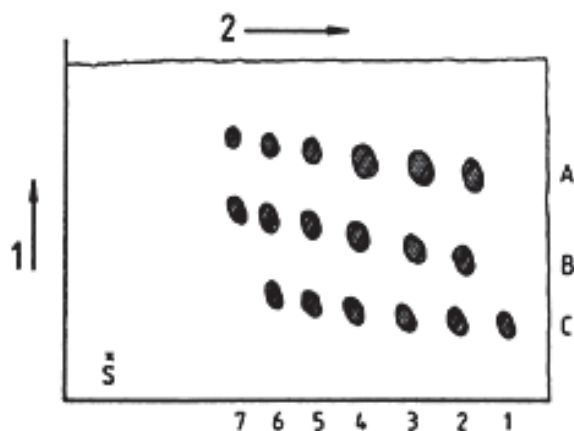


Figure 2 Two-dimensional TLC of homologous alkenyl resorcinols. (From Ref. 34.)

Table 11 R_f Values of Phenolic Acids on Various Layers: Silica Gel (SG), Cellulose, Polyamide^a

Acid	SG ^b				Cellulose				Polyamide IX
	I	II	III	IV	V	VI	VII	VIII	
Benzoic	1.0	0.50	0.79	0.50	—	—	—	—	0.80
2-Hydroxybenzoic	1.0	0.40	0.76	0.76	0.70	0.57	0.44	0.62	0.20
3-Hydroxybenzoic	0.65	0.20	—	—	0.37	0.63	0.58	0.70	—
4-Hydroxybenzoic	—	—	0.62	0.70	0.27	0.59	0.52	0.70	0.48
2,3-Dihydroxybenzoic	—	—	—	—	—	—	—	—	—
3,4-Dihydroxybenzoic	0.35	0.10	0.45	0.76	—	—	—	—	0.19
3,5-Dihydroxybenzoic	0.15	0.05	—	—	—	—	—	—	—
2,4-Dihydroxybenzoic	0.20	0.20	—	—	—	—	—	—	—
2,6-Dihydroxybenzoic	—	—	—	—	—	—	—	—	—
2,5-Dihydroxybenzoic	0.50	0.15	0.55	0.75	0.29	0.37	0.32	0.54	0.07
3,4,5-Trihydroxybenzoic	0.10	0.01	0.25	0.84	0.09	0.33	0.31	0.46	0.07
3-Methoxybenzoic	—	—	—	—	0.66	0.67	0.49	0.65	—
4-Methoxybenzoic	—	—	—	—	0.56	0.62	0.49	0.53	—
3,4-Dimethoxybenzoic	—	—	—	—	0.53	0.32	0.50	0.66	—
3-Methoxy-4-hydroxybenzoic	1.0	0.35	—	—	0.26	0.14	0.21	0.39	—
3,5-Dimethoxy-4-hydroxybenzoic	0.95	0.30	—	—	—	—	—	—	—

2-Hydroxy-5-methoxybenzoic	—	—	—	—	0.07	0.44	0.42	0.60	—
<i>trans</i> -Cinnamic	1.0	0.45	0.78	0.41	0.64	0.57	0.44	0.54	0.83
2-Hydroxycinnamic	0.80	0.25	0.66	0.59	0.34	0.39	0.29	0.51	0.47
3-Hydroxycinnamic	0.75	0.20	0.62	0.64	0.43	0.26	0.33	0.52	0.54
4-Hydroxycinnamic	0.75	0.20	0.63	0.67	0.34	0.33	0.28	0.47	0.50
Coumarin	—	—	—	—	0.46	0.43	0.56	0.72	—
3-Methoxy-4-hydroxycinnamic	1.0	0.30	0.63	0.69	0.28	0.21	0.19	0.43	0.66
3,4-Dihydroxycinnamic	0.40	0.10	0.45	0.75	0.11	0.16	0.23	0.36	0.24
4-Methoxycinnamic	—	—	—	—	0.63	0.09	0.14	0.32	—
3-Hydroxy-4-methoxycinnamic	0.95	0.30	0.58	0.67	—	—	—	—	0.61
3,5-Dimethoxy-4-hydroxycinnamic	0.95	0.35	—	—	—	—	—	—	—
Chlorogenic	—	—	—	—	0.08	0.45	0.49	0.68	—
Isochlorogenic	—	—	—	—	0.05	0.04	0.10	0.26	—
3,4-Dihydroxydihydrocinnamic	—	0.10	0.37	0.75	0.18	0.64	0.70	0.76	0.30

^aFor solvents I-IX, see text.

^bFor type of silica gel in each case, see text.

Source: Columns I and II adapted from Ref. 17; columns III, IV, IX adapted from Ref. 103.

formic acid (70.5:25:4.5:1) proceeding to 100% dichloromethane (113a). Two new acylated flavonol monoglycosides (kaempferol rhamnosides) from *Platanus acerifolia* buds were separated and examined by TLC on cellulose (113b).

The high-performance TLC separation of catecholamines is important with regard to pharmaceutical preparations, metabolic studies, and the identification of drugs of abuse. Reversed-phase TLC was used (102) with silanized silica gel layers OPTI-UP C12, SIL C18-50, and RP-18 in certain cases impregnated with *N*-dodecylpyridinium chloride (NDPC) or dodecylbenzene sulfonic acid (DBS) and with layers of ammonium tungstophosphate (AWP). The R_f values are given in Table 18. Sixteen visualizing agents were studied for use in the TLC separations of adrenaline, dopamine, phenylephrine, metaraminol, fenoterol, and bithionol (102a).

Numerous natural phenolic products, including cannabinoids (114), vitamin E (115), morphine alkaloids (116), toxins such as gossypol (117), tetracyclines (118), phenolic steroids (119), phenolic glycosides (120), for example, glucopyranosyl sinapate and other phenolic compounds from

Table 12 R_f Values of Aromatic Carboxylic Acids on Cellulose and on Silica Gel G (With and Without Silver Oxide)^a

Compound	Silica gel (Ag ₂ O-impregnated)				Cellulose	
	SiO ₂ +Ag ₂ O I	SiO ₂ II	SiO ₂ +Ag ₂ O III	SiO ₂ IV	1-D V	2-D VI
Benzoic acid	0.70	0.77	0.73	0.81	—	—
2,3-Dihydroxy	—	—	—	—	0.53	0.60
2,4-Dihydroxy	0.65	0.83	0.69	0.85	0.38	0.57
2,5-Dihydroxy	—	—	—	—	0.60	0.51
2,6-Dihydroxy	0.90	0.92	0.92	0.94	0.81	0.28
3,4-Dihydroxy	—	—	—	—	0.91	0.43
3,5-Dihydroxy	—	—	—	—	0.29	0.31
2-Hydroxy	0.82	0.88	0.86	0.90	—	—
3-Hydroxy	0.59	0.78	0.68	0.82	—	—
4-Hydroxy	0.50	0.80	0.64	0.85	—	—
3,5-Dinitro	0.88	0.91	0.91	0.91	—	—
2,3,4-Trihydroxy	—	—	—	—	0.22	0.17
2,4,6-Trihydroxy	—	—	—	—	0.44	0.18
3,4,5-Trihydroxy	—	—	—	—	0.19	0.23
4-Hydroxy-3-methoxy	—	—	—	—	0.28	0.74

^aSolvents: for I, II, ethanol–ethyl acetate–conc. ammonia (9:3:2); for III, IV, ethanol–water–conc. ammonia (10:1.2:1.6); for V, isopropanol–conc. ammonia–water (8:1:1); for VI, anisole–glacial acetic acid–water (70:29:1).

Source: Data for I, II, III, IV adapted from Ref. 107; for V, VI from Ref. 104.

rapeseed oil (120a), salicin in the bark of willow (120b), anthocyanins (121), and other natural product groups have all been studied by TLC. The identification of substances separated by TLC can be achieved reliably only by chemical and spectroscopic means.

C. Chromatographic Systems

In the TLC of phenols a number of different chromatographic systems have been used, but none of these appears to be uniquely preferred. Although self-prepared silica gel G plates have proved adequate for the great majority of applications, the availability of a variety of commercially prepared plates and uniform adsorbents has enabled formerly less-used techniques such as reversed-phase HPTLC methods to emerge. These have considerable significance, particularly with regard to pH control in the development stage

and to detergent impregnation methods. Ascending development has been generally used in all systems. As much information as possible is included here in the tabulated information on R_f values; nevertheless there is little doubt that in those cases where special development chambers have been stipulated as being essential, the original literature should be consulted. For complex mixtures either multiple development or two-dimensional methods are undoubtedly called for, particularly for quantitative studies. The scope of TLC applied to phenol separations is likely to benefit from developments in other areas, which will improve its control and applicability vis-à-vis HPLC.

D. Detection Methods

This section describes spray reagents and the development of colored derivatives in situ using impregnated plates designed to produce a colored substance.

Table 13 R_f Values of Phenolic Aldehydes on Silica Gel G and on Cellulose

R_f values on silica gel G				
Benzaldehyde	CH ₃ C ₆ H ₅ – CHCl ₃ –Me ₂ CO (5:3:2)	CH ₃ OC ₆ H ₅ – CHCl ₃ –Me ₂ CO (5:3:2)	CHCl ₃ – Me ₂ CO (8:2)	CH ₃ OC ₆ H ₅ – CH ₃ OH (8:2)
3,4-Dihydroxy	0	0.01	0	0.10
4-Hydroxy-3,5-dimethoxy	0.09	0.11	0.13	0.27
2,4-Dihydroxy	0.12	0.14	0.11	0.37
3-Methoxy-4-hydroxy	0.20	0.23	0.26	0.46
4-Hydroxy	0.26	0.33	0.39	0.53
3-Hydroxy-4-methoxy	0.32	0.45	0.53	0.66
3-Ethoxy-4-hydroxy	0.36	0.38	0.48	0.60
2-Hydroxy-3-methoxy	0.45	0.47	0.55	0.73
R_f values on cellulose				
Benzaldehyde	2% HCO ₂ H	20% KCl	i-PrOH– NH ₄ OH–H ₂ O (8:1:1)	10% AcOH
2,4-Dihydroxy	0.37	0.43	0.54	0.65
2,5-Dihydroxy	0.38	0.52	0.62	0.71
3,4-Dimethoxy	0.94	0.45	0.66	0.78

4,6-Dimethoxy-2-hydroxy	0.90	0.08	0.18	0.38
3-Hydroxy	0.79	0.57	0.69	0.78
4-Hydroxy	0.64	0.56	0.68	0.76
3,4-Dihydroxy	0.36	0.50	0.60	0.68
2-Hydroxy	0.63	0.52	0.63	0.71
3,5-Dimethoxy-4-hydroxy	0.46	0.48	0.62	0.73
3-Methoxy-4-hydroxy	0.62	0.53	0.63	0.73
3-Methoxy-2-hydroxy	0.61	0.49	0.61	0.74

Source: Adapted from Refs. 108 and 205.

1. Spray Reagents

The chromogenic detection systems used by investigators are indicated for the tabulated data on R_f values. Although with silica gel G F₂₅₄, examination at both short and long wavelengths is necessary, it has tended to displace the former charring procedures and fluorescent sprays such as Rhodamine 6G or dichlorofluorescein. The range of colors observable in the charring process is often highly characteristic but may not outweigh the environmental objections to this method. Newer spray reagents are thorium nitrate (detection limit 1–3 μg) (122) and zinc chloride–potassium ferricyanide for aminophenols (123). A number of spray reagents for halogenated phenols and nitrophenols have been studied (31). Ten newer visualizing reagents for monohydric phenols on silica gel, kieselguhr, and polyamide have been applied. The best and most universal were bromocresol green, bromothymol blue, and brilliant cresyl blue (31a). New visualizing agents for polybasic phenols and chlorophenols were described (31b). Ammonium ceric nitrate, 5% in acetone solution, together with 5% hydroxyamine hydrochloride in 80% acetone gives specific brown spots with phenols and indoles, the range of detection being 1–10 μg (124). The color reactions of 21 polyhydric phenols were described (125). Phenols separated on silica gel or silica gel impregnated with potassium phosphomolybdates were detected with a spray based on a 1:1

Table 14 R_f Values of Hydroxybenzophenones and Hydroxydiphenylmethanes on Silica Gel G F₂₅₄

Compound	C ₆ H ₆	C ₆ H ₆ –ligorin (9:1)	C ₆ H ₆ –p ^a (9:1)	C ₆ H ₆ –EtOH–p ^a (8:1:1)	CCl ₄ –MeOAc–p ^a (8:1:1)
Benzophenone	0.53	0.48	0.77	0.86	0.80
2-Hydroxy	0.63	0.60	0.59	0.89	0.53
4-Hydroxy	0	0	0.09	0.35	0

2,2-Dihydroxy	0.44	0.41	0.17	0.61	0.11
4,4-Dihydroxy	0	0	0	0.08	0
2,4-Dihydroxy	0.06	0.03	0	0.21	0
Diphenylmethane	0.76	0.78	0.85	0.92	0.90
2-Hydroxy	0.33	0.28	0.55	0.71	0.48
4-Hydroxy	0.13	0.12	0.47	0.70	0.36
2,2-Dihydroxy	0	0	0.11	0.52	0
4,4-Dihydroxy	0	0	0.26	0.56	0.10
Benzhydrol	0.18	0.17	0.64	0.87	0.64

^ap=piperidine.

Source: Adapted from Ref. 109.

fresh aqueous mixture of 1% 4-(*N,N*-diethylamino)aniline and 2% sodium carbonate solution (125a). Various spray reagents were evaluated for isomeric dimethylphenols, methylphenols, and phenol (36, 37, 126). Nine spray reagents were studied in the TLC of chlorophenol isomers (127). 3-Methyl-2-benzothiazolone hydrazone hydrochloride was used as a spray for dihydric phenol derivatives from lichens (128). Phenolic acids give a wide range of colors with diazotized 4-nitroaniline (104). The same reagents and diazotized sulfanilic acid were used with aromatic aldehydes, phenolic alcohols, flavonoids, and coumarins (110). Monohydric phenols give a range of yellow to brown colors with a spray of potassium persulfate containing silver nitrate (129) followed by warming. Qualitative detection methods have generally involved the use of chemical techniques, and physical methods have generally been used only in quantitative work.

2. Chromogenic Reaction Techniques

Somewhat different chromogenic procedures have involved preliminary reaction of phenols with 4-(5-fluoro-2,4-dinitro-1-phenylazo)-*N,N*-dimethylaniline (130) and coupling with 4-benzoylamino-2,5-diethoxyaniline (Fast Blue Salt BB) (131) followed in each case by separation of the azo

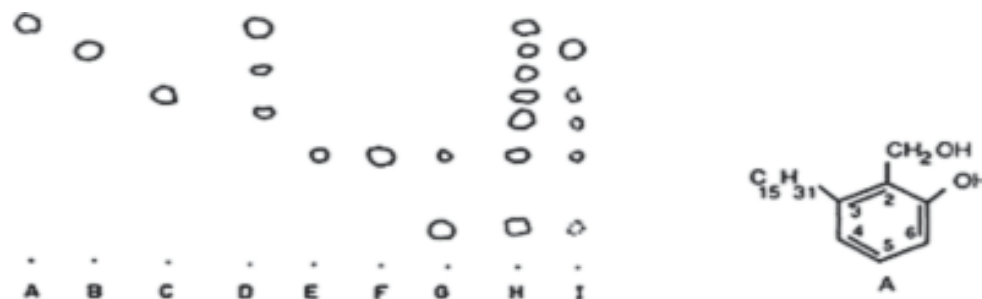


Figure 3 TLC of methylols of 3-pentadecylphenol. (From Ref. 32.)

Table 15 R_f Values of Homologous Alkylsalicylic Acids and Their Derivatives on Silica Gel G^a

Compound	R_f	Compound	R_f
2-Carboxy-3-pentadecylphenol	0.56 (B)	3-Pentadecylcatechol	0.57 (C)
6-Carboxy-3-pentadecylphenol	0.44 (B)	6-Methylol-3-pentadecylanisole	0.66 (C)
2-Methylol-3-pentadecylphenol	0.39 (B)	2-Methylol-3-octylphenol	0.31 (C)
6-Methylol-3-pentadecylphenol	0.37 (C)	6-Methylol-3-octylphenol	0.22 (C)
6-Methylol-3-pentadecylanisole	0.24 (C)	2-Methylol-3- <i>n</i> -butylphenol	0.29 (C)
6-Carbonylmethoxy-3-pentadecylphenol	0.70 (C)	6-Methylol-3- <i>n</i> -butylphenol	0.15 (C)
		2,6-Di(di- <i>n</i> -propylaminomethyl-3-pentadecylphenol	0.51 (C)
6-Di- <i>n</i> -propylaminomethylpentadecylphenol	0.84 (D)		
4,6-Di(di- <i>n</i> -propylaminomethyl)3-pentadecylphenol	0.66 (D)		

^aSolvents: B, CHCl₃-EtOAc-HCO₂H (95:5:2); C, CHCl₃-EtOAc (90:10); D, EtOAc-light petroleum (40–60°C)-NH₄OH (0.88) (50:50:7).

Source: R_f values for amino compounds from Ref. 121a.

Table 16 R_f Values of Phenolic Wood Extractives on Silica Gel G (I, II, III) and on Cellulose (IV)^a

Compound ^b	Solvent			
	I	II	III	IV
R—CO—CO—CH ₃	0.47	0.52	0.83	0.74
R—CO—CH(OH)CH ₃	0.30	0.37	0.77	0.72
R—CH(OH)CH(OH)CH ₃	0.14	0.30	0.84	0.72
R—CH(OH)CH(OH)CH ₂ OH	0.02	0.06	0.50	0.86
R—CO—CH ₂ CH ₃	0.44	0.61	0.85	0.59
R—CH(OH)CH ₂ CH ₃	0.30	0.50	0.90	0.79
R—CH ₂ CH ₂ CH ₃	0.59	0.70	0.93	— ^c
R—CH ₂ COCH ₃	0.44	0.60	0.93	0.85

R—CH ₂ CH(OH)CH ₃	0.29	0.42	0.85	0.69
R—COCH ₂ CH ₂ OH	0.20	0.32	0.77	0.65
R—CH(OH)CH ₂ CH ₂ OH	0.10	0.18	0.75	0.82
R—CH ₂ CO—CH ₂ OH	0.32	0.43	0.70	0.88
R—COCH ₃	0.39	0.58	0.93	0.70
R—CH(OH)CH ₃	0.31	0.45	0.93	0.79
R—CHO (vanillin)	0.40	0.59	0.93	0.75
R—CH ₂ OH	0.35	0.40	0.72	0.82
R—CO ₂ H	0.23 (S)	0.23 (S)	0.17 (S)	0.42
R—CH ₂ CH—CH ₂	0.61	0.67	0.93	—
R—CH—CHCH ₃	0.57	0.65	0.93	—
R—CH=CHCHO (coniferaldehyde)	0.47	0.55	0.93	0.49
R—CH=CHCH₂OH (coniferyl alcohol)	0.37	0.40	0.76	0.65
R—CH=CHCO₂H (ferulic acid)	0.15 (S)	0.13 (S)	0.35 (S)	0.31

^aI, C₆H₆-C₂H₂OH (150:22); II, C₆H₆-Me₂CO (3:2); III, MeOH-CHCl₃ (3:7), IV, 2% aq. AcOH. S, streaked.

^b(R=3-methoxy-4-hydroxyphenyl).

^cUndetected.

Source: Adapted from Ref. 110.

Table 17 *R_f* Values of Hydroxy- and Methoxyflavones on Silica Gel G

Flavone	C ₆ H ₆ -MeOH-AcOH (43:3:2)	CHCl ₃ - <i>n</i> -C ₆ H ₁₄ - MeOH (40:40:3)
5,7-Dihydroxy	0.60	0.47
5-Hydroxy-7-methoxy	0.84	0.85
5,7,4'-Trihydroxy	0.20	0.07
5,7-Dihydroxy-4'-methoxy	0.57	0.44
5,7,3',4'-Tetrahydroxy	0.05	0
5,7,4'-Trihydroxy-6-methoxy	0.24	0.11
5,4-Dihydroxy-6,7-dimethoxy	0.38	0.32
5,6,3',4'-Tetrahydroxy-7-methoxy	0.04	0.02
5,7,3',4'-Tetrahydroxy-6-methoxy	0.12	0.04
5,3',4'-Trihydroxy-6,7-dimethoxy	0.23	0.14
5,4'-Dihydroxy-6,7,3'-trimethoxy	0.44	0.53

5-Hydroxy-6,7,3',4'-tetramethoxy	0.58	0.83
5,4'-Dihydroxy-6,7,8-trimethoxy	0.44	0.47
5,3',4'-Trihydroxy-6,7,8-trimethoxy	0.30	0.25
5,4'-Dihydroxy-6,7,8,3'-tetramethoxy	0.49	0.62
5-Hydroxy-6,7,8,3',4'-pentamethoxy	0.63	0.88

Source: Adapted from Ref. 113.

compounds produced. Diazotized 4-nitroaniline, or the preferred reagent, anthraquinone-1-diazonium chloride (Fast Red Salt AL) (131), were reacted with phenol, the isomeric methylphenols, and dimethylphenols, and the colored azo products were separated on silica gel G impregnated with potassium carbonate (86). Using this technique, separations otherwise impossible with the free phenols were achieved. A wide scope exists for quantification procedures with greatly improved sensitivity. 4-(*N,N*-Dimethylaminophenyl)-4'-azobenzoates of various phenols have also been used (132).

E. Quantification

Most studies in TLC have been qualitative, and considerable experimentation may be necessary to obtain quantitative results, although excellent commercial layers and new equipment have mitigated the problem. Phenolic compounds have been analyzed by spectrophotometry "off the plate" at 725 nm (133) by means of Folin-Ciocalteu reagent, essentially sodium tungstate and molybdate with lithium sulfate (134). Chlorophenols have been quantified after reaction with dansyl chloride and separation (135). Channel TLC (linear relationships between spot lengths and concentrations of 1–8 $\mu\text{g}/\text{spot}$) has been used (136). The phenols in cashew nut shell liquid have been quantified by "off-the-plate" and "on-the-plate" methods with a flying-spot scanning procedure and densitometry (237) and the distribution of unsaturated compounds by TLC/MS (138). Catecholamines and their metabolites in urine have been quantified (12) and determined by "on-the-plate" fluorimetry (139). Densitometric determinations of vanillylmandelic acid (4-hydroxy-3-methoxymandelic acid) by direct urine application, TLC separation, and derivatization (139a), and of vanillylmandelic acid, homovanillic acid, and 5-hydroxyindoleacetic acid in urine by a simple TLC method that has been compared with the more elaborate HPLC method have been described (139b). Hindered phenols have been analyzed by densitometry (140) and a quantitative method proposed for BHT in chewing gum bases (140a). The antiseptic 4-chloro-3,5-dimethyl-phenol in synthetic products has been determined by TLC scanning (140b). Semiquantitative determinations have been made of the coupling products of 4,4'-diazidostilbene-2,2'-disulfonic

Table 18 R_f Values of Catecholamines and Their Relatives^a

Compound	OPT1- UPC ₁₂		Sil C ₁₈	Sil C ₁₈ + 14% NDPC		RP-18+ 4% HDBS	AWP+ CaSO ₄
	a	b	c	d	e	f	g
1. 1-Phenylethylamine	0.29	0.28	0.54	0.65	0.26	0.30	0.42
2. 2-Phenylethylamine PE	0.29	0.27	0.51	0.56	0.18	0.24	0.28
3. 2-(4-Hydroxyphenyl)ethylamine	0.54	0.47	0.68	0.70	0.44	0.66	0.42
4. 2-(3, 4-Dihydroxyphenyl)ethylamine	0.66	0.61	0.74	0.68	0.16	0.75	0.42
5. 2-(3-Methoxy-4-hydroxy) PE	0.44	0.26	0.66	0.72	0.46	0.62	0.21
6. 3,4-Dihydroxy- α -(methylaminomethyl)-benzyl alcohol	0.70	0.68	0.77	0.67	0.26	0.88	—
7. 3,4-Dihydroxy- α -(aminomethyl)benzyl alcohol	0.84	0.84	0.83	0.67	0.25	0.87	0.57
8. 1-Methyl-2-phenylethylamine	0.26	0.18	0.48	0.55	0.14	0.24	0.17
9. α -(Aminoniethyl)benzyl alcohol	0.50	0.47	0.62	0.38	0.44	0.46	
10. 2-(3, 4-Dimethoxyphenyl)ethylamine	0.14	0.10	0.50	0.67	0.34	0.44	0.06
11. 3-Methoxy-4-hydroxy- α -(aminomethyl)-benzyl alcohol	0.69	0.55	0.79	0.78	0.70	0.77	0.40
12. 4-Hydroxy- α -(aminomethyl)benzyl alcohol	0.78	0.74	0.79	0.76	0.70	0.78	0.56
13. α -(1-Aminoethyl)benzyl alcohol	0.39	0.28	0.55	0.56	0.29	0.22	0.41
14. <i>threo</i> - α -(1-Aminoethyl)benzyl alcohol	0.34	0.23	0.54	0.56	0.21	0.32	0.44
15. α -(1-Methylaminoethyl)benzyl alcohol	0.26	0.16	0.53	0.55	0.16	0.29	0.27
16. 4-Hydroxy- α -(methylaminomethyl)benzyl alcohol	0.63	0.54	0.72	0.75	0.52	0.74	0.48
17. 3-Methoxy-4-hydroxy- α -(methylamino-methyl)benzyl alcohol	0.53	0.35	0.69	0.75	0.52	0.72	0.27
18. 2-(5-Hydroxy)phenylethyldimethylamine	0.29	0.16	0.60	0.52	0.13	0.54	0.17

^aSolvents: a, 1 M AcOH+3% KCl in H₂O; b, 1 M HCl+3% KCl in H₂O; c, 1 M AcOH in H₂O-MeOH (20%); d, 1 M AcOH in H₂O-MeOH (30%); e, 0.5 Na₂CO₃ in H₂O-MeOH (30%); f, 1 M AcOH+1 M HCl in H₂O-MeOH (40%); g, 1 M NH₄NO₃ in H₂O. 3, tyramine; 4, dopamine; 5, 3-methoxytyramine; 6, epinephrine (adrenaline); 7, noradrenaline; 8, amphetamine (benzedrine); 11, *nor*-metanephrine; 12, octopamine; 13, *nor*-ephedrine; 14, *nor*-pseudoephedrine; 15, ephedrine; 16, synephrine; 17, metanephrine; 18, hordenine.

Source: Adapted from Ref. 102.

acid with phenols (59) and with fast dye salts (141). Detection limits were found with various spray reagents (127). Phenols occurring in water were quantified by in situ TLC (142) and by coupling with diazotized 4-nitroaniline (21). A comprehensive study of the chromatographic properties of 39 phenols by reversed-phase TLC has been applied to the screening of drinking water (21a). Vanadium pentoxide and dichlorofluorescein were used for the in situ determination of phenols in water (143). TLC and HPLC methods for the analysis of phenolic compounds in wine have been compared. The agreement was satisfactory, with a recovery of 94–104% (143a).

Chromatographic (TLC, GC-MS) and spectrophotometric methods for the determination of pentachlorophenol in wood have been compared (21b). Phenolic acids in plant material have been quantified (92), and medicinally interesting phenolic acids have been separated from Lamiaceae (*Lycopus europaeus*) by multiple gradient development (92a), by densitometry (53), and by UV spectrophotometry (53a).

Phenolic compounds contained in pharmaceutical preparations were studied quantitatively (144). Paracetamol has been determined in drug preparations that also contained pseudoephedrine and chlorpheniramine maleate (144a). A rapid method was described for paracetamol in serum by TLC scanning (144b) and for *p*-aminophenol in paracetamol (144c). Ferulic acid (4-hydroxy-3-methoxycinnamic acid) of Chinese pharmaceutical interest has been TLC quantified in Antike capsules (144d), in *Leonurus* herb (144e), and in *Arenaria kansuensis* (144f).

Phenolic compounds such as 9-tetrahydrocannabinol in Indian hemp were quantified by spectrophotometry of the derived antipyrylquinoneimine dyes of the colored product with Fast Blue B (145), catechin in cube gambir (146) by densitometry, phenolic compounds in *C. triquetra* by fluorodensitometry (147), and usnic acid in *Usnea florida* by densitometric HPTLC (147a). Mangiferin in Chinese Jingzu Gaintaishi capsules (147b) and thymol and carvacrol in thyme oil have been determined by TLC densitometry (147c) and the method compared with a colorimetric procedure.

F. Application of TLC to the Analysis of Phenolic Compounds in Biochemical, Environmental, Industrial, and Consumer Products

In this section some of the major work in the last 30 years on the applications of TLC of phenolic materials has been selected and summarized in Table 19. Work of a more basic type has outpaced applied work, as can be seen from Table 20, which lists the distribution of some of the publications on mono- and polyhydric phenols and phenolic acids over the same period of time. It is probable that more work has been carried out than has been published.

II. AROMATIC CARBOXYLIC ACIDS

There is considerable overlap between work on phenolic acids and that on aromatic carboxylic acids. Relatively few aromatic acids other than benzoic acid and the isomeric dicarboxylic acids are of major industrial significance. Hydroxybenzoic acids, as either salicylic, 4-hydroxybenzoic, or gallic acid (3, 4, 5-trihydroxybenzoic acid), tend to appear under both phenolic and aromatic acid classifications. In this section, as with the previous

one on phenols, a summary is given of the systems that have been used and the applications to TLC in the analysis of derivatives of aromatic acids.

A. Chromatographic Properties

In this section the chromatographic properties of aromatic acids are summarized as shown by their R_f values on a variety of layers and in various solvents. Table 21 lists some of the systems that have been employed. Figures 4 and 5 depict the influence of percent formic acid in aqueous or diethyleneglycol monoethyl ether solution, respectively, together with the solvent benzene in both cases, on the R_f values on silica gel G for some substituted aromatic acids (156), namely, (1) benzoic and (2) 2-chloro-, (3) 3-chloro-, (4) 4-chloro-, (5) 2-hydroxy-, (6) 3-hydroxy-, (7) 4-hydroxy-, (8) 2-nitro-, (9) 3-nitro-, (10) 4-nitro-, (11) 2-amino-, and (12) 4-aminobenzoic acids. Guinchard et al. (156) examined the influence of the type of chromatographic tank, and the figures shown relate to the use of a conventional TLC tank. The best separations were obtained with little water or glycol either present. By contrast, Fig. 6 shows the influence of a variety of solvents on the separation on silanized silica gel (RP-8) in the presence of the ion-pair reagent tetramethylammonium bromide of eight 2-substituted benzoic acids having hydroxy, acetoxy, carboxy, nitro, methyl, amino, and chloro groups (162).

An ion-pair reagent was not essential for separation, and it was found that the optimum concentration was probably below 0.01 mol/L. Different chain lengths up to C_4 caused minor modifications in the order of R_f values for the eight acids. For the aromatic dicarboxylic acids in chloroform-tetrahydrofuran (2:1) on Silufol (175), the R_f order found was isophthalic acid (0.5), terephthalic acid (0.49), 2-hydroxyethyl isophthalate (0.33), 2-hydroxyethyl terephthalate (0.35), and the bis ester (both 0.18). C_2 and cyanopropyl-bonded high-performance silica gel layers in conjunction with ion-pair reagents were employed to separate dihydroxybenzoic acids (175a, 175b). Derivatization of alkanecarboxylic acids with fluorescent agents such as 4-bromomethyl-7-methoxycoumarin has been described, an application that could be relevant to arylpropionic acids and relatives (175c).

Table 19 Examples of Applications of TLC to Phenolic Separations in Biological, Environmental, and Industrial Products

Subject group	Phenolic type	Adsorbent	Solvent	Detection	System	Ref.	Observations
Lindane metabolism in urine	Chlorophenols	Silufol	$n\text{-C}_6\text{H}_{14}$ – C_6H_6 – EtOAc (6:4:1)	$\text{AgNO}_3/\text{Me}_2\text{CO}$, UV	Adsorption, 1-D	57	Sensitivity 1 ppm (combined GC/TLC)
Catecholamine metabolism (urine)	Phenolic acids	Silica gel F_{254}	CHCl_3 – PrOH – HCO_2H (50:10:5)	Fluorimetric	Adsorption, 1-D	12 139	HPTLC also used
Cigarette	Phenols	Micropol	C_6H_6 –	Diazotized	Adsorption	71	Catechol

smoke condensate		yamide	MeOH–AcOH (45:8:4)	benzidine	1-D	148	located 36 compds. screened
Water	Nitro phenols	Silufol	—	—	Adsorption	149	Toxicity discussed
Water	Phenols	Silica gel–Al ₂ O ₃ (60:40)	(1) C ₆ H ₆ (2) i-Pr ₂ O	Diazotized sulfanilic acid	Adsorption, 2-D, sandwich tank	35 150	ppb Phenols in surface waters
Pharmaceutical preps.	Phenolic compounds	Silica gel	C ₆ H ₆ –Me ₂ CO (3:1)	Antipyrine quinoneimine	HPTLC	144	Guaiacol determined
Beverages (apple juice)	Plant phenols	Cellulose	(1) HCO ₂ H–H ₂ O (2:98) (2) BuOH–AcOH–H ₂ O (30:35:15)	Diazotized sulfanilic acid	Partition 2-D	10	Precolumn used (polyamide)
Beer	Beer phenolics	Polyamide–cellulose (80:20)	48 different types	—	Adsorption	26	Continuous aging Change noted
Medical pharmaceutical rubber	Phenolic antioxidants	Silica gel	C ₆ H ₆ , C ₆ H ₆ –C ₆ H ₅] ₄ (75:25) C ₆ H ₆ –EtOAc–Me ₂ CO (100:5:1)	<i>N</i> -Chloro-3,6dichloro- <i>p</i> -benzoquinonemonooxime	Adsorption	3	Eight hindered phenols found (in 102 samples)
Elastomers	Phenolic antioxidants	Silica gel	C ₆ H ₆ and C ₆ H ₆ –C ₆ H ₁₄ (8:1)	<i>N</i> -Chloro-3,6dichloro- <i>p</i> -benzoquinone monooxime	Adsorption	151	Phenols and phosphites
Edible fats	NDGA, quercin, silica gel phenolic acids	Silica gel	CHCl ₃ –C ₆ H ₆ –MeOH–LP (60:20:15:5)	Exposure to air	Adsorption	18	Sensitive for detection of the compounds in fats
Hair dyes	Phenols and arylamines	Silica gel H	CHCl ₃ –C ₆ H ₆ –BuOH–AcOH (40:10:7:1)	4-Me ₂ NC ₆ H ₄ –CHO	Adsorption	152	Analysis of phenols and arylamines in oxidation dyes: four

			BuOH– (HOCH ₂) ₂ – AcOH–H ₂ O (10:2:1:10)				solvents examined; quantitative determination
Pesticides	Organophosphorus compounds	Silica gel G	C ₆ H ₁₄ – xylene– EtOAc– Me ₂ CO (50:15:5:18)	Exposure to I ₂	Adsorption	153	Silica gel impregnated with a metal salt or phenol
Herbicides	Chlorophenols, aromatic amines	Several	Several	Reagents or UV	Adsorption	154	45 compounds, prelim, enrichment f rom water a nd separation into (a) neutral/ basic, (b) acidic fractions
Phenolform aldehyde resins	Phenol	Silica gel G F ₂₅₄	CHCl ₃ – CCl ₄ (7:1)	Me ₂ CO– BuNH ₂ (1:1) spray	Adsorption	155	Reaction with 2,4-dinitrobenzen esulfonylchloride, then TLC (3% phenol detectable)

Table 20 Subject Distribution of Specific TLC
Studies 1966–2001

Analytical interest	Worldwide publications
Monohydric phenols	102
Polyhydric phenols	14
Phenolic acids	58
Phenolic antioxidants	14
Phenols and the environment	14
Phenols and beverages	8
Metabolic studies	9
Clinical studies	3
Phenolic resins	6
Phenolic and medicinal products	26
Phenolic plasticisers and elastomers	4
Pesticides	4
Cosmetic products	3

Herbicides	3
Fossil fuel aspects	2
Pyrolysis studies	3
Surfactants	2
Organic reactions	8

B. Application of TLC to the Analysis of Aromatic Carboxylic Acids Used as Preservatives or as Medicinal Agents

Benzoic acid, alkyl 4-hydroxybenzoates, and 3,4,5-trihydroxybenzoates are used as preservatives, and salicylic acid and its acetyl derivatives appear in numerous pharmaceutical preparations. The following references summarize some of the investigations on the analyses of aromatic carboxylic acid products. The simultaneous detection of benzoic acid, citric acid, and lactic acid in soft drinks (168) and quantitative HPTLC determination of sorbic, benzoic, and dehydroacetic acids in beverages, without extraction or cleanup (168a), of benzoic and sorbic acid preservatives (168b), and of benzoic acid in textile fibers (169), in Chinese traditional medicine from *Daemonorops draco* (170), and in foods (171–173) has been effected, and fluorimetric detection and quantification (173a) used. TLC of vanillic acid in Chinese natural sources (173b) and of 4-azido-2,3,5,6-tetra-fluorobenzoic acid in mixtures (173c) have been studied. Alkyl 4-hydroxybenzoates have been separated by circular HPTLC (174), on Silufol (preferred visualization, iodine vapor) (175), by reflectance spectrophotometry at 272 nm on sil G HR UV plates (176), and by remission photometry (177). Hydroxybenzoic acids in grape leaves, berries, and wine have been separated on micropolyamide layers (178), and preservatives in wine (179) on polyamide layers containing a fluorescent pigment. Silanized silica gel H (F₂₅₄) has been employed for the quantitative analysis of alkyl 4-hydroxybenzoates by “off-the-plate” differential spectrophotometry (180). Salicylic acid and its derivatives have been analyzed by a variety of TLC procedures (181–190), and both free salicylic acid and aspirin (190a) and acetylsalicylic acid in pharmaceutical products have been analyzed by HPTLC (190b). Sources of error have been discussed (191) and manipulative problems examined (192). Eighteen new visualizing reagents were examined for the detection of aromatic carboxylic acids after TLC on silica gel, on a mixture of silica gel and kieselguhr, and on polyamide (192a).

In environmental applications, rosmarinic acid (193), gallic acid in fermentation liquors (193a), and humic acid in river water (194) have been investigated. Gallic acid and bergenin in *Bergenia ligulata* have been determined by HPTLC (194a) and quantitatively in Lihoule tablets by TLC scanning (194b). In the Chinese folk medicine Jiuniuzao, the total tannins and gallic acid

Table 21 TLC Separation of Aromatic Carboxylic Acids

Aromatic carboxylic type	Adsorbent	Solvent	Detection	Ref.	Observation
Amino, chloro, hydroxy, nitro	Silica Gel H F ₂₅₄	Benzene-formic acid (various proportions)	UV	156	The separation depends on percent water in solvent
Terephthalic, isophthalic, and esters	Silica gel G	CHCl ₃ -THF (2:1)	UV	158	—
Carboxylic from coal oxidation	Silufol	MeOH-H ₂ O-NH ₃ , i-PrOH-H ₂ O-NH ₃	UV	159	Rapid analysis achieved
Hydroxybenzoic	Silica gel G with Fe ³⁺	Water	Visible	160	%Fe ³⁺ not stated
Hydroxybenzoic	Alumina	Water	Bromocresol green (UV)	94a	Results compared with Fe ³⁺ on silica gel
Alkoxybenzoic	Silica gel	MeOH, 0.1 M phosphate buffer MeCN, 0.1 M phosphate buffer		161	<i>R_m</i> values correlated with %MeOH, MeCN
Hydroxy, dihydroxy, dinitro	Silica gel+Ag ₂ O	EtOH-EtOAc-NH ₄ OH (9:3:2) EtOH-H ₂ O-NH ₄ OH (100:12:16)	—	107	Order of <i>R_f</i> values in both solvents: 2,6-di-OH>3,5-dinitro>2-OH>H>2,4-di-OH>3-OH>4-OH
2-Me, Cl, OH, H, OAc, NO ₂ , CO ₂ H, NH ₂	Silica gel RP-8 F ₂₅₄ +R4NX	Me ₂ CO-H ₂ O (various proportions)	UV	162	<i>R_f</i> values decrease and increase with ion-pair reagents as H ₂ O increases
Phenoxy, alkanoic	Silica gel RP (HPTLC)	—	1,7-Dichlorofluorescein	163	For partition H ₂ O, HCONH ₂ or liq. paraffin; detection limit 0.5–10 µg
Hydroxy	Silufol U F ₂₅₄	ClCH ₂ CH ₂ Cl-C ₆ H ₅ -HCO ₂ H	UV	164	TLC separation and densitometry

(50:49:9)					11 acids identified
Aromatic carboxylic type	Adsorbent	Solvent	Detection	Ref.	Observation
Di- and poly acids	Cellulose with addn. of silica gel H or polyvinyl alcohol or glass powder	EtOH-25% NH ₃ -H ₂ O-Et ₂ NH (100:16:12:0.5) <i>n</i> -C ₅ H ₁₁ OH-HCO ₂ H-H ₂ O (48.8:48.8:2.4)	AgNO ₃ /vanillin reagent	165	—
Plant acids	BaSO ₄ /CaSO ₄	—	—	166	Good TLC material for detection and quantification of carboxylic acids
2-, 3-, 4-Subst. benzoic acids	Polyamide	Aqueous α -cyclodextrin	—	167	18 acids separated; <i>R_f</i> values dependent on α -cyclodextrin concentration

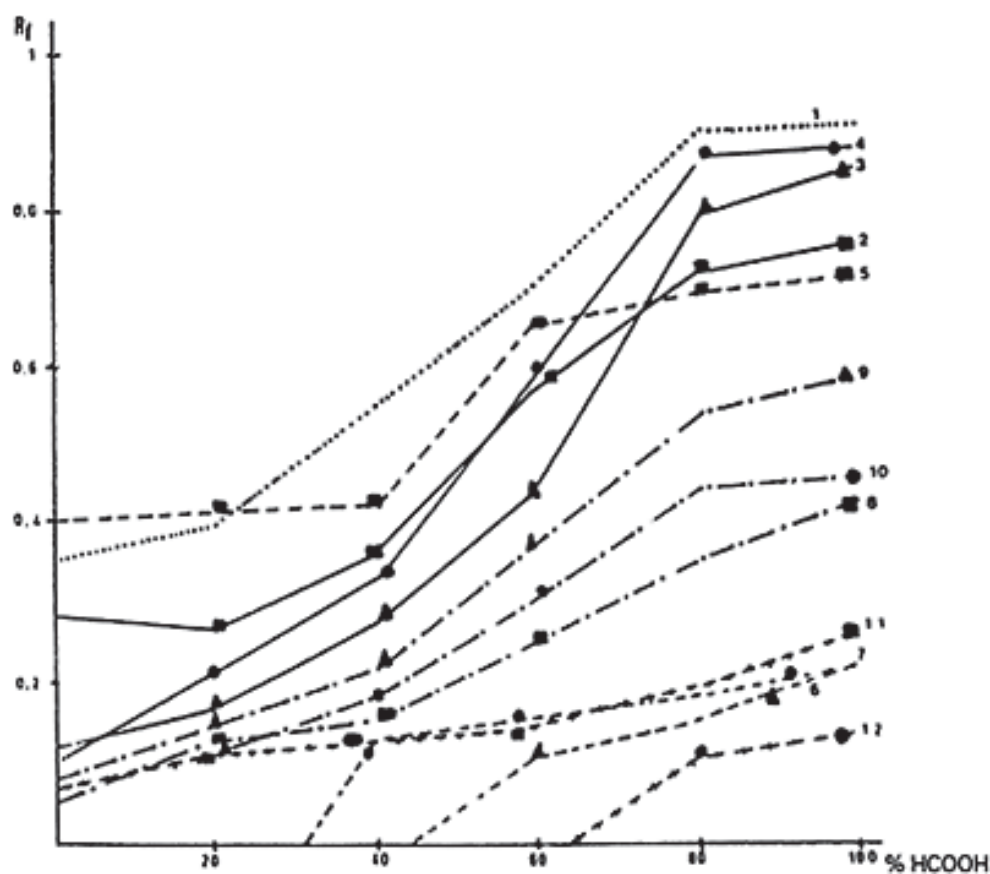


Figure 4 R_f values of aromatic carboxylic acids (1–12; see text) in benzene with aqueous formic acid. (From Ref. 156.)

have been analyzed by TLC scanning (194c). Chlorogenic acid in Xuandong powder, obtained from *Scrophularia ningpoensis*, has been analyzed quantitatively by TLC (194d) and separated by TLC in four kinds of eucommias (194e). It has also been determined in Yidan tablets by TLC scanning (194f). The occurrence of hippuric, mandelic, and phenylglyoxylic acids in urine has been studied (195). The TLC of derivatives of phenolic acids less common in plants, such as phloretic, veratric, homoprotocatechuic, and homogentisic acid (195a), and of phenolic acids in *Polygonum* species (195b) have been studied. Orsellinic acid and methyl orsellinate, accompanied by lichesterol, have been isolated by TLC from a Korean source, *Umbilicaria esculenta* (195c). 2-Aminobenzoic acid, β -D-(glucopyranosyl-1)-2-aminobenzoate, and a disaccharide relative in cell suspension cultures of *Solanum mammosum* have been determined by TLC densitometry (195d). 4-Aminobenzoic acid, which can be produced by hydrolysis of the pharmaceutical procaine, has been determined by HPTLC (195e). Certain racemic 2-arylpropionic acids have been resolved by two-dimensional TLC on silica gel impregnated with (–)-brucine (195f).

III. INDOLES

Indole structures exist in many natural products as simple compounds such as skatole or tryptophan or in many groups of complex alkaloids in the auxins and indole dyes. This section is concerned largely with the thin-layer chromatography of simple indoles and derivatives. The TLC

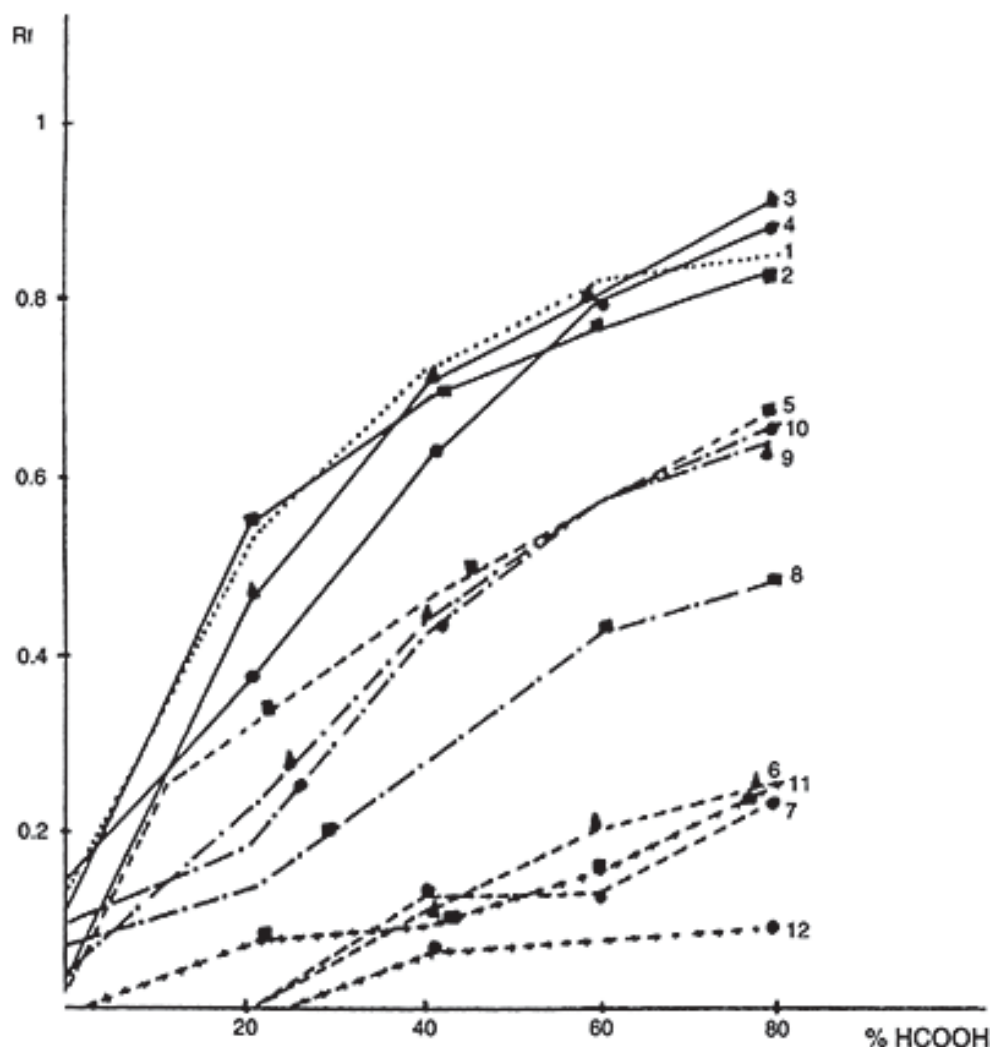


Figure 5 R_f values of aromatic carboxylic acids (1–12; see text) in benzene with formic acid in $\text{HOCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OEt}$. (From Ref. 156.)

of indole compounds and metabolites under adsorption conditions has been reviewed extensively (196), and in this section the newer adsorbents and procedures that have been introduced in the last two decades are listed.

A. Chromatographic Properties

As with most groups of compounds, silica gel has generally proved to be the most widely used adsorbent, although cellulose, alumina, and poly amide layers have sometimes had important uses. The main contributions are summarized in Table 22. The R_f values for alkylindoles, hydroxyindoles, and a series of 3-substituted derivatives on silica gel are listed in Table 23 with five solvent systems (197, 203). Table 24 records the R_f values for

some of the same compounds on cellulose with two of the same solvents. The R_f values of a group of 3-substituted indoles on silanized silica gel with and without the addition of *N*-dodecylpyridinium chloride are shown in Table 25. In addition to these types of layers, polyamide has been employed (205). Impregnation of silica

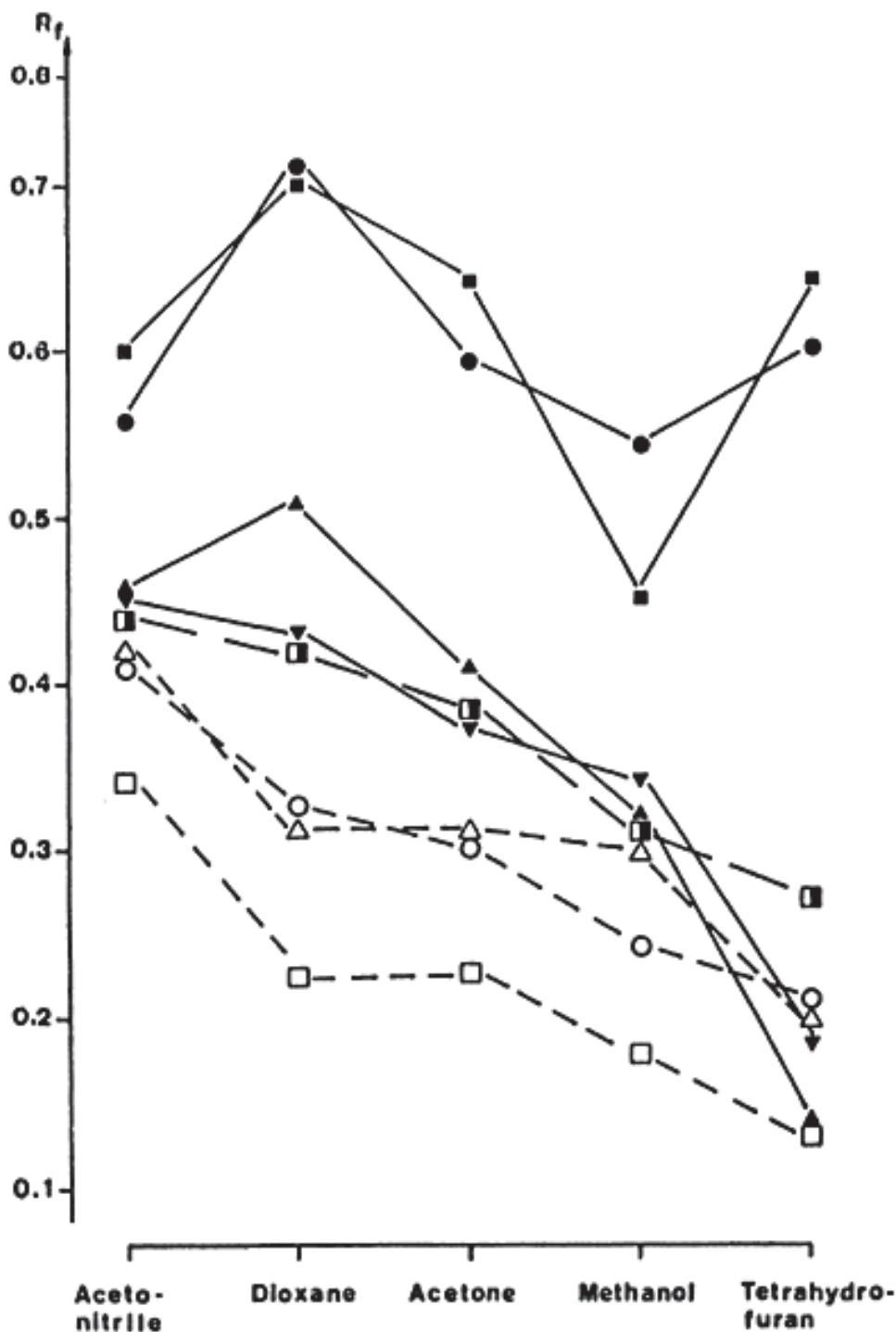


Figure 6 R_f values of 2-substituted benzoic acids in selected solvents. The

solvent composition organic component–water (40:60 v/v) with addition of 0.1 M tetramethylammonium bromide (*pK* value in parentheses): (○) benzoic acid (4.19); (▲) 2-hydroxy- (2.97); (◼) 2-acetoxy- (3.5); (■) 2-carboxy(2.91/5.59); (●) 2-nitro- (2.16); (□) 2-methyl- (3.91); (Δ) 2-amino- (6.97); and (♥) 2-chlorobenzoic acid (2.92). (From Ref. 162.)

Table 22 TLC Systems and Conditions for the Separation of Indole Derivatives

Compounds	Adsorbent	Solvent ^a	System	Detection	Ref.	Observations
Simple indole derivatives	Silica gel Silica gel F ₂₅₄	CHCl ₃ – MeOH– AcOH (A) (75:35:5) (50 min) CHCl ₃ –96% AcOH (B) (95:5) (40 min) CH ₃ CO ₂ Me– i-PrOH–MH ₃ (C) (45:35:20) (20 min) i-ProOH– NH ₃ –H ₂ O (D) (100:5:10) (20 min)	1-D ads.	(1) van Urk (2) Prochazka (3) Ehrlich	197	Solvent B gave best separation. Silica gel F ₂₅₄ commercial plates gave better results than self-prepared layers. Van Urk: 4-Me ₂ NC ₆ H ₄ CHO (1 g) in cone. HCl (50 mL), added. Prochazka: 35% formalin (10 mL) and 25% HCl (120 mL) treated with ethanol (20 mL). Ehrlich: 4-Me ₂ NC ₆ H ₄ CHO (1 g) in conc. HCl (25 mL) and methanol (75 mL). Plate warming required.
Indoles and auxins as 2,4-dinitrophenyl derivatives	Silica gel	11 solvent systems, e.g., i-Pr ₂ O– HCONMe ₂ (80:20)	1-D ads.	Self-indicating	198	Indole derivative (10 mg) in CH ₂ Cl ₂ (2 mL) and 99% HCOOH (1 mL)+2,4(NO ₂) ₂ C ₆ H ₃ SCl equimolar proportion and kept 1–2 h at 25°C. No change in color with Ehrlich. Salkowski 2,4,7-trinitrofluorene-9-one or 2,3-dichloro-5,6-dicyanobenzoquinone.

Tryptophan, indole, indole-3-acetic acid in presence of 18 other amino acids	Silica gel	BuOH–AcOH–H ₂ O	1-D ads.	20 vol % H ₃ PO ₄ in 95% EtOH (40 min at 110°C)	199	Method more convenient and as sensitive as using Me ₂ NC ₆ H ₄ CHO.
Indole compounds	Silica gel	CHO ₃ –AcOH (95:5); MeCO ₂ Me–i-PrOH–NH ₃ (45:35:20)	1-D ads.	Glyoxylic acid	200	Characteristic different colors useful for identification of tryptophan metabolites.
Indole derivatives	Silanized silica	Six solvents: three with SiO ₂ C ₁₈ , three with SiO ₂ C ₁₈ +4% N-DPC	Reversedphase+ 4% NDPC	Ehrlich	201	Untreated Sil C ₁₈ -50 and the same impregnated with 4% N-DPC. Effect of pH examined in the six solvents used.
Indole and catechol	Cellulose	BuOH–(5 M)AcOH (100:35) (A); Me ₂ CO–(2.5 M)AcOH–MeCOEt (1:1:2) (B)	1-D	4-Dimethylaminocinnamaldehyde	202	Tryptophan metabolites were separated, identified, and determined densitometrically.
Natural and synthetic indoles	Silica gel	CHCl ₃ –CCl ₄ –MeOH (50:25:25)	1-D	UV fluorimetry	203	In situ fluorescence spectroscopy used in the excitation range (max 285–310 nm) and emission (max 345–360 nm). Spraying the plates with 0.1 N H ₂ SO ₄ and Me ₂ SO enhanced indole fluorescence.
Biogenic indole compounds	Cellulose or Al ₂ O ₃	Sodium citrate or sodium acetate and sodium	1-D	Phosphorimetry	204	After development, the plates are completely dried under N ₂ and dinned in molten

iodide
solutionsparaffin wax.
Phosphorescence
stable for 3 h.^aRequired development time in parentheses.**Table 23** R_f Values of Indole Derivatives on Silica Gel^a

Compound	Solvent system					Van Urk Ehrlich
	A	B	C	D	E	
Indole	0.88	0.78	0.86	0.80	—	Violet
2-Methylindole	0.90	0.68	0.92	0.75	—	Pink
3-Methylindole	0.91	0.77	0.87	0.73	—	Blue
5-Methylindole	0.95	0.73	0.94	0.77	0.91	Pink
5-Hydroxyindole	0.76	0.22	0.88	0.76	0.89	Mauve
5-Methoxy indole	—	—	—	—	0.93	—
5-Aminoindole	—	—	—	—	0.93	—
(±)-Tryptophan	—	—	—	—	0.87	—
(±)-5-Hydroxytryptophan	0	0	0.16	0.15	0	Blue-gray
Tryptamine	0.24	0.15	0.76	0.42	—	Blue
5-Hydroxytryptamine (serotonin)	0.09	0	0.53	0.17	0	Blue
5-Hydroxy-2-methylindole	—	—	—	—	0.91	Blue
3-Dimethylaminomethylindole	0.16	0	0.74	0.08	—	Light beige
(±)-Acetyl-5-methoxytryptamine (melatonin)	—	—	—	—	0.87	—
Indole-3-aldehyde	0.77	0.17	0.83	0.62	—	Pink
<i>N</i> -Acetyl-5-hydroxy tryptamine	—	—	—	—	0.81	—
3-(2-Hydroxyethylindole (tryptophol)	—	—	—	—	0.79	—
5-Methoxytryptophol	—	—	—	—	0.92	—
5-Hydroxytryptophol	—	—	—	—	0.79	—
Indole-3-acetic acid	0.90	0.36	0.96	0.76	0.37	Blue
5-Hydroxyindole-3-acetic acid	0.51	0.04	0.25	0.26	0.23	Blue
5-Methoxyindole-3-acetic acid	—	—	—	—	0.39	—
3-(3-Indolyl)propanoic acid	0.77	0.51	0.53	0.40	—	Blue
4-(3-Indolyl)butanoic acid	0.81	0.35	0.39	0.35	—	Blue

3-(3-Indolyl)propenoic acid	0.77	0.51	0.53	0.40	—	Blue
(±)-2-Hydroxy-3-(3-indolyl)propanoic acid	0.45	0.09	0.56	0.35	—	Blue
(±)-5-Methyltryptophan	0.90	0	0.3	0.27	—	Blue

Solvents: A, chloroform–methanol–acetic acid (75:20:5); B, chloroform–95% acetic acid (95.5); C, methyl acetate–isopropanol–ammonia (45:35:20); D, isopropanol–ammonia–water (100:5:10); E, chloroform–carbon tetrachloride–methanol (50:25:25).

Source: Columns A, B, C, D adapted from Ref. 197; column E from Ref. 203.

gel G with a variety of sodium salts has been investigated for the separation of aromatic amines, including indole (205a). Biogenic amines, including serotonin, have been examined on silica gel in 23 solvents with detection by means of thiourea (206). A simple and sensitive method for the analysis of norepinephrine, serotonin, and dopamine in rat brain has been quantified (206a). Melatonin (acetyl-5-methoxytryptamine) in Mian Erkang capsules, a Chinese herbal medicine, has been analyzed by HPLC and TLC (206b). Spray reagents have been studied extensively. The Van Urk-Salkowski reagent was examined with 79 indole derivatives on silica gel and was found to be both sensitive and specific for indoles (207). Paraformaldehyde dissolved in slightly alkaline ethanol and the solution neutralized with acetic acid afforded a sensitive spray reagent enabling fluorimetry to be carried out (208). Indoles with α,β -unsaturated aldehydes under acidic conditions give blue-violet salts (209). An initial spray with a 5% solution of ammonium ceric nitrate followed by a second spray with 5% hydroxylamine hydrochloride in 80% acetone gives brown colorations with a wide variety of indoles (124), the sensitivity being between 1 and 10 μg . The R_f values, fluorescence, and minimal detectable concentration of nine hydroxy- and methoxyindoleamines on silica gel by the use of the spray reagent *o*-phthalaldehyde in the presence of

Table 24 R_f Values of Indole Derivatives
(Tryptophen Metabolites)

Compound	Cellulose (MN 300)		4-DMCA ^b Color
	A ^a	B ^a	
(±)-5-Hydroxytryptophan	0.15	0.35	Blue
(±)-Tryptophan	0.33	0.50	Lilac
5-Hydroxy-3(2-aminoethyl)indole (serotonin)	0.35	0.65	Blue
5-Hydroxyindole-3-acetic acid	0.85	0.98	Blue
3-(2-Aminoethyl)indole (tryptamine)	0.71	0.65	Lilac
<i>N,N</i> -Dimethyltryptamine	0.72	0.71	Lilac
<i>N,N</i> -Dimethylserotonin (bufotenine)	0.47	0.75	Blue
5-Methoxyindole-3-acetic acid	0.98	0.98	Blue
Indole-3-acetic acid	0.98	0.98	Lilac

<i>N</i> -Acetyl-5-methoxy tryptamine	0.98	0.98	Blue

^aFor solvents A and B, see Table 23.

^b4-DMCA=4-dimethylaminocinnamaldehyde.

Source: Adapted from Ref. 202.

hydrochloric acid (210) or sulfuric acid (210A) have been described. Formaldehyde with hydrochloric acid has been used for the detection of indole-3-acetic, propionic, and butanoic acids with a pH gradient layer developed with benzene–ethanol (90:10) (211). Indole was separated from carbazole and diphenylamine on silica gel, copper sulf ate-impregnated silica gel, alumina, and cellulose with aqueous and nonaqueous systems (212). The color reactions with six different spray reagents following two-dimensional separations on silica gel H were studied (213) for a wide range of indoles and skatole on cellulose detected by their fluorescence with 2,4-dinitrophenyl-pyridinium chloride (213a). Indole and oxindole alkaloids were separated in 10 neutral and 10 alkaline solvents (214). Indole alkaloids from *Tabernaemontana hilariana* (Apocynaceae) were qualitatively separated and identified (214a). A reagent, 1,2,3,4-tetrahydro-1,4-dioxo-2,2,3,3-tetrahydronaphthalene, has been described for the TLC detection of biogenic amines (214b), and a method for obtaining ninhydrin from oxolin was also listed.

B. Application of TLC of Indoles in Botanical, Biomedical, Biosynthetic, and Medical Studies

Increasing use has been made of thin-layer chromatography methodology involving a whole range of interests. Spectrodensitometric TLC (215) and TLC luminescence studies (216) have been made. Metabolic pathways (217), enzyme inhibition methods for the detection of traces of amines (217a), chemical aspects (218), and HPLC/TLC procedures for identification (219) have been examined. Botanical applications have been studied (220), and biosynthetic and microbiological experiments (221) and horticultural studies (222) have been fundamentally aided through TLC of the indole systems involved. Indoles in physiological processes have been identified (223, 224). Methodology for the multidetection and semiquantitative determination of eight biogenic amines in foods and a rapid quantitative procedure have been described (225, 225a). Adsorption and partition chromatographic TLC methods have been comprehensively studied for the analysis of biogenic amines and alkaloids and their derivatives (225b). A procedure has been developed for the determination of eight biogenic amines in fish (226). The methodology for the separation, detection, and quantitative determination of catecholamines, 5-hydroxytryptamine, and their me-tabolites in biological samples has been reviewed (227).

It is doubtful whether the early pioneers in thin-layer chromatography, such as Egon Stahl, could have had any realization of the extent to which in just one area—phenols, aromatic acids,

Table 25 R_f Values of indole Derivatives on Silanized Silica Gel Untreated and With 4% Af-Dodecylpyridinium Chloride (NDPC)^a

Compound	Sil C ₁₈ -50			Sil C ₁₈ -50+4% (NDPC)		
	A	B	C	D	E	F
Indole	0.16	0.13	0.97	0.07	0.07	0.82
3-(2-Aminoethyl)indole (tryptamine)	0.47	0.02	0	0.22	0.22	0.02
3-(3-Alanyl)indole (tryptophan)	0.64	0.67	0	0.32	0.39	0
5-Hydroxy-3-(2-aminoethyl)indole (serotonin)	0.62	0.08	0	0.42	0.43	0
Indole-2-carboxylic acid	0.34	0.78	0.63	0.05	0.11	0.11
Indole-5-carboxylic acid	0.69	0.89	0.75	0.17	0.33	0.37
3-Indolylacetic acid	0.62	0.84	0.61	0.10	0.21	0.13
5-Hydroxy indole-3-acetic acid	0.78	0.92	0.18	0.22	0.40	0.02
3-(3-Indolyl)propanoic acid	0.22	0.77	0.75	0.07	0.15	0.24
4-(3-Indolyl)butanoic acid	0.14	0.69	0.18	0.05	0.10	0.36
3-Indolylglyoxylic acid	0.61	0.82	0	0.10	0.21	0
3-(3-Indolyl)lactic acid	0.59	0.80	0.04	0.10	0.23	0
3-(3-Indolyl)propenoic acid	0.15	0.74	0.64	0.06	0.13	0.22
3-Indolylacetamide	0.41	0.36	0.34	0.25	0.25	0.16
Ethyl 3-indolyl acetate	0.09	0.07	0.98	0.04	0.04	0.75
Indole-3-aldehyde	0.25	0.18	0.60	0.12	0.12	0.30
3-Indolylacetaldehyde	0.32	0.22	0.96	0.11	0.10	0
3-(2-Hydroxyethyl)indole	0.29	0.23	0.74	0.14	0.14	0.42
3-Indolyl lpropanone	0.24	0.18	0.95	0.11	0.12	0.70
3-Indolylacetonitrile	0.23	0.17	0.95	0.09	0.09	0.68
Isatin(2, 3-dioxoindole)	0.42	0.40	0.78	0.26	—	0.50

^aSolvents: A, water–ethanol–acetic acid (59:40:1); B, 0.1 M ammonia in 40% methanol; C, hexane–ethyl acetate–acetic acid (72:27:1); D, 0.1 M ammonia+0.1 M ammonium chloride in 40% methanol; E, 0.5 M ammonia+0.5 M ammonium chloride in 40% methanol; F, hexane–ethyl acetate–acetic acid (67:32:1).

Source: Adapted from Ref. 201.

and indoles—their methodological studies would be applied. It is in the exploitation of that initial work in current interdisciplinary studies that TLC will foster new achievements.

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30 Steroids

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I. INTRODUCTION

The purpose of this chapter is to present advances in the techniques and applications of steroid thin-layer chromatography (TLC) starting with late 1993, the last year covered in the chapters on this topic in the first and second editions of this Handbook (1, 2). Readers are referred to these earlier chapters for detailed discussions and extensive tabular data on sample preparation, layers, mobile phases, detection, identification, quantification, and applications of steroid TLC based on papers published up to 1994. The references given in this chapter are not comprehensive but were chosen selectively to update the material presented in the earlier editions.

Steroids are natural and synthetic compounds with a cyanopentanoperhydrophenanthrene skeleton and include bile acids, androgens, estrogens, corticosteroids, ecdysteroids, sterols (cholesterol), and vitamin D. The latter two are given only relatively minor coverage in this chapter because their TLC analysis is described further in the chapters on lipids (Chap. 22) and lipophilic vitamins (Chap. 23), respectively.

Thin-layer chromatography continues to be an important method for the determination of steroids because of its inherent advantages: Many samples can be analyzed simultaneously and quickly at relatively low cost, minimal cleanup of samples is required because layers are not reused, and multiple separation techniques and detection procedures can be applied. Detection limits are often in the low nanogram range, and quantitative densitometric methods are accurate and precise. Preparative layer chromatography (PLC) on thick layers allows up to several grams of steroidal substances to be isolated.

An encyclopedia chapter on steroid analysis by TLC was published recently (3). A review on chromatographic procedures, including TLC, for phytoecdysteroids included lists of numerous mobile phases and detection spray reagents (4). A database was presented (5) that contains information on TLC methods for steroids published in pharmacopeias of various countries, including the United States, United Kingdom, and China.

II. SAMPLE PREPARATION

Steroids and their metabolites are analyzed by TLC in a variety of samples such as biological (clinical) samples, plants, pharmaceutical formulations, and raw materials and synthesis and biotransformation products of importance in steroid production. Classical liquid-liquid extraction techniques and solvent partitioning and column chromatography cleanup methods have been used most often to prepare test samples to be applied on TLC plates. In some analyses, steroidal conjugates in samples or extracts are cleaved by enzymatic and/or nonenzymatic hydrolysis methods before or after TLC.

Heating under vacuum was found to accelerate the removal of extractives from plant biological matrices without significant modification in a study of the separation and characterization of steroids in biomass vacuum pyrolysis oils using TLC and gas chromatography coupled with mass spectrometry (GC/MS) (6).

Solid-phase extraction (SPE) with a silica gel column and diethyl ether eluent was used to prepare cholesterol oxidation product samples for silica gel high-performance TLC (HPTLC) analysis using hexane–diethyl ether–ethanol (30:70:3) as the mobile phase. Zones were detected with a 0.2% ethanolic solution of 2,7-dichlorofluorescein as well as under 254 nm ultraviolet (UV) light (7). The use of different numbers of Sep-Pak C-18 cartridges was examined for SPE purification of radiolabeled steroids extracted from blood, liver tissue, and feces prior to TLC assay; the results indicated that recoveries were best when four to six cartridges were used (8).

Mulja and Indrayanto (3) stated that steroid derivatives formed directly on the layer or in solution prior to TLC or HPTLC can often be better separated than the free compounds. They described reactions for acetylation (using acetic anhydride and pyridine), benzylation (benzoyl chloride), propionylation (propionyl chloride), and trifluoroacetylation (trifluoroacetic anhydride). They also stated that steroid critical pairs (such as saturated or unsaturated steroids with one double bond) can be separated better by spotting with 0.1% bromine solution or *m*-chlorperbenzoic acid in chloroform directly at the origin; in the latter case, epoxidation of the double bond occurs. Additional sample preparation methods for particular analyses are described in later sections of this chapter, especially Section VI.

III. LAYERS AND MOBILE PHASES

The following sorbents have been applied for the TLC of steroids according to a recent review (3). Silica gel TLC and HPTLC layers are by far the most frequently used, including those with organic binder, gypsum binder (G layer), no binder (H layer), or fluorescent indicator (phosphor) for fluorescence quenching detection (F-layer), and preparative (P) layers. Silver nitrate is added to silica gel to facilitate separation of compounds differing in unconjugated double bonds (argentation TLC), and impregnation with boric acid or formamide has provided special selectivity for particular steroid separations. Bonded silica gel phases (C-18, C-8, C-2, CN, NH₂, and diol), cellulose (unimpregnated or impregnated with 1,2-propanediol), kieselguhr, alumina (unimpregnated or deactivated with 2.5% acetic acid), polyamide, magnesium oxide, and Celite have also been reported occasionally for separations of steroids in TLC.

Pretreatment with ethanolic ascorbic acid solution has been used to retard oxidation of estrogens on silica gel layers.

Three papers reported fundamental studies of structure—retention relationships using the following layers and mobile phases that are valuable for steroid separations: estradiol and estrone derivatives on silica gel 60 layers with 20–40% ethyl acetate in cyclohexane and 10–30% ethyl acetate in benzene as mobile phases and on C-8 and C-18 modified silica gel layers with methanol–water (8:2) and acetonitrile–water (8:2) mobile phases (9); 18 steroids on alumina layers using dichloroethane–dioxane mixtures as mobile phases (10); and 22 steroids on silica gel 60 with mobile phases that are 20% ethyl acetate or 10% acetone or acetonitrile plus a mixture of hexane–benzene in variable volume proportions (100:0 to 10:90) (11). These papers present tables of R_m values for scores of mobile phases that can be converted to R_f values through the equation $R_m = \log(1/R_f - 1)$ and used to predict separations of the steroids studied.

Although TLC is most often used for separations of steroids, HPTLC layers characterized by smaller sorbent material particle sizes and a reduced layer thickness are being applied more frequently each year. For example, simple extraction followed by a rapid silica gel HPTLC analysis was applied to survey the use of 39 steroidal hormones in cattle fattening, such as estradiol, testosterone, clostebol acetate, nandrolone, stanozolol, and fluoxymesterone, based on analysis of injection site tissue samples (12). The following mobile phases were suggested for separation of the by-products of steroid synthesis on silica gel HPTLC plates: hexane–diethyl ether–dichloromethane (5:9:6), chloroform–hexane–acetone (5:4:1), chloroform–acetone (9:1), cyclohexane–ethyl acetate–ethanol (24:16:1), toluene–methanol–water (1:15:4), and hexane–dichloromethane–acetonitrile (8:2:1) (13).

Ultrathin (10 μm) layers made from a monolithic structure based on a silica gel matrix have been described by workers from Merck (14). These layers do not contain separate particles, nor is binder needed to fix the layer to the glass plate backing. They have meso- and macropores, with fine capillaries penetrating the layer. The separation of steroids by ultrathin layer chromatography (UTLC) with these layers is shown in Fig. 1.

The following modified method for preparation of layers for argentation TLC was described (15). A silica gel 60 layer is developed with an aqueous solution of silver nitrate (2.0 g) in distilled water (5 mL); the plate is dried and activated with a heat gun for 2 min. R_f values for 32 steroids and triterpenes on these layers, of which silver nitrate constituted about 25% of the adsorbent, are shown in Table 1.

An improved technique was described for argentation TLC separation of most of the androgen C-19 steroids formed by mammalian cells that can be used for analysis of plasma, urine, amniotic fluid, and cell culture medium (16). Silica gel plates were dipped into a 4.06% solution of silver nitrate in acetone–water (90:8.5), air-dried for 3 min, and heated for 60 min at 80°C. Sample extracts and standards were spotted and developed with toluene–acetone–chloroform (8:2:5). R_f values are shown in Table 2. Other TLC systems were needed to separate two pairs of compounds that are especially difficult to resolve, as shown in Table 3. Argentation TLC was also applied to the isolation and characterization of a C-18 neutral steroid, estra-5(10),7-diene-3,17-diol, from pregnant mare urine and allantoic fluid (17).

Separations of the conjugated bile acids taurocholic acid, glycocholic acid, taurodeoxycholic acid, taurochenodeoxycholic acid, glycochenodeoxycholic acid,

glycodeoxycholic acid, tauroursodeoxycholic acid, glyoursodeoxycholic acid, tauroolithocholic acid, and glycolithocholic acid were compared on different silica gel and C-18 bonded-phase silica gel reversed-phase layers with variation in development temperature and in the ionic strength, pH, and components of the mobile phase. Separations obtained under various conditions were tabulated. Good resolution with compact zones was obtained using silica gel 60 developed with chloroform–methanol–acetic acid (85:20:9) or hexane–toluene–methanol–*n*-butanol–ethyl acetate–acetic acid (77:25:47:30:22:18) at room temperature, but none of the systems separated all 10 compounds. Zones were detected by dipping into a solution of manganese dichloride (200 mg) in water–methanol–sulfuric acid (15:15:1) and heating at 120°C for 30 min (18). The interaction of 15 steroidal drugs with a water-soluble β -cyclodextrin polymer was studied by reversed-phase TLC on silica gel F layers impregnated with paraffin and developed in a sandwich chamber with mobile phases consisting of methanol (20–27.5%)–water–cyclodextrin (CD) (0–15 mg/mL) with or without 0.1 M NaCl (19). A similar study of the retention of 18 steroidal drugs was carried out with mobile phases composed of methanol (0–55–water–hydroxypropyl- β -CD (2.5–20% or dimethyl- β -CD (1–50%) (20).

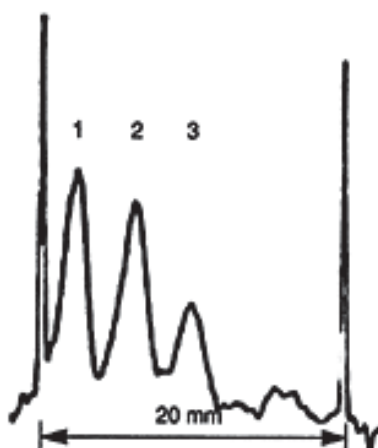


Figure 1 Densitogram of the separation of hydrocortisone (1), progesterone (2), and testosterone (3) by silica gel UTLC. The application volume was 0.5 μ L of a 0.1% solution in methanol–chloroform (4:1), the mobile phase was ethyl acetate–cyclohexane (3:2), and the development distance and time were 2 cm and 45 s, respectively. Video documentation was performed with a ProViDoc, and plates were evaluated

with a Camag TLC-Scanner II.
(Adapted from Ref. 14.)

Table 1 R_f Values of Steroids and Triterpenes

Mixture	R_f	Mobile phase
Cholesterol	0.37	Hexane–ethyl acetate (3:1)
Cholestanol	0.47	
3 β -Acetoxycholest-5-ene	0.48	Hexane–diethyl ether (10:1)
3 β -Acetoxycholestane	0.62	
3 β -Acetoxycholest-8(14)-ene	0.42	Hexane–diethyl ether (5:1)
3 β -Acetoxycholest-14-ene	0.27	
4 β -Methylcholest-2-ene	0.43	Hexane
4-Methylcholest-3-ene	0.33	
4-Methylcholest-4-ene	0.55	
Cholest-4-ene	0.68	Hexane–toluene (10:1)
Cholest-3-ene	0.57	
Cholest-2-ene	0.54	
Cholesta-3,5-diene	0.32	
(20 <i>R</i>)-Diacholest-13(17)-ene	0.46	Hexane–toluene (10:1)
(20 <i>S</i>)-Diacholest-13(17)-ene	0.61	
4,4-Dimethylcholest-5-ene	0.35	Hexane
4,5 α -Dimethylcholest-3-ene	0.64	
(20 <i>R</i>)-4,4-Dimethyldiacholest-13(17)-ene	0.46	Hexane–toluene (10:1)
(20 <i>S</i>)-4,4-Dimethyldiacholest-13(17)-ene	0.61	
Lupeol	0.42	Hexane–ethyl acetate (3:1)
Dihydrolupeol	0.53	
Betulin	0.39	Hexane–ethyl acetate (2:1)
Dihydrobetulin	0.50	
Lup-2-ene	0.34	Hexane
γ -Lup-3(4)-ene	0.50	
γ -Lup-3(5)-ene	0.29	
Lupa-2,20(29)-diene	0.37	Hexane–toluene (10:1)
γ -Lupa-3(4),20(29)-diene	0.46	
19 α (H)-28-Norlup-17-ene	0.81	Hexane
19 β (H)-28-Norlup-17-ene	0.70	
28-Norlup-16-ene	0.51	
28-Norlup-17(22)-ene	0.38	

Source: Ref. 15.

Table 2 Chromatographic Separation of Androsterone/ Dehydro-*epi*-androsterone and 5 α -Androstane-3 α ,17 β -diol/ 5 α -Androstane-3 β , 17 β -diol

C ₁₉ steroids	R_f^a
Androsterone	0.40 $\pm$.01 ^b
5 α -Dihydrotestosterone	0.44 $\pm$.01 ^b
5 α -Androstane-3 α ,17 β -diol	0.31 $\pm$.01 ^c
5 α -Androstane-3 β ,17 β -diol	0.24 $\pm$.01 ^c

^a R_f values are the mean $\pm$ SD of five independent determinations.

^bAluminum oxide 60 neutral F₂₅₄ layer developed with toluene–acetone–chloroform (8:2:5).

^cHard-layer silica gel plate containing a preadsorbent zone developed with benzene—acetone (4:1).
Source: Ref. 16.

In both studies, R_m values that allow calculation of R_f values were tabulated for all compounds in each mobile phase. A series of polar, ionic, and hydrophilic double conjugates of bile acids amidated at the C-24 carboxyl group with glycine or taurine and sulfonated or glucosylated at hydroxyl groups in the 5/3-steroidal nucleus were separated by two-dimensional (2-D) inclusion reversed-phase (RP)-HPTLC with tetra-*n*-butylammonium phosphate (TBAP) and methyl- β -cy-clodextrin (Me- β -CD) mobile phase additives. Complete separation of the hydrophilic bile acid conjugates, particularly the recalcitrant pairs of chenodeoxycholic acid and deoxycholic acid bile acid conjugates in each group, was achieved by developing with methanol-water-0.5 M TBAP (90:10:5–75:25:5) in the first direction and the same mobile phase containing 5 mM Me- β -CD in the second direction (21).

The TLC behavior of glucocorticosteroids was determined on silica gel 60 HPTLC plates with 12 different mobile phases. Optical evaluation of the plates after treatment with four different spray reagents revealed that the optimum combination for separation and detection was chloroform-methanol (92:8) or chloroform-acetone (90:10) mobile phase and 2,4-dihydroxybenzaldehyde.

Table 3 Chromatographic Behavior of C₁₉ Steroids on Silver-Impregnated Silica Gel Layers^a

Compound	R_f^b ($n=6$)
Androst-5-ene-3 β ,17 β -diol	0.25 $\pm$ 0.01
5 α -Androstane-3 β ,17 β -diol	0.28 $\pm$ 0.01
5 α -Androstane-3 α ,17 β -diol	0.29 $\pm$ 0.01
Testosterone	0.36 $\pm$ 0.01
Dehydro- <i>epi</i> -androsterone	0.40 $\pm$ 0.01

Androsterone	0.44±0.01
5 α -Dihydrotestosterone	0.46±0.01
Androst-4-ene-3,17-dione	0.56±0.02
5 α -Androstane-3,17-dione	0.68±0.01

^aMobile phase: toluene-acetone-chloroform (8:2:5).

^b R_f values are the mean±SD of six independent determinations.

Source: Ref. 16.

hyde-sulfuric acid-acetic acid reagent (Tables 4–6). The other spray reagents shown in Tables 5 and 6 had different advantages and disadvantages that are described in the paper (22). The specificity of the chromatographic conditions was assessed by analysis of 35 anabolic steroids and 10 other veterinary drugs, and no significant interferences were found.

Bufadienolides have been separated using one- and two-dimensional (Fig. 2) multiple development TLC. Silica gel GF plates were used, and 20 different mobile phases were tested in this study. Detection was under 254 nm UV light and by spraying with a 5% solution of sulfuric acid in ethanol or a solution of cerie sulfate in sulfuric acid followed by heating to produce zones with different characteristic colors that aided identification (23).

Sterols from Nicotianeae (Solanaceae) seeds were separated on silica gel with hexane-diethyl ether-acetic acid (90:10:1); the sterol fraction was purified by PLC on silica gel-silver nitrate (8:2) layers (24). Other mobile phases used for sterol TLC separations on silica gel are chloroform-methanol (2:1) (25), chloroform-butyl acetate (3:1) (25), chloroform-acetone (9:1) (26), and chloroform-ethyl acetate (9:1) (27). Silica gel developed with benzene-acetone (8:2) was used for the TLC identification of β -sitosterol in olive oil (28).

Vitamins D₂ and D₃ were separated on silica gel 60 F layers with benzene-methanol (9:1) mobile phase and on kieselguhr F layers impregnated with paraffin oil and developed with methanol-water or acetonitrile-water (95:5). Respective R_f values in the latter two reversed-phase systems were 0.56, 0.48 and 0.50, 0.41 (29). Vitamins D₁ and D₂ were determined in fruit pulp by TLC on paraffin oil-impregnated corn starch layers with acetone-concentrated acetic acid (3:2) as the mobile phase (30).

Additional layer-mobile phase combinations reported in the recent literature for steroid separations are given in many of the following sections of this chapter. The *Handbook of Chromatography* volume on steroids (31) contains sections on androgens, bile acids and alcohols, cardenolides, ecdysteroids, estrogens, pregnanes and corticoids (C-21), sterols and cholesterol, and vitamin D, including extensive R_f data tables for many layer-mobile phase combinations and reagents for compound detection.

Table 4 R_f Values of 18 Corticosteroids After Elution with the Two Most Suitable Mobile Phases

Corticosteroid	Chloroform–methanol (92:8)	Chloroform–acetone (90:10)
1. Prednisolone	0.40	0.010
2. Dexamethasone	0.45	0.021
3. Triamcinolone	0.29	0.054
4. Beclomethasone	0.43	0.043
5. Prednisone	0.50	0.043
6. Betamethasone	0.38	0.021
7. Triamcinolone diacetate	0.62	0.13
8. Methylprednisolone acetate	0.56	0.10
9. Hydrocortisone	0.39	0.021
10. Prednisolone acetate	0.36 and 0.47	0.27 and 0.076
11. Hydrocortisone caponate	0.54 and 0.61	0.25 and 0.17
12. Fludrocortisone acetate	0.39 and 0.63	0.16 and 0.027
13. Dexamethasone acetate	0.41	0.2 and 0.027
14. Methylprednisolone hemisuccinate	0.14	0
15. Beclomethasone dipropionate	0.59 and 0.91	0.34 and 0.24
16. Triamcinolone acetonide	0.48	0.23 and 0.2
17. Betamethasone valerate	0.67	0.24
18. Cortisone acetate	0.60	0.18

Source: Ref. 22.

Table 5 Colors of Corticosteroid Spots After Reaction with Three Spray Reagents^a

Corticosteroid	Color			
	Reagent 1	Reagent 2	Reagent 3	Reagent 3 F ₂₅₄ (with UV)
1	Gray-violet	Violet	Brown	—
2	Blue-green	Violet	Violet	—
3	Grayish	Violet	Orange	—
4	Blue	Violet	Violet-gray	—

5	Brown-yellow	Violet	Orange	—
6	Blue	Violet	Violet	—
7	Gray-blue	Violet	Yellow	—
8	Gray-violet	Violet	Violet-pink	—
9	Greenish	Violet	Green	—
10	Violet	Violet	Violet-brown	—
11	Brown-green	Violet	Green	Green-yellow
12	Pink	Violet	Brown-pink	Brown-green
13	Blue-green	Violet	Violet-gray	Green-violet
14	Violet	Violet	Brown	—
15	Blue	Violet	Violet-gray	—
16	Indigo	Violet	Orange	Green
17	Blue	Violet	Gray	Green-violet
18	Yellow-gray	Violet	Orange	Bluish

^aParent solutions were prepared in methanol (200 ng/mL). Spray reagents were prepared as follows: *Reagent 1*. A 1:1 mixture of (a) a solution of 2,4-dihydroxybenzaldehyde (0.25 g) in glacial acetic acid (50 mL) and (b) a mixture of sulfuric acid (95–97%, 12.5 mL) and glacial acetic acid (37.5 mL).

Reagent 2. A 1:1 mixture of (a) a solution of tetrazolium blue (0.25 g) in methanol (50 mL) and (b) a solution of sodium hydroxide (10 g) in water (50 mL).

Reagent 3. A solution of sulfuric acid (95–97%, 1 mL) in methanol (9 mL).

Reagent 4. Comprised three separate solutions: (a) zirconium(IV) oxide chloride GR (5 g) in water (100 mL); (b) alizarin sulfonide acid (0.14 g) in water (100 mL); and (c) sodium hydroxide (0.5 M). Color development with reagents 1–3 was achieved by heating the plates in an oven at 85–90°C for 10 min. With reagent 4, plates were immersed in solution (a) for 10 s, dried with a warm stream of air, sprayed with solution (b), dried in air, and sprayed with solution (c).

Source: Ref. 22.

Table 6 Detection Limits (ng) for Seven Corticosteroids After Reaction with Spray Reagents 1 and 2 of Table 5

Compound	Reagent 1	Reagent 2
Dexamethasone	300	100
Prednisolone	400	150
Triamcinolone	200	75
Beclomethasone	60	40
Prednisone	300	100
Betamethasone	50	30

Source: Ref. 22.

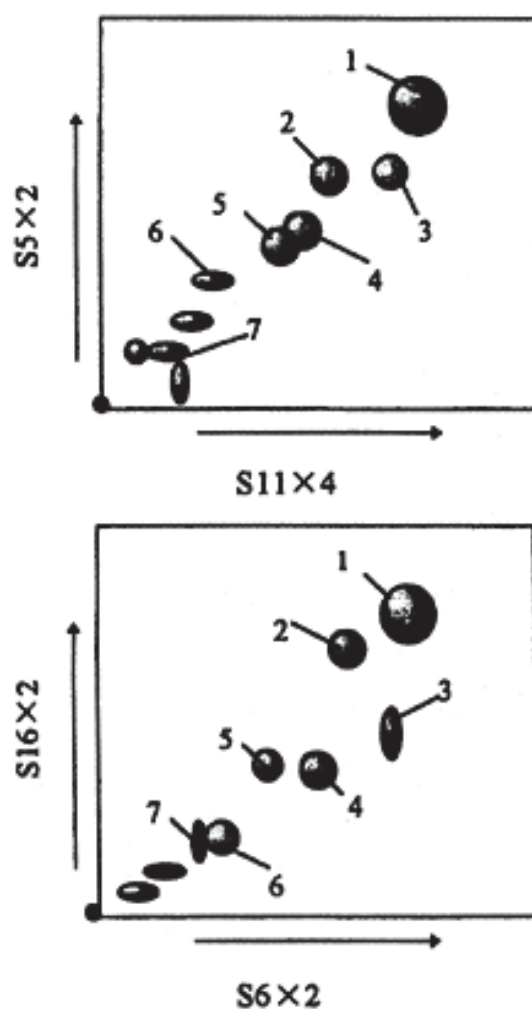


Figure 2 Representation of multiple development 2-D separations of bufadienolides in the chloroform extract of the Chinese traditional drug Ch'an Su. Top: Twofold development with S5 followed by fourfold development with S11. Bottom: Twofold development with S16 followed by twofold development with S6. S5: Hexane–acetone (7:3)+3% *N,N*-dimethylcyclohexylamine (DMCA). S6: Hexane–acetone

(7:3)+3% triethylamine (TEA). S11: Hexane–dichloromethane–acetone (8:2:1)+3% TEA. S16: Hexane–dichloromethane–methanol (20:10:1)+3% DMCA. 1, Resibufogenin; 2, cinobufagin; 3, bufalin; 4, bufotalin; 5, cinobufotalin; 6, telocinobufagin; 7, gamabufotalin. (Adapted from Ref. 23.)

IV. DEVELOPMENT TECHNIQUES

Steroids are usually analyzed by single, ascending capillary action development in a glass chamber, but other techniques have also been used. For example, cholesterol, neutral lipids, and phospholipids were determined by horizontal development of silica gel layers with petroleum ether–diethyl ether–acetic acid (80:20:2), and these compounds were also chromatographed in a sandwich chamber (32). A DS-type sandwich chamber was used for sterol TLC on silica gel, silica gel–kieselguhr, aluminum oxide, and C-18 bonded silica gel layers, and overpressured layer chromatography (OPLC) was also used to achieve optimum separations (26).

The essential steps of displacement TLC and its applications for the separation of plant ecdysteroids were described (33), including optimization of the mobile phase composition, mobile phase flow rate, and preelution before displacement development.

One-dimensional multiple development with a change in entry position was applied to the separation of eight major androgens derived from skin or prostate metabolism (testosterone, an-drostenedione, dihydrotestosterone, androsterone, epiandrosterone, androstenedione, 3- α -androstanedione, 3- β -androstanediol) on a silica gel layer with concentrating zone using dichloromethane–diethyl ether (9:1) mobile phase in a horizontal DS-MP chamber and detection by exposure to iodine vapor (34).

Two-dimensional TLC analysis, in which a single sample applied in the corner of a plate is developed with two complementary mobile phases at right angles, was described for the separation of bufadienolides cited in Section III (23) and in the following papers. Regioselective and stereospecific androgen metabolites were separated on silica gel with chloroform–acetone (9:1) followed by dichloromethane–ethyl acetate–methanol (14:4:1) in direction 1 (multiple development) and chloroform–ethyl acetate in direction 2; detection was under 245 nm UV light or by exposure to iodine vapor. The procedure was used for identification of specific forms of cytochrome P450 in crude microsomal preparations as well as for monitoring the purification of new forms of the enzyme and alterations in the catalytic activities of mutagenically altered forms of the enzyme (35). Anabolic steroids used as growth promoters in cattle fattening were determined by 2-D TLC with chloroform–acetone (9:1) in the first dimension followed by cyclohexane–ethyl acetate–methanol (117:78:11) in the second. Detection was by spraying with 10% sulfuric acid in methanol, heating at 95°C, and inspection under 366 nm UV light (36). Virtually identical conditions were reported for the determination of synthetic estrogens

in illegal veterinary formulations, except that the proportions of the second mobile phase were 31:8:1 (37).

Five major unconjugated bile acids (cholic acid, chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid, and lithocholic acid) and their glycine conjugates were separated by 2-D inclusion RP-TLC with aqueous methanol in the first dimension and the same solvent containing Me- β -CD in the second. Detection was by spraying with 20% phosphomolybdic acid (PMA) in methanol and heating at 110°C (38).

Two dimensional silica gel and reversed-phase C-18 bonded-phase silica gel TLC were used to screen plant samples for ecdysteroids, giving satisfactory separation even in the presence of substantial impurities and for complex mixtures (see Figs. 3 and 4) (39). Production of steroid metabolites was determined based on comparison of standard steroid zones and sample zones on 2-D chromatograms in a study of two steroidogenic pathways present in the chicken ovary; it was found that the theca layer prefers the delta-5 pathway and the granulosa layer, the delta-4 pathway (40).

Overpressured layer chromatography (OPLC) (Chap. 7) was used for the determination of norethisterone on HPTLC silica gel with 4 mL of butyl acetate–chloroform (17:3) after a prerun with 4 mL of hexane. Detection was by spraying with 10% ethanolic sulfuric acid solution, and semiquantitative evaluation at 366 nm was by densitometry. Impurities (6- α -hydroxy-, 6- β -hydroxy-, 10-hydroxy-, 6-keto-, 17-*epi*-norethisterone, and nordione) were separated and detected below 0.05% (41). The mobile phase chosen for the silica gel OPLC of allylestrenol, desogestrel, and ethynodiol diacetate was cyclohexane–butyl acetate–chloroform (86:7:7), whereas toluene–ethyl acetate–chloroform (5:1:4) was used for levonorgestrel, estrone, estradiol, ethinylestradiol, norethisterone, and 3-ketodesogestrel (42). The use of OPLC for pharmaceutical purity testing was studied using silica gel and cyclohexane–ethyl acetate–chloroform (3:1:1) as mobile phase. The method was compared with official British and European pharmacopeia monographs, and only the OPLC method was able to separate all impurities, i.e., 6-hydroxy derivatives (both isomers), the oxo derivatives (6-oxo- and 16-oxo-), and 9(11)-dehydro and the 17-*epi*-derivatives from the estrone and estradiol. The OPLC method was preferred to HPLC for purity testing process validation because it showed a short analysis time and low solvent consumption (one-tenth as much as HPLC) (43).

V. DETECTION OF ZONES

In addition to this section, other sections in this chapter also contain information on steroid TLC detection methods, e.g., Sections III (Table 5), IV, VI, and IX.A. Descriptions of more than 450 TLC detection reagents, including many for steroids, are available in Volume II of the *Handbook of Chromatography* (44). New detection reagents for steroids are updated biennially in the review

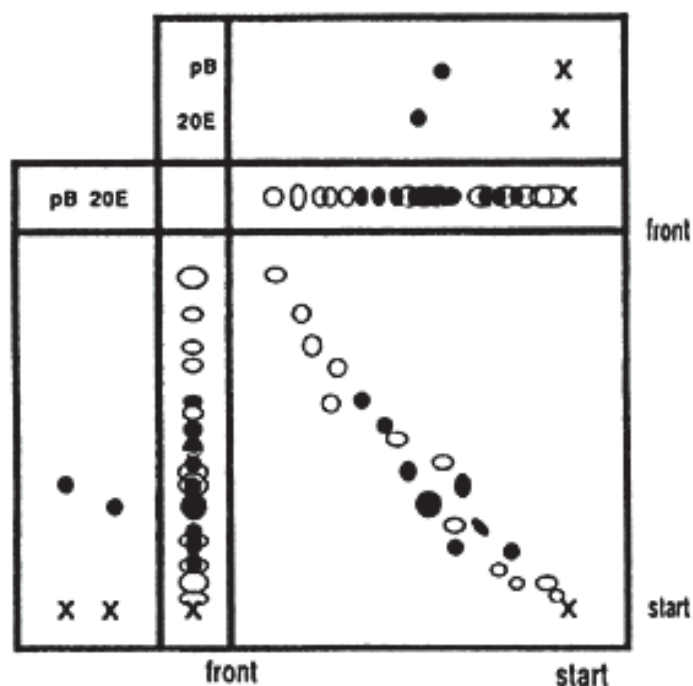


Figure 3 Result of 2-D TLC on a silica gel F layer of an extract of *Silene italica* spp. *nemoralis*. The same sample and samples of 20-hydroxyecdysone (20E) and polypodine B (pB) were spotted on side tracks and subjected to one-dimensional TLC. Developments in the first (bottom to top) and second (right to left) directions were performed with chloroform–methanol–benzene (25:5:3) and toluene–acetone–96% ethanol–25% aqueous ammonia (100:140:32:9), respectively. The zones were initially detected under 254 nm UV light and then sprayed with vanillin–sulfuric acid reagent and observed in daylight and under 366 nm UV light. Observation of specific violet fluorescence at 366 nm, then black zones when the plate was sprayed, is indicative of ecdysteroids.

Open circles denote other components.
(Adapted from Ref. 39.)

of planar chromatography written by Sherma in the ACS journal *Analytical Chemistry* (45). Books on physical and chemical detection methods in TLC have been published (46, 47) that include descriptions of reagents and methods for steroid visualization.

In their recent article on steroid TLC, Mulja and Indrayanto (3) reviewed the following detection methods. Sulfuric acid is a very widely used detection reagent for steroids; characteristic color and fluorescence response (366 nm UV light) can be produced with a short heating at a relatively low temperature, and permanent black zones appear after longer heating at a higher temperature if layers can withstand the charring reaction (e.g., silica gel G). Other generally applicable, destructive chromogenic and fluorogenic reagents for detection of steroids are antimony trichloride (Carr–Price reagent) (for vitamin D, cardenolides, bufadienolides, triterpenoids); aromatic aldehyde-acids (sapogenin steroids, steroid alkaloids, ketosteroids; PMA (reducing and unsaturated steroids, cholesterol and cholesterol ester, bile acids); chlorosulfonic acid–acetic acid (cardenolides), phosphoric acid (results similar to sulfuric acid) (48); *m*-dinitrobenzene (ketosteroids); and phthalic acid-*p*-phenylenediamine (oxo-steroids). Many steroids absorb UV radiation around 254 nm and can be detected nondestructively by viewing under light of this wavelength on a plate with an F-layer. Iodine and iodine-KI are nondestructive detection reagents that react reversibly with many steroids.

Anisaldehyde (5 mL) in glacial acetic acid (50 mL) and sulfuric acid (97%, 1 mL) was used to detect steroids and terpenes in Baltic amber as deep violet zones after heating to 105°C; resin ethanol extracts were developed on silica gel 60 F plates with *n*-hexane–benzene–methanol (2:6:1) mobile phase (48a).

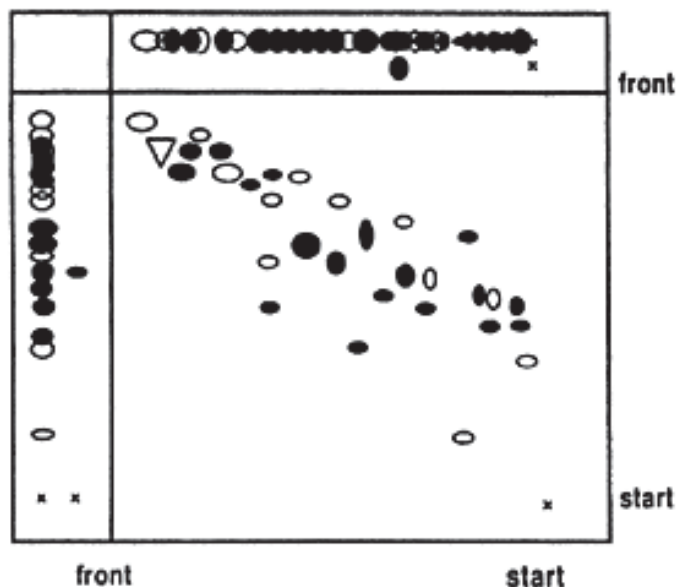


Figure 4 Two-dimensional TLC on a C-18 F-layer of the same extract as in Fig. 3. Developments in the first

(bottom to top) and second (right to left) directions were performed with tetrahydrofuran–water (45:55) and methanol–water (55:45), respectively. The same sample and a mixture of 20E and pB were spotted on the side tracks and subjected to one-dimensional TLC. 20E and pB were not separated. Detections were as in Fig. 3. (Adapted from Ref. 39.)

The detection reagent antimony trichloride was used for identification of marinobufagenin separated by TLC from a mixture of steroids from *Bufo marinus* venom (49). A 1:4 solution of antimony pentachloride was used as a spray reagent for detection of vitamins D₁ and D₂ as zones of different colors on a white background; visible mode densitometry was used to quantify the detected vitamins (30).

Identification of bile acids in carnivore feces was facilitated by detection using 50% aqueous sulfuric acid rather than 5% anisaldehyde reagent after TLC on silica gel 60 F layers (50). The latter produced bile acid zones that all had the same color, whereas sulfuric acid produced zones of different colors: cholesterol, red; lithocholic acid and ursocholic acid, brown; deoxycholic acid, cholic acid, and hyodeoxycholic acid, yellow; chenodeoxycholic acid, green; and hyocholic acid, fluorescent under 254 nm UV light.

Androgen-derived C-19 steroids were successfully detected on silver nitrate-impregnated silica gel layers by spraying with a solution containing 5% ammonium molybdate and 5% sulfuric acid in water and slowly heating until blue spots appeared (16).

Thermochemical activation is a method for detection of several classes of compounds on amino-bonded silica gel layers without a derivatization reagent, simply by heating the layer and viewing the resultant fluorescent zones under 366 nm UV light. Conditions recommended for the thermochemical activation of steroids were heating for 7 min at 220°C, with resultant detection limits of 500, 375, and 75 ng (visual) and 375, 150, and 75 ng (photometric with a densitometer) for estradiol, prednisolone, and progesterone, respectively (51). This approach was applied successfully to the detection of a group of steroidal hormones developed with (a) chloroform–ethanol–formic acid (5:1:1.2), (b) chloroform–methanol (95:5), or (c) chloroform–1-propanol–formic acid (50:10:5) mobile phases (Fig. 5). In addition to the compounds in Fig. 5, estradiol, estradiol benzoate, estriol, estrone, methyltestosterone, and pregnanediol and -triol were detectable (52). Enhancement of the detection sensitivity of steroids by thermochemical activation was obtained by measuring fluorescence in situ after dipping into hexane–paraffin (2:1) and/or using a filter that cut emission wavelengths below 400 nm; the detection limit was about 5 ng/spot (53).

Detection reagents for cholesterol and cholesterol derivatives in adsorption, argentation, and partition TLC were studied in detail (54, 55), including bromothymol blue, thymol blue, phenol

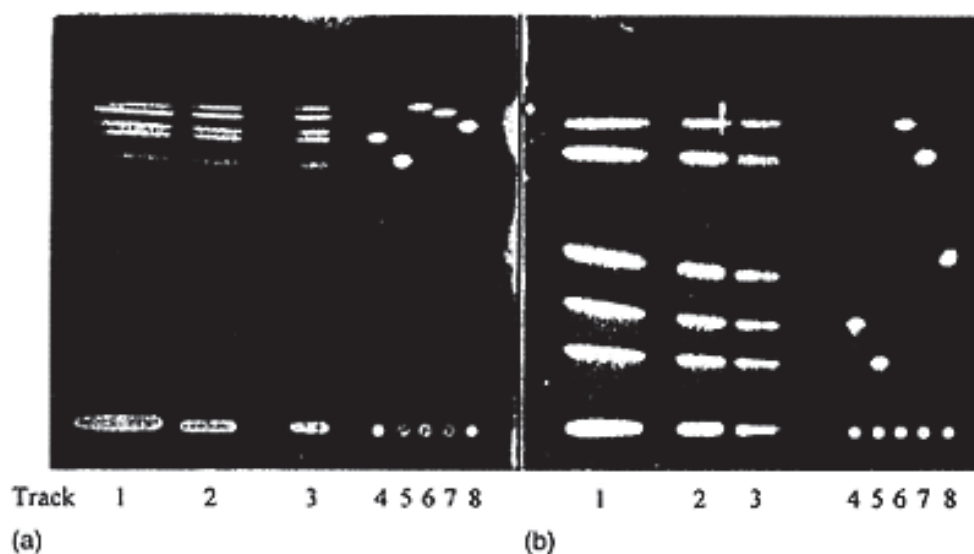


Figure 5 Separation and fluorimetric detection of selected steroids using thermochemical activation. (a) Chromatogram developed with mobile phase (a); (b) chromatogram developed with mobile phase (b) (see text). Tracks 1–3, mixture of progesterone, testosterone, Reichstein's substance S, cortisone, and hydrocortisone; 4, cortisone; 5, hydrocortisone; 6, progesterone; 7, testosterone; 8; Reichstein's substance S. 2.5 μg or 1.5 μg of each compound was spotted. (Adapted from Ref. 52.)

red, helasol green, bromocresol green, Brilliant Cresyl Blue, bromophenol blue, neutral red, aniline blue, alkaline blue, Brilliant Green, Sudan I, Sudan II, Sudan III, and Sudan IV. R_m values, limits of detection, and colors of the cholesterol spot and background were tabulated for layers consisting of silica gel, basic and neutral aluminum oxide, silica gel+silver nitrate, and silica gel–kieselguhr +paraffin oil. The best reagents were found to be aniline blue, bromophenol blue, helasol green, and alkaline blue. Spraying with 5% ethanolic PMA and heating for 15 min at 115°C is an excellent method for visualizing cholesterol, cholesterol esters, and other sterols as blue zones on a light yellow background with a detection limit of about 200 ng (56).

Sterols have been detected by spraying with 2,7-dichlorofluorescein solution (24, 28) or berberine (0.2% in ethanol) (27) and then viewing under 366 nm UV light.

Vitamins D₂ and D₃ were detected on silica gel F and paraffin oil-impregnated kieselguhr F plates by observation under 254 nm UV light or use of 0.005% aqueous new fuchsin solution detection reagent (29).

A major use of TLC is to identify unknown steroids or to confirm the identity of steroids initially detected by gas chromatography (GC) or high-performance column liquid chromatography (HPLC). Qualitative identification of unknown zones is based on characteristic colors produced by a selective detection reagent combined with R_f values relative to standard steroids. In general, at least one spectroscopic or chromatographic method must be used in addition to TLC results in order to make a valid statement of identity. TLC has been combined off-line and online with many different spectrometric methods for compound identification. (See Chaps. 1, 8, and 9.) A specific example of confirmation of steroid identification is the study of anabolic compounds in injection sites (57). Silica gel HPTLC was used to show different zones recognizable as the esters of a certain anabolic compound, e.g., testosterone or estradiol, without giving the exact identity of the ester. Hydrolysis of the extract and re-spotting on HPTLC plates confirmed that the zones were esters because of the absence of such zones on the plate after hydrolysis. The residues are then seen in their unesterified forms. Identification was possible by re-spotting on reversed-phase plates or using GC/MS.

Thin-layer chromatography coupled with tandem mass spectrometry (TLC/MS-MS) has been applied to ecdysteroid analysis in two studies described by Wilson and Morden (58). The first involved TLC of a plant extract on aluminum-backed silica gel HPTLC plates developed with chloroform-ethanol (4:1). After separation, the track to be measured was removed from the plate and glycerol and dimethyl sulfoxide (DMSO) cosolvent was applied prior to attaching the track to a commercial TLC/MS probe. Mass spectrometry and MS-MS spectra of individual components were obtained by liquid secondary ion mass spectrometry (LSIMS) (30 keV cesium ions) using a tandem mass spectrometer. In the second, insect egg extract was analyzed with similar methodology except that samples were introduced manually after scraping the appropriate layer material and adding glycerol and cosolvent. Mass spectra obtained for polypodine B and 2-deoxyecdysone are illustrated in the Wilson and Morden paper.

VI. QUANTITATIVE ANALYSIS

Steroids have been quantified by TLC using scraping and elution of separated analyte zones in sample and standard chromatograms, usually followed by visible or UV spectrometry or fluorimetry. An unspotted layer area adjacent to the zones is analyzed simultaneously and identically as a blank for background correction.

More common today is *in situ* quantification by optical densitometry with a slit-scanning densitometer (see Chaps. 5 and 10). Compounds that absorb UV light (e.g., corticosteroids) are scanned directly with the UV mode of the densitometer on a nonfluorescent layer at the wavelength of maximum absorption, or more commonly as quenched zones on an F-layer containing a fluorescent indicator at 254 nm. Compounds that are detected as colored zones with a post-chromatographic derivatization reagent are scanned in the visible mode at the wavelength of maximum absorption in the 370–700 nm range. Compounds that become fluorescent by pre- or postchromatographic reaction

with a fluorogenic reagent or by thermal activation are scanned with the fluorescence mode of the densitometer using a mercury or deuterium UV source lamp for excitation and visible range emission measurement. Reflectance scanning is most often performed. If they are to be meaningful, the results of quantitative analyses should be validated in terms of factors such as accuracy, precision, detection limit, range of applicability, linearity, specificity (resolution), and robustness.

The remainder of this section contains references to papers that have been published on the quantitative determination of steroids in various samples by TLC-densitometry.

Steroid sapogenins (diosgenin, hecogenin, manogenin), steroid alkaloids (solasodine, tomatidine), and total steroids have been assayed by visible absorbance-reflectance scanning after detection of zones with anisaldehyde-sulfuric acid reagent. The method was found to be faster, simpler, and cheaper than HPLC or GC (3).

Corticosteroids and their esters (e.g., betamethasone dipropionate, dexamethasone, hydrocortisone, testosterone) in pharmaceutical preparations of creams and ointments were identified and quantified by silica gel TLC for the purpose of quality control. Mobile phases were chloroform-ethyl acetate (2:1) for esters and chloroform-ethyl acetate (1:1) for free corticosteroids, and detection was with tetrazolium blue chromogenic reagent. Densitometry was performed at 240 nm before spraying with the reagent or at 520 nm after spraying (59).

Three papers (60–62) reported method development and results for the determination of the ecdysteroids 20-hydroxyecdysone and polypodine B from *Serratula tinctoria* and *Serratula wolffii* (Asteraceae) using silica gel layers, successive development with dichloromethane-ethanol (4:1) and chloroform-methanol-benzene (25:5:3), and reflectance densitometry at 254 nm. Plant material was extracted with methanol, and extracts were cleaned up on a polyamide column prior to TLC. Seasonal dependence of the steroid levels in leaves was studied.

Cholic acid and deoxycholic acid were determined in commercial bile salts on silica gel with isooctane-ethyl butyrate-acetic acid-water (18:5:3:1) as mobile phase. Detection was by spraying with 30% sulfuric acid in ethanol and heating at 110°C, and quantification was by scanning at 385 nm (63). Bile acids were quantified in artificial Niu Huang by TLC on silica gel with chloroform-diethyl ether-acetic acid (2:2:1), detection with 10% PMA acid in ethanol, and visible absorbance densitometry at 540 nm (64).

Palmityl- β -sitosterol, lupenone, 24-methylene-cycloartenol, and β -sitosterol were determined in Chinese drugs on silica gel with chloroform-benzene (9:1) mobile phase. Detection was by spraying with 5% ethanolic PMA acid and heating, and quantification by densitometry at 625 nm (65).

Cholesterol and oxygenated cholesterol derivatives were quantified in blood plasma by alkaline hydrolysis (KOH-ethanol), extraction with chloroform-0.15 M NaCl (3:1), and SPE on a 500 mg silica gel column eluted with hexane-2-propanol (99.5:0.5) to recover cholesterol and (7:3) for the oxysterols. Separation was performed on silica gel and silanized silica gel (Merck) plates in an SDII horizontal chamber (Chromdes, Lublin, Poland) with the mobile phases chloroform-acetone (9:1) and *n*-hexane-diethyl ether-methanol (38:2:1), respectively. After spraying with Liebermann-Burchard reagent, the zones were quantified by reflectance densitometry at 360 nm (66).

Cholesterol, cholesterol esters, and other lipids were determined in animal tissues by extraction with chloroform–methanol (2:1) containing 0.01% butylated hydroxytoluene to avoid oxidation and silica gel 60 HPTLC-densitometry (67). Extract and standard solutions were applied as bands with a Linomat IV (Camag, Wilmington, NC USA); the layer was developed with petroleum ether–diethyl ether–acetic acid (80:20:3); and compounds were detected by dipping into a solution composed of manganese chloride (1 g), methanol (150 mL), concentrated sulfuric acid (10 mL), and water (150 mL) and heating at 110°C for 45 min. Although this was called a charring reagent in the paper, zones were reportedly pink on a white background. Scanning was performed at 250 nm in the reflectance mode. R_f values of cholesterol and cholesterol ester were 0.23 and 0.76, respectively; the detection limit was 20 ng; and sample throughput was 20–24 samples per plate.

Cholesterol sulfate (CS) was estimated in blood plasma and erythrocyte membranes from individuals with Down's syndrome and diabetes mellitus Type I by HPTLC-densitometry. From the results it was postulated that differences in CS levels may contribute to changes of erythrocyte properties in these pathological states (68).

Cholesterol was determined in liquid egg yolk before and after its reduction by extraction with β -cyclodextrin HPTLC on silica gel layers developed with petroleum ether–diethyl ether–acetone (90:10:1) in an unsaturated chamber, detection by spraying with 10% PMA in ethanol and heating at 100°C, and quantification by densitometry at 580 nm (Fig. 6). Comparison with

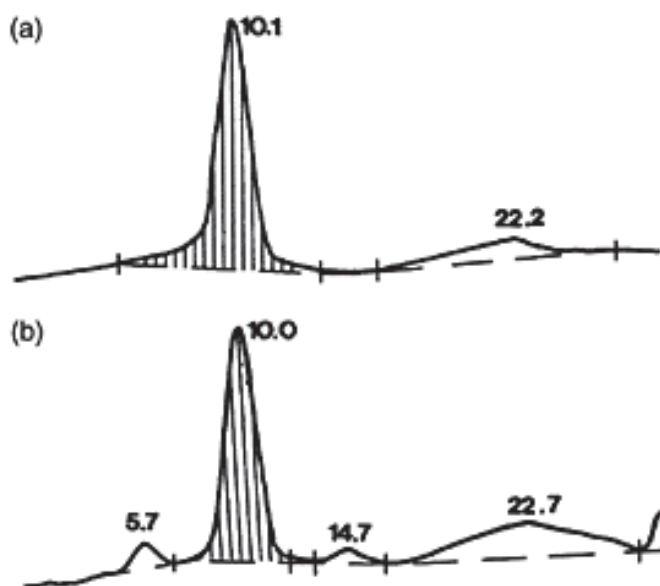


Figure 6 Densitograms obtained after HPTLC of cholesterol, (a) Standard solution (2 μ L of a 400 ng/ μ L solution applied with a Linomat IV). (b) Egg yolk extract sample. (Adapted from Ref. 69.)

HPLC showed that HPTLC was faster and less expensive and equally accurate for high and low levels of cholesterol (69).

Vitamin D₃ was quantified at ppb levels in cod liver oil by addition of ethanol, 2% orthophosphoric acid solution, and hexane followed by saponification. The hexane layer was evaporated and reconstituted and applied with standard aliquots as bands using a Linomat IV to silica gel 60 F₂₅₄ plates. Dual ascending development was performed with *n*-hexane for 5 cm in an unsaturated twin-trough chamber (Camag) and then for 7 cm with cyclohexane–diethyl ether (1:1) in a saturated chamber. Densitometry was carried out at 268 nm using peak height and linear regression. All operations were done as much as possible in the dark away from any source of UV radiation to retard compound decomposition (70).

VII. PREPARATIVE LAYER CHROMATOGRAPHY

Preparative layer chromatography (PLC) involves separation and recovery of larger amounts of substances on layers thicker than those used in analytical TLC for the purpose of identification, structure determination, quantitative analysis, or determination of chemical or biological properties of the purified fraction (see Chap. 11). Examples of the use of PLC for steroids were the study of 3- β -hydroxysteroid dehydrogenase activity in human osteoblastlike cells (purification of androstenedione) (71) and purification of the glucocorticoid receptor-mineralocorticoid receptor modulator-2 from rabbit liver (72).

Free sterols from jojoba oil were purified by PLC on silica gel layers developed four times for 15 cm each with hexane–diethyl ether (17:3). Detection was by spraying with 0.01% rhodamine 6G in ethanol and viewing under 366 nm UV light (73). The PLC of 7-hydroxysterols was carried out on silica gel with hexane–ethyl acetate (7:3) mobile phase and C-18 bonded silica gel with 95% aqueous methanol (74).

VIII. THIN-LAYER RADIOCHROMATOGRAPHY

Thin-layer radiochromatography (TLRC), or radio-TLC (Chap. 12), is used widely for separation, identification, and measurement of radioisotopes in studies involving steroids. This section contains selected references illustrating the use of the major TLRC methods, such as the digital autoradiograph and analyzer imaging system (16), and applications involving steroid radioisotopes. Many of the metabolism studies and miscellaneous applications cited later in Sections IX.B and IX.C, respectively, also made use of TLRC.

Metabolites of the neuroactive steroid 3- α -hydroxy-1-pregnen-20-one in rat pituitaries were identified by TLC and autoradiography (75).

In a study of hypothalamic aromatase cytochrome P450 and 5- α -reductase enzyme activities in pregnant and female rats, the 5- α -reductase rates were determined by TLC and scintillation counting of the isolated 5- α metabolites (76). Progesterone metabolites were detected by autoradiography and quantified by liquid scintillation counting in a metabolism study in human saliva in vitro (77).

In situ measurement of separated zones containing radioisotopes on TLC plates by scanning with a linear analyzer was used in studies of bacterial steroidogenesis by

periodontal pathogens and the effect of bacterial enzymes on steroid conversions by human gingival fibroblasts in culture (78), the influence of insulin-like growth factor (IGF) on C-19 steroid conversions by human gingiva and in cultured gingival fibroblasts (79), and the effect of minocycline on the metabolism of androgens by human oral periosteal fibroblasts and its inhibition by finasteride (80). A digital autoradiography (DAR)-TLC method was described (81) for direct quantification of the major sebaceous neutral ^{14}C -labeled lipids, including sterol esters and free sterols; the precision of the overall methodology, evaluated from a pool of neosynthesized lipids, was 15% for the range of 50–100 disintegrations per minute (dpm) and 8.5% for 300–2000 dpm. The beta-Imager 1200 radiodetector from Biospace (Paris, France), which is based on the concept of parallel avalanche detection, was evaluated in a study of steroid metabolism (82). It allowed detection of post-chromatographic radiosignals of 2.0 dpm and quantitative measurement in the range of 5–50 dpm for ^{14}C . Figure 7 shows the pattern of testosterone metabolism from three human hair follicles incubated in the presence of [4- ^{14}C]testosterone using direct DAR with this radiodetector.

Radioactive zones were measured by radioisotope image analysis in order to quantify the neurotoxic steroidal alkaloid zygacine in *Zigadenus venenosus* (death camas) (83) and determine steroid levels and metabolism in relation to early gonadal development in the tilapia fish, *Oreochromis niloticus* (Teleostei: Cyprinoidei) (84).

Concentrations of cortisol in guinea pig urine were determined by HPTLC and TLC-radio-immunoassay (TLC-RIA) (85).

Thin-layer radiochromatography was also used in studies of steroidogenesis in the ovarian follicle of the medaka (*Oryzias latipes*), a daily spawner, during oocyte maturation (86); expression in *Escherichia coli*, and characterization of a bile acid-inducible 3- α -hydroxysteroid dehydrogenase from *Eubacterium* sp. strain VPI-12708 (87); fecal metabolites of infused ^{14}C -progesterone in domestic livestock (88); and plasma-sulfated C-21 steroid levels during the periovulatory period in female common wolffish reared at three different temperatures (89).

IX. APPLICATIONS OF TLC AND HPTLC TO STEROID ANALYSIS

The references cited in this section give further evidence of the great importance of TLC analysis in current steroid research and illustrate the diversity of the topics of the studies that have involved TLC.

A. Qualitative Identification

Silica gel TLC or HPTLC was used to qualitatively identify ecdysteroids in *Serratula* plants [dichloromethane–ethanol (85:15) and chloroform–methanol–benzene (25:5:3) mobile phases; detection under 254 nm UV light] (90); β -amyrenonol, citrostadienol, and β -sitosterol in oil of seabuckthorn seed, *Hippophae rhamnoides* [petroleum ether–diethyl ether (7:3), detection by exposure to iodine vapor] (91); budesonide with prednisolone acetate, dexamethasone acetate, and betamethasone 17- α -valerate as reference substances [methyl ethyl ketone–toluene (2:3), detection

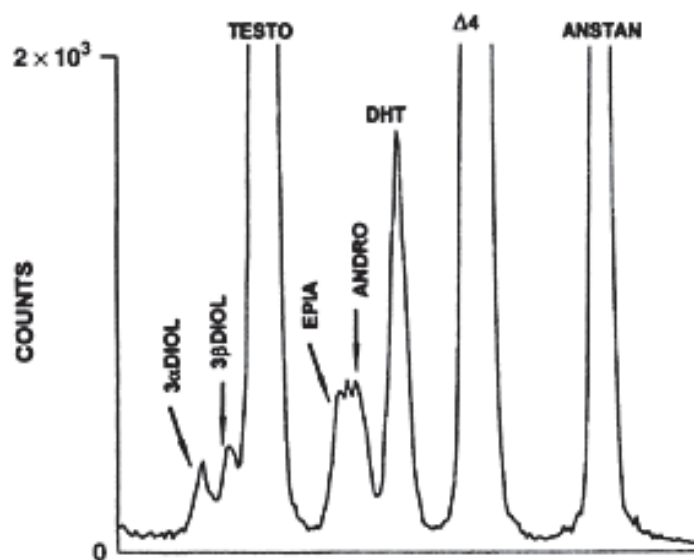


Figure 7 Digital audioradiographic scan of a chromatogram showing the pattern of testosterone metabolites obtained from three human hair follicles. Incubation medium was spotted on a silica gel plate and developed twice to a distance of 20 cm with dichloromethane–diethyl ether (9:1) in a vertical tank with lateral channels (Desaga, Weisloch, Germany). 3 α DIOL, 3 α -androstenediol; 3 β DIOL, 3 β -androstenediol; TESTO, testosterone; ANDRO, androsterone; EPIA, epiandrosterone; DHT, dihydrotestosterone; Δ 4, androstenedione; ANSTAN, androstenedione. (Adapted from Ref. 82.)

in daylight and then under 366 nm UV light after spraying with 20% ethanolic sulfuric acid and heating at 110°C] (92); ethinyl estradiol with methyltestosterone, norethisterone, progesterone, estradiol benzoate, testosterone propionate, and mestranol as reference substances [toluene–ethyl acetate (7:3), detection under 254 nm UV light and then under 366 nm UV light after detection with sulfuric acid reagent] (93); and betamethasone 17 α -valerate with prednisolone acetate, hydrocortisone acetate, and dexamethasone acetate as reference substances [methyl ethyl ketone–toluene (2:3), detection under 254 nm UV

light and then under 366 nm UV light after detection with sulfuric acid reagent] (94). The latter three procedures are approved for testing of pharmaceutical identity and quality.

A semiquantitative OPLC purity test was described for a steroid bulk drug substance used as the active ingredient in contraceptive products (95). The automatic Personal OPLC BS-50 instrument (see Chap. 7) was used with HPTLC silica gel sheets presealed for OPLC and cyclohexane–ethyl acetate–chloroform (3:1:1) as the mobile phase. The R_f values of the monitored compounds follow: 6-hydroxy derivative, 0.05; starting material, 0.16; 6-dehydro derivative, 0.36; main component, 0.40; 5-methoxy derivative, 0.59; ketal derivative, 0.80. The intermediate precision (RSD) of the R_f values measured on different plates at different times in two laboratories ranged from 3.01% to 4.17% ($n=13-32$).

B. Metabolism Studies

Steroid metabolites were identified by silica gel TLC or HPTLC in the published studies cited in this section. Readers should consult the individual references for details of the sample preparation, mobile phases, layers, and detection/quantification methods: androgen and estrogen metabolism during sex differentiation in single-sex populations of the Nile tilapia (*Oreochromis niloticus*) (96); progesterone and the oligodendroglial lineage: stage-dependent biosynthesis and metabolism (97); progesterone metabolism in human endometrial and gland cells in culture (98); in vitro effects of γ -hexachlorocyclohexane on in vitro biosynthesis and metabolism of steroids in goldfish (*Carassius auratus*) (99); in vivo steroid metabolism in embryonic and newly hatched steelhead trout (*Oncorhynchus mykiss*) (100); metabolism of testosterone by dermal papilla cells cultured from human pubic and axillary hair follicles and the relation to hair growth in 5- α -reductase deficiency (101); steroid metabolism in theca externa cells from preovulatory follicles of the domestic hen (*Gallus domesticus*) (102); gonadal in vitro androstenedione metabolism and changes in some plasma and gonadal steroidal hormones during sex inversion of the protandrous sea bass (*Lateolabrax japonicus*) (103); androstenedione metabolism in the indifferent stage of bovine gonad development (104); factors influencing testosterone metabolism by anuran larvae (105); progesterone metabolism by the filamentous fungus *Cochliobolus lunatus* (106); and corticosteroid metabolism in human immortalized keratinocytes (107).

C. Additional Miscellaneous Applications

Thin-layer chromatography was among the analytical methods covered in a review of the roles of natural sex steroids and their xenobiotic analogs in animal production (108).

Qualitative and/or quantitative analysis of steroids by TLC or HPTLC was applied in the following biological, biochemical, biotechnological, and biomedical studies: election of *Mycobacterium* sp. strains with the capacity to biotransform high concentrations of β -sitosterol based on TLC analysis of biotransformation products after extraction from bacterial cultures with methanol and ethyl acetate (109); steroid profiles in gonad and blood tissue samples from cultured jundia [*Rhamdia quelen* (Quoy and Gaimard)] (110); gonadotropin-induced steroidogenic shift toward maturation-inducing hormone in Japanese yellowtail during final oocyte maturation (111); steroidogenic pathways to

17,20- β -dihydroxy-4-pregnen-3-one and 17,30- β -21-trihydroxy-4-pregnen-3-one in the ovarian follicles of bambooleaf wrasse (*Pseudolabrus sieboldi*) (112); model structure and substrate specificity of 17- β -hydroxysteroid dehydrogenase from *Cochliobolus lunatas* (113); in vivo evidences of early neurosteroid synthesis in the developing rat central nervous system and placenta (114); effects of cytochrome P450 inhibitors and of steroidal hormones on the formation of 7-hydroxylated metabolites of pregnenolone in mouse brain micro-somes (115); estrone potentiation of myeloid cell differentiation (116); inhibition of dehydroepiandrosterone 7- α - and 7- β -hydroxylation in mouse liver microsomes (117); structure—function relationships of aldosterone synthase and 11- β -hydroxylase enzymes and implications for human hypertension (118); seasonal changes in the production of two novel and abundant ovarian steroids in the channel catfish (*Ictalurus punctatus*) (119); hydroxylation of pregnenolone at the 7- α - and 7- β -positions by mouse liver microsomes and effects of cytochrome P450 inhibitors and structure-specific inhibition by steroidal hormones (120); biosynthesis in vivo and excretion of cortisol by fish larvae (121); steroidogenesis in the ovarian follicles of the medaka (*Oryzias latipes*) during vitellogenesis and oocyte maturation (122); follicle-stimulating hormone-induced estradiol and progesterone production by bovine antral and mural granulosa cells cultured in vitro in a completely defined medium (123); branchial excretion of the maturation-inducing steroid 17,20- β -dihydroxy-4-pregnen-3-one from rainbow trout (*Oncorhynchus mykiss*) (124); testosterone extragonadal secretion by European quail maintained on short days (125); toxic effects of chronic cocaine consumption on the female reproductive endocrine functions (126); attenuation of serum androgen levels in patients with polycystic ovary syndrome and inhibition of ovarian steroidogenesis in vitro by a low-dose ketoconazole (127); reduction of aromatic steroidal A rings by lithium in ethylamine (128); pharmacokinetics and biliary excretion of osaterone acetate, a new steroidal antiandrogen, in dogs (129); and regulation of vitamin D1 α -hydroxylase in a human cortical collecting duct cell line (determination of 25-, 1,25-, and 24,25-dihydroxyvitamin D₃ by TLC) (130). Readers should consult the individual references for details of the sample preparation and TLC/HPTLC procedures.

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31

Synthetic Dyes

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I. INTRODUCTION

Synthetic dyes are mainly derivatives of aromatic hydrocarbons for which the chief source is coal tar. The term “synthetic dyes” has therefore been regarded in common usage as being synonymous with coal tar dyes. This is no longer strictly true, however, because the aromatic hydrocarbons are being manufactured in increasingly large quantities from petroleum.

A. Classification, Applications, and Colors

Dyes may be classified according to either their chemical constitution or their application to textile fibers and for other coloring purposes. To some extent there is common ground between the two methods of classification, because certain groups of dyes, such as sulfur colors must be applied by methods that depend on their chemical character. Further, the dyeing properties are useful for subdividing a large group of dyes such as the azo or anthraquinone dyes. Thus, the basic classification “anthraquinonoid dyes” indicates the fundamental nucleus from which all the dyes in the series are derived, but for detailed consideration it is convenient to divide the group into cellulose acetate, mordant, acid, and vat dyes.

The dyes are also classified according to the chromophores or essential color-producing groups that are present, but color—constitution relationships are now known to be extremely complex, and for a systematic treatment of the chemistry of dyes a classification based primarily on characteristic structural units is more satisfactory. The azo group, the anthraquinone nucleus, and heterocyclic ring systems (e.g., pyrazolone, thiazole, acridine, thiazine, oxazine) are examples of structural units that provide a simple basis of division and subdivision. Methods of preparation and application are also useful for classification. Although the nitro group occurs in numerous dyes, the class name “nitro dyes” is restricted to the nitrophenols and the nitroarylamines, in which the nitro group is a vital factor in the production of color and dyeing properties. Dyes that do not justify treatment in separate chemical classes on account of their limited numbers and

indefinite constitution have been classified as "miscellaneous dyes." For detailed classification, Venkataraman's text on synthetic dyes should be consulted (1).

Dyes are used for a wide variety of purposes, but their application to textile, foods, and leather is most important. The color of the dye depends on a number of factors. For example, with the azo group as the characteristic chromophore, the possible variations that influence the color and dyeing properties are (a) the number and position of the azo groups, (b) the nature of the aromatic nuclei, and (c) the end number and position of the sulfonic groups. It is obvious that various combinations of these factors can be used to obtain a given effect and that the color of the dye will ultimately depend on the constitution of the molecule as a whole.

B. Identification, Analysis, and Evaluation

Identification of a commercial dyestuff as a known dye or mixture of dyes is important for users of dyestuffs, public analysts, and chemical examiners in customs laboratories. Because of the very large number of dyestuffs marketed under a much larger number of names, the problems of identification and analysis have become exceedingly complex. The chemical identity of a dye is of interest to the color user, because two dyes that give the same shades upon dyeing may differ substantially in fastness properties. However, it is often difficult to establish the chemical identity of a dye. It is clear from a consideration of the number and variety of synthetic dyes that their analysis, especially in mixtures, can never become a simple routine. Because many of the distinguishing tests depend on color reactions, an important requirement for success in dyestuff analysis is the possession of a complete range of authentic samples with which a direct comparison can be made. Direct chemical methods of analysis are frequently impractical and of very limited value in dealing with commercial dyes. Colorimetric and spectroscopic methods, and dye trials or evaluation procedures based on the application of the dyes, are therefore widely employed.

Some commercial dyestuffs, such as basic dyes, azoic coupling components (particularly the amines), and a few of the anthraquinonoid acid and vat dyes, are of high purity, but dyes in general are not marketed in the pure state. Manufacturers and users of dyestuffs are interested in products of standard quality that give reproducible results in application rather than in their chemical purity. Two types of impurities or substances other than the essential tinctorial constituent occur in commercial dyes: (a) by-products of the various reactions by which the dye is synthesized and (b) substances (e.g., sodium chloride and sulfate derivative dispersing agents) added subsequently for standardization. Among the latter may be one or more dyes added to the main dyestuff for shading purposes. The separation of a mixture of two or more dyes presents much more intractable problems than the separation of a dye from inorganic or colorless organic substances.

Partition between immiscible solvents, using a fractionation procedure based on a counter-current principle, is sometimes effective for the separation of dyes in a mixture (2). Separation of dyes by means of immiscible solvents has been thoroughly investigated for the limited range of dyes used for coloring foods, drugs, and cosmetics (3, 4). Mixtures of dyes in aqueous solution can be separated by frothing with gelatin at

different pH values (5). Basic and acid dyes can be separated by means of ion-exchange resins (6).

C. Chromatography of Dyes

The advantages of chromatography are now well-known. Its value as a test for purity; ability to separate a complex mixture of compounds having only minor differences in structure; rapidity, simplicity, and ready availability of the equipment; applicability to micro quantities for purposes of isolation, identification, and quantitative estimation of the constituents of a mixture, as well as large quantities for preparative purposes; and usefulness in isolating very small quantities of solutes from very large volumes of solutions are worth mentioning.

A review of the literature up to 1973 indicates that very little work had been done on the separation of synthetic dyes. Venkataraman et al. (7) separated acid dyes by using semicircular filter paper with a short stem in the middle, water being used for adsorption and development. The method is useful for acid dyes only. Mottier and Poterat used both radial (8) and ascending (9) chromatography with loose layers of alumina to separate some food dyes, both synthetic and natural. Montag (10) used silica gel G plates for the separation of Martius Yellow, dimethylaminoazobenzene, Ceres Yellow, Ceres Orange, Sudan Red G, Ceres Red BB, and indophenol. Fujii and Kamikura (11) studied the separation of 15 oil-soluble dyes on silica gel using 12 different solvents. Sudan G, azobenzene, *p*-aminoazobenzene, Butter Yellow, *p*-methoxyazobenzene, and *p*-hydroxyazobenzene were separated with 1,2-dichloroethane. Xylene or pentachloroethane would separate Oil Yellow AB and Oil Yellow OB. Chloroform separated Quinoline Yellow SS from the remaining dyes.

Copius-Peereboom (12) chromatographed a series of 12 dyes along with several natural pigments on silica gel G, aluminum oxide G, and kieselguhr G with six different solvent systems. Copius-Peereboom and Beekes (13) chromatographed 19 oil-soluble dyes using a number of systems: on polyamide with chloroform-methanol-water (5:15:1), on silica gel with hexane-ethyl acetate (9:1), on kieselguhr G with cyclohexane, on aluminum oxide G with hexane-ethyl acetate (19:1), and on silver nitrate-impregnated silica gel G with chloroform-petroleum ether-acetic acid (75:25:0.5).

Waldi et al. (14) separated a series of dyes on silica gel G layers using a solvent consisting of chloroform-acetone-isopropanol-sulfurous acid (5-6% SO₂) (3:4:2:1). Stier and Specht (15) chromatographed a series of xanthene stains for histological work on silica gel G layers using *n*-propanol-formic acid (8:2) as the solvent system. Horobin and Goldstein (16) investigated the impurities in 11 Alcian Blue and Alcian Green samples by chromatographing on cellulose using *n*-butanol-water-acetic acid (3:3:1) as mobile phase in open or closed tanks. Horobin and Murgatroyd (17) compared paper chromatography, thin-layer chromatography (TLC) on silica gel H F₂₅₄, and thin-layer electrophoresis on agar for the resolution of histological dye components and found TLC to be a superior technique for these separations.

Waldi et al. (14) also separated a series of indicators on a 1:1 mixture of silica gel G and alumina G using a solvent composed of ethyl acetate-methanol-5 N ammonium hydroxide (6:3:1). Aliotta and Roso (18) chromatographed 12 indicator dyes on silica gel

or silica gel impregnated with phthalate buffer (pH 6.0), using water-saturated butanol as a solvent. Chiang and Yeh (19) chromatographed 12 common indicator dyes on polyamide–silica gel (1:5.2) using both acidic and basic solvent systems containing 0.5–7% sodium chloride to reduce hydrogen bonding.

Perdih (20) separated cosmetic azo pigments on air-dried silica gel G with benzene–nitrobenzene (5:1). Deshusses and Desbaumes (21) used *n*-propanol–ammonia (9:1) with silica gel G to separate 11 dyes used in lipsticks. Brown (22) reviewed the chemistry of hair dyes on silica gel thin layers with petroleum ether–acetone–ammonia (sp. gr. 0.88) (30:10:1). Meckel et al. (23) chromatographed nine direct azo dyes into a number of constituents on silica gel and alumina layers prepared with 2.5% sodium carbonate.

Loger et al. (24, 25) chromatographed 19 basic dyes for polyacrylonitrile fibers on alumina with ethanol–water (5:2) and another 11 dyes on silica gel G with pyridine–water (1:2) as the solvent system. Arsov et al. (26) chromatographed 23 basic dyes on silica gel with various solvents. Takeshita et al. (27) chromatographed 15 basic dyes on polyamide layers with five solvents. Shenai et al. (28) chromatographed direct, acid, basic, reactive, and 1:2 metal complex dyes and their mixtures on thin layers of BaSO₄ with different eluting systems. In the present chapter the work carried out on the TLC separation of synthetic dyes during the period 1973 to 2001 is reviewed, along with the relevant data from selected papers.

II. PREPARATION OF SAMPLES AND THIN LAYERS

The TLC of synthetic dyes consists of extracting the dyes from the samples and then subjecting them to TLC analysis. Various methods are used for the extraction of dyes from various sources depending on the nature of the source, ion-pair extraction (29, 30) being the most important technique. Some methods for sample preparation are given below.

A. Foodstuff Dyes

Samples of azo dyes from foodstuffs are prepared by extraction: 5–10 g of food sample is mixed with 2–5 mL of methanolic 0.1 M hexadecylpyridinium chloride, and the resulting ion pair with anionic azo dyes is extracted in 10 mL of CH₂Cl₂. The dye can be back-extracted by shaking with dilute HClO₄ (31–35). For the general class of dyes, the food sample can be treated with aqueous 0.5% NH₃, and the mixture then extracted with butanol. The aqueous phase is acidified, then extracted with butanol–Amberlite LA-2 (19:1). The aqueous phase (containing some water–soluble dyes) is removed. The combined organic phase is diluted with butanol–hexane and extracted three times with aqueous 2% NH₃. The organic phase is treated with acetic acid, and the acetic acid phase and organic phase may be analyzed by TLC (36–39).

Dyes from fruit and gums can be extracted by dissolving the sample in hot water and incubating the resulting solution at 50°C for 3 h with amylo-1,6-glucosidase solution in acetate buffer (pH 4.5). The mixture is then applied to a polyamide column, and the dyes are eluted with acetone–water–concentrated aqueous NH₃ (40:9:1). The eluate is evaporated to dryness, and the residue is dissolved in water (1 mL) (40). The chewing

gum sample (3 g) can be extracted at 75°C with water (15 mL) and then with five 15 mL portions of H₂O, each mixed with 0.5 mL of aqueous 25% NH₃. The combined extracts are acidified with anhydrous CH₃COOH and passed through a column of 125 g of polyamide. The column is washed with hot water–acetone (65°C). The dyes are eluted with aqueous 70% methanol–aqueous 25% NH₃ (49:1) at 55°C. The eluate is concentrated to 5 mL at 75°C; then H₂O (20 mL) and anhydrous acetic acid (0.1 mL) are added (41).

Dyes from pudding samples can be isolated by adsorption on wool or on polyamide, from which they can be extracted with aqueous methanolic NH₃ (42). Dyes from powdered species can be extracted by shaking 10 g of sample in 50 mL of H₂O. The color is extracted over 30 min, and the solution is then concentrated to 1 mL. The concentrated solution is diluted with methanol, and this solution can be analyzed for dyes (43).

Drinks (filtered or centrifuged if cloudy) are acidified and applied to a C₁₈ Sep-Pak cartridge from which the colors are eluted with ethanol made alkaline with aqueous NH₃. Solid foods are extracted by maceration with aqueous 50% acetone or ethanol made alkaline with Na₂B₄O₇ solution, and the solids are separated by centrifuging (after addition of Celite 545). The solvent is evaporated in a rotary evaporator at 40°C, and the residual solution is acidified with HCl and applied to a Sep-Pak cartridge as before (44).

Dyes can be extracted from oils and fats and from chocolates in light petroleum (boiling range 40°–60°C. The light petroleum is shaken with dimethylformamide (I), and phase I is separated and mixed with an equal volume of water. A portion of solution I is applied to a column (25 cm×15 mm) packed with polyamide powder MN5C6. The column is washed with water to remove solution I. The dyes can be eluted with various solvents (45).

Dyes from meat samples are extracted with light petroleum and subsequently with aqueous methanolic 0.5% sodium dodecylsulfate. The light petroleum extract is concentrated by dissolving in methanol before separation (45a).

B. Leather Dyes

Leather dyes are isolated from the sample by extraction prior to their separation. Samples of leather (100 g cut into strips) that have been dyed can be extracted under reflux for 2–3 h with 100 mL of aqueous 50% dimethylformamide (46, 47). Acid dyes from leather are extracted with aqueous NH₃ solution. The extraction system is based on use of concentration gradient with aqueous NH₃ (47a).

C. Fiber Dyes

The fiber to be examined can be extracted in a sealed melting point tube with 100 mL of aqueous NH₃ in a boiling water bath for 40 min. Any color change is noted, and the extract can be analyzed for dyes (48, 49). The extraction from fibers must be nondestructive, leaving the fiber intact for further analysis. Sometimes the dye content of synthetic fibers can be extracted with a solvent in which the dyes are soluble. For example, the dye contents of polyester acrylics, nylons, and acetates that are soluble in hexafluoropropane-2-ol can be extracted with this solvent (50). The scheme for extraction

from fibers was modified by Home and Dudley (51). Standardization of TLC systems for comparison of fiber dyes was reported by Laing et al. (51a).

Azoic dyes from cotton fibers can be extracted with anhydrous acetic acid by heating in a sealed tube at 100°C and separated on Kiesel-gel 60 F₂₅₄ TLC plates using chlorobenzene–1,2-dichloroethene–acetone (20:20:1) as mobile phase (51b). Disperse, cationic, and other dyes can be extracted from fibers with DMF/chlorobenzene, 50% formic acid, and NH₃ solution, respectively (51c). Wiggins et al. (51d) investigated the use of thin-layer chromatography in the analysis of reactive dyes released from wool fibers. Bulk samples of dyed wool and single dyed fibers were dissolved in 0.75 M NaOH at 40°C for 24 h with subsequent addition of methanolic 0.3 M citric acid and centrifugation, and the resulting solutions were analyzed by TLC on Kiesel-gel 60 F₂₅₄ aluminum-black plates with propan-1-ol–aqueous NH₃–methanol–H₂O (6:4:3:1) as mobile phase. The additional information obtained from TLC outweighed the disadvantage of destruction of the fibers.

D. Dyes from Alcoholic Products

Dyes can be isolated from alcoholic products by the wool yarn method (52). The dye is extracted from an acidified sample of lime or ethanol onto wool yarn, which is then extracted in 10% aqueous NH₃ (53, 54). Alternatively, liquor samples (10 mL) can be evaporated and the residue dissolved in 40% ethanol (1 mL). This solution can be analyzed for dyes (55, 56).

E. Cosmetic Dyes

Lipsticks are sometimes applied directly to TLC plates (57). Fat and fat-soluble colors are extracted from samples of cosmetics with hexane, after which the organic dyes are extracted with dimethylformamide in the presence of H₃PO₄ (58).

Cosmetics and food dyes are extracted from tablet coating formulations, releasing the dyes from their lakes by treatment with 85% H₃PO₄, then dissolving in methanol and making alkaline with concentrated aqueous NH₃ (59).

Dyes are extracted from soaps by dissolution in methanol or in CH₂Cl₂ and subsequent TLC (60). Alternatively, the soaps are fused with formic acid and the fatty acids are extracted into heptane. Oil-soluble dyes and some pigment dyes are separated from fatty acids by back-extraction into formic acid. After dilution of the filtrate, 30% NaOH solution is added, the mixture is extracted with CHCl₃, and the extract is washed with H₂O (61).

Dyes can be extracted from mouthwashes and toothpastes in either light petroleum or CH₂Cl₂ (62).

Nail lacquers are digested with ethyl acetate, and the digest is extracted with aqueous 50% dimethylformamide. The lower dimethylformamide phase is separated and, after extraction with high petroleum to remove fat, is mixed with polyamide powder, which adsorbs the dye. The powder is packed in a column and washed with methanol. The dye is then eluted with concentrated aqueous NH₃–methanol (1:19) (63).

F. Dyes from Miscellaneous Sources

The extraction of dyes from capsules and sugar-coated tablets can be carried out by dissolving a suitable number of empty capsules in 5 mL of 10% CH_3COOH and passing the mixture through a column (1 cm diameter) containing 1.5 g of alumina. The gelatin is removed by passing 10 mL of H_2O through the column with gentle suction. The coloring matter is then eluted with 1% aqueous NH_3 . The eluate is carefully evaporated to dryness, and the residue is dissolved in a few drops of methanol and examined by TLC (64). Dyes from paints can be extracted with CH_2Cl_2 (65).

Dyes from powdered pencil lead or pencil writing on plain white paper can be extracted with acetone, and portions of extract can be separated on silica gel G plates using toluene–cyclohexane (1:1), butanol–ethanol– H_2O –anhydrous CH_3COOH (70:30:30:0.5), or butanol–ethanol– NH_3 (4:1:1) as mobile phase (65a). Reports on the extraction and separation of dyes from different types of inks and other marking materials are also available (65b–65e). Zlotnick and Smith (65f) reviewed the components of different types of inks and their identification by using a number of separation techniques including thin-layer chromatography.

Koprivanac et al. (65 g) studied the production of monochlorotriazine reactive dyes using an anthraquinone reactive blue as a model. The stages of the process were monitored with TLC. Twelve chromatographic systems were investigated, and the most favorable mobile phase for TLC of the studied compounds was chloroform–isopropyl alcohol–ammonium hydroxide (2:4:1).

Separation of oil-soluble synthetic colors from annatto were studied by Biswas et al. (65h). Annatto extract was shaken with hexane and aqueous 70% acetonitrile, and the aqueous layer was diluted with water and reextracted hexane or CHCl_3 . The combined extracts were concentrated by evaporation and analyzed by TLC on silica gel plates with acetic acid– CHCl_3 –acetone (2:50:50) as mobile phase. After development the plates were sprayed with concentrated H_2SO_4 , causing the spots corresponding to the synthetic oil-soluble colors to turn from red to blue and from yellow to red or deep orange.

Azo dyes, metal complexes, and tryptophan enantiomers were analyzed by TLC on cellulose layers using aqueous mobile phases comprising 1 M NaCl –cyclodextrin polymer (65i). Development was carried out at 20–22°C. The polymers eluted the azo dyes better than an α -cyclo-dextrin solution although the α -cyclodextrin monomer generally gave better separations than any of the polymers. Attempts to use the polymers as nonspecific desorbing agents for colored inks were unsuccessful.

Two dicationic zeolites, each containing sodium and tetramethylammonium cations, were synthesized by different methods (65j). These zeolites were studied as stationary phase layers on glass plates (20×20 cm) in the TLC separation of a synthetic mixture of five standard basic dyes. Two multicomponent mobile phases were used, benzene– CHCl_3 –methanol (6:4:1) and benzene– CHCl_3 –methanol–acetic acid (4:3:3:1), and detection was by IR spectrophotometry. The two zeolites behaved similarly and were satisfactory for the chosen dye separations.

Twenty-three lichen species mixed with norstictic acid and atronorine as reference compounds were subjected to TLC on silica gel 60 F_{254} with toluene–dioxane–acetic acid (45:15:2), *n*-hexane–diethyl ether–formic acid (13:10:2), or toluene–acetic acid (20:3) as

mobile phase. Twenty-six compounds including 20 dyes and dye precursors, e.g., depsides and depsidones, were identified from the TLC R_f values and visualization test results and their UV spectra (65k).

G. Preparation of Thin Layers

Thin-layer chromatographic plates of adsorbents can be prepared as described by Stahl (66) by mixing the adsorbent with the required amount of water or solvent and spreading the solution on plates with the help of a Quickfit spreader or applicator. The plates are first dried, then activated for a few hours. For example, put 60 g of the adsorbent alumina neutral in a 500 mL conical flask, add 75 mL of distilled water, stopper it, and shake vigorously for a minute until a uniform slurry is obtained. Pour the slurry immediately into the applicator and spread it into a uniform thickness of 0.40 mm. Air-dry the plates for 30 min and activate them in an oven at $102 \pm 3^\circ\text{C}$ for an hour. Cool the plates in a desiccator at room temperature before use. To prepare the impregnated plates, known amounts of impregnant can be mixed with the adsorbent. Precoated plates are also available, and a comparison of these plates for separation of dyes has been reported (66a).

Berezkin and Markov (66b) suggested the use of plates bearing paper preadsorption layers for thin-layer chromatography. Chromatographic paper on glass and polymer plates as a preadsorption layer increases the volume preadsorption zone and also simplifies the introduction of sample for the analysis. The application range of the TLC plates with flexible support is increased as these plates with concentrating layers were studied for the first time. Zhang and Cai (66c) investigated the effect of the binder contents (sodium carboxymethyl cellulose) on homemade coated thin-layer plates. The characteristics of the plates were investigated using an automatic CS-920 TLC scanner with a dyestuff mixture or a mixture of 2-amino-4,6-dinitrotoluene and 4-amino-4,6-dinitrotoluene as analyte and with toluene (or benzene)–ethyl acetate–methanol (80:2:1), respectively, as mobile phase. Results showed that the plate prepared with aqueous 1% CMC solution had good TLC performance.

The dyes are spotted 2 cm above the edge of the plate in 2–3 μL portions of their solutions. The chromatograms are developed until the solvent front has migrated 10–12 cm. Multicomponent solvent systems should be thoroughly shaken in a separately funnel before use.

III. TLC OF DYES ON UNTREATED PLATES

Considerable work has been carried out on the TLC separation of various classes of synthetic dyes on untreated plates. Dyes from various sources, namely, foodstuffs (fruits, oils, spices, al-coholic products), coated tablets, leather, fibers, cosmetics, etc., are first extracted and then applied to the layers. About 200 mL of developing solvent in a chromatographic development tank is then used to develop the chromatograms up to 10 cm. The data for the different developing solvents and adsorbents (stationary phases) used for the separation of a variety of synthetic dyes, along with specific characteristics of the separation procedure, if any, are tabulated in Table 1. Details of specific separations follow.

A. Cationic Dyes

Cationic dyes—Acridine Orange, Crystal Violet, Janus Green B, Methyl Violet, Neutral Red, Pyronin B, Pyronin Y (G), Safranin, Victoria Blue B, and Victoria Blue 4R—commonly used in histology, were studied by TLC on the Marshall and Lewis system (115). Marshall also separated some Sudan dyes, used for the histological staining of fats, on silica gel TLC sheets using benzene–CHCl₃ (10:1) as the mobile phase (115).

Owing to the poor results obtained by LC separation of the dyes Victoria Blue R, methylene blue, and fluorescein because of the similar colors of the blue dyes, a replacement dye mixture was prepared comprising Oil Red O, Victoria Blue R, and fluorescein. Preliminary studies were done using TLC with ethyl acetate or 95% ethanol as developing solvent and densitometric scanning at 370–700 nm (115a).

Wakasmundzka (115b) studied the retention behavior of 16 heteroazophenol dyes in normal-phase systems by TLC. Solutions of each of the 16 dyes, from four different heterocyclic systems, viz., 1,3,4-thiadiazole, 1,2,4-triazole, and benzimidazole conjugated with pyrocatechol, and β - and γ -resorcylic acids were applied to plates (10×20 cm) coated with alumina basic 60 E HF₂₅₄ (0.25 mm thickness). The mobile phases were ternary mixtures of ethyl acetate, THF, methanol, or propan-2-ol in CH₂Cl₂ containing 2% acetic acid. The most selective systems were those containing methanol or propan-2-ol as polar modifiers.

B. Food Dyes

The developing systems used for the separation of food colors are recorded in Table 1. Some typical R_f values of water-soluble dyestuffs (72) are recorded in Table 2 along with the $v_{\max}$ value and percent recovery of each dye. Slightly soluble food dyes can be studied at elevated temperatures (102). Twenty-two high-boiling organic solvents were used as eluents, including hydrocarbons and esters. Using the selected solvents, indigo was chromatographed on a silica gel layer at 150°C. The data are recorded in Table 3.

Sherma (115c) reviewed TLC analysis of a number of agricultural products, foods, beverages, and plant constituents from mid-1995 to mid-1999. Techniques and applications for a wide range of analyte and sample matrix types were covered, with specification of the particular layers, mobile phases, detection methods, and quantification conditions in many cases.

Soluble dyes from spices were separated and identified on alumina plates using methanol–liquor ammonia (8:2 v/v) as mobile phase (43). Bright-colored spots of respective coal tar dyes are separated and observed with the following R_f values: Rhodamine B, 0.93; Metanil Yellow, 0.87; erythrosine, 0.83; Fast Red E, 0.71; carmoisine, 0.69; Sunset Yellow FCF, 0.66; tartrazine, 0.46; Ponceau 4R, 0.32; and Amaranth, 0.22.

Food dyes permitted in Japan were investigated under fast atom bombardment (FAB) and liquid secondary ion (LSI) MS conditions with the use of various materials. The mobile phase was 10% Na₂SO₄ solution–methanol–ethyl methyl ketone (7:2:2) for xanthenes and 10% Na₂SO₄ solution–methanol–acetonitrile (10:3:3) for other dyes (102a). Seven permitted coloring materials used in foods and pharmaceutical preparations in Egypt were separated by two-dimensional TLC on cellulose layers (102b).

Oka et al. (102c) studied the identification of illicit dyes in foods by thin layer chromatography coupled with fast atomic bombardment and mass spectroscopy (TLC/FAB-MS). These dyes were extracted and transferred onto pure wool in a medium of aqueous acetic acid; the wool was then transferred into methanol. Glass plates coated with octadecyl sulfate (ODS) were used for TLC with mobile phases of aqueous 5% Na₂SO₄-methanol-acetonitrile (10:3:3) or aqueous 5%

Table 1 Solvent Systems and Adsorbents for Synthetic Dyes

Source	Dyes obtained	Stationary phase (adsorbent)	Mobile phase(s) [solvent system(s)]	Other techniques/ visualization, etc.	Ref.
Food	Brilliant Blue FCF and Amaranth	Zeolite Scolecite	Acetone	—	67
	Indigo Carmine, Cochineal Amaranth, Orange Yel- low, and Tartrazine	Magnesia	15% sodium citratemethanol (4:1)	—	68
	Fluorescein in erythrosine	Silica gel	Ethanol-CHCl ₃ - formic acid	Determined spectrophotometrically at 493 nm	69
Foodstuff	Tartrazine, orange, Yellow, Cochineal Red, amaranth, Brilliant Black BN	Silica gel	—	Limits of detection in ppm reported	70
		Silica gel/poly amide gel	Ethyl acetate-methanol-25% aq. NH ₃ (33:10:10), MeOH-EtOH-isoamyl alcohol-aq. 25% NH ₃ (15:10:5:3)	Dyes extracted with 1% NH ₃ -ethanol (2:3)	70a
	10 dyes extracted from meat	Silica gel G	Butan-2-ol-isoamyl alcohol-pyridine-ethanol-aq. 25% NH ₃ (3:3:3:4:6)	—	70b
	Xanthene dyes and	Aluminum-backed silica gel	Aq. 10% Na ₂ SO ₄ -	Spectrum also shown	70c

	triphenylmethane dyes	impregnated with 10% liquid paraffin soln.	MeOH– ethyl methyl ketone (7:7:2), aq. 10% Na ₂ SO ₄ – MeOH– acetonitrile (10:3:3)		
	Quinoline Yellow, erythro-cin, chrysoin, along with seven red dyes and four other dyes	Cellulose/Silufol and silica gel 60 G	Ethyl acetate– MeOH–conc. aq. NH ₃ (3:1:1) plus aq. 67% propanol for cellulose plates only	Detection limit also reported	70d
	Ten food coloring dyes	Silica gel H	EtOH– diethylamine– CHCl ₃ – aq. 12.5% NH ₃ (6:6:5:3)	—	70e
	Tetrazine, Brilliant Blue, Amaranth, Coccine, and erythrosine	Polyamide	SDS–Triton X-100– aq. NH ₃ soln. (1:5:1)	Spots quantified by densitometry	70f
Fruits	—	Silica gel 60	Propan-2-ol–conc. aq. NH ₃ – ethanol (77:13:10)	Spots analyzed by photoacoustic spectrophotometer	40
Mustard	Tartrazine, chrysoin, Quino-Line Yellow, Naphthol Yellow, Auramine O, Sudan Yellow, Dimethyl Yellow	Silica gel 60 F ₂₅₄	CHCl ₃ –acetic acid (9:1) on cellulose ethanol–butanol–pyridine–H ₂ O (1:7:6:6) benzene	HPTLC	71
Tablet coatings	Twenty dyes are separated	Silica gel	Ethyl acetate– methanol– H ₂ O–conc. aq. NH ₃ (30:8:7:1)	—	59
	Edible acid dyes	Silica gel or cellulose	(a) Butanol–ethyl methyl ketone–aq. 25% or 1% NH ₃ –H ₂ O (4:2:1:1) (b) Ethyl acetate– methanol– aq. 25% NH ₃ (45:10:7)	Densitometry of chromatograms	38
Alcoholic products	Acid-fast dyes	Cellulose layers	Ethyl acetate– butanol– pyridine– water (5:5:6:5)	HPTLC also carried out	53

Textiles	Five natural dyes	Silica gel G	Benzene–ethyl acetate–formic acid (74:24:1)	Methanolic 10% KOH as spray reagent	73
Fiber	(a) Disperse dyes	Silica gel 60 F ₂₅₄	(a) Toluene–pyridine (4:1) (b) Hexane–ethyl acetate–acetone (5:4:1) (c) Toluene–methanol–acetone (20:2:1)	Viewed in UV light at 254 nm	49
	(b) Acid dyes	Silica gel 60 F ₂₅₄	CHCl ₃ –H ₂ O–methanol–conc. aq. NH ₃ (11:1:7:1)	Viewed in UV light at 254 nm	49
	Six reactive, six direct, six vat, and six sulfur dyes	Kiesel-gel	Several mobile phases for each type of dye	Extraction procedure given	86
	Fifty-six commercial dyes (acid and basic)	Silica gel	Ten solvent systems evaluated mathematically	—	74
Source	Dyes obtained	Stationary phase (adsorbent)	Mobile phase(s) [solvent system(s)]	Other techniques/ visualization, etc.	Ref.
	Thirty-three purple-dyed wool fibers distinguished	Camlab Sil G	First direction: butanol–H ₂ O–aq. NH ₃ acetone (5:1:2:5) Second direction: aq. NH ₃ –CHCl ₃ –H ₂ O–methanol (2:11:1:7)	Two-dimensional TLC performed and method of extraction reported	48
	Cationic dyes red, pink, blue, and yellow	Silufol	Butanol–acetic acid–H ₂ O (1:4:5)	Separated dyes extracted with DMF and determined spectrophotometrically	74a
Leather	Acid and direct dyes	Silica gel microplates or cellulose	(a) CHCl ₃ –propan-2-ol–H ₂ O (1:3:1) (b) Propan-2-ol–aq. NH ₃ –H ₂ O (7:1:1)	Two-dimensional TLC used if separation incomplete	47
Cosmetics, lipsticks	Oil-soluble unsulfonated	Silica gel 60	Ethyl acetate–methanol–ac	—	57

	colors		30% NH ₃ (5:1:1)		
	Twenty organic dyes	—	—	—	77
	Red dyes	Silylated silica/AG 50 WX ₄	0.5 M acetic acid in 50% methanol	—	78
	—	—	Cyclohexane–pentanol–conc. HCl (13:6:1)	Located by laser light	82
Soaps	Organic dyes	Kiesel-gel	Ethyl acetate–pyridine–H ₂ O (11:5:4)	Method of extraction given	60
Mouth and dental agents	Lipophilic dyes	Silica gel 60	(a) Ethyl acetate–pyridine–H ₂ O (11:5:4) (b) Ethyl acetate–pyridine–H ₂ O–1% HClO ₄ (in methanol)–aq. 25% NH ₃ (11:5:4:4:2)	0.1 M hexadecylpyridinium chloride used as locating reagent	62
Paints	Soluble dyes	Aluminum sheets coated with silica gel 60 F ₂₅₄	Various solvents reported	Extraction procedure given	60
Hair	Oxidative hair dyes	Silica gel	(a) Toluene–acetone–CHCl ₃ (8:7:5) (b) Benzene– <i>sec</i> -butyl alcohol–H ₂ O (2:1:1)	—	79, 80 80a
	<i>p</i> -Phenylenediamine, resorcinol, etc.	Silica gel	CHCl ₃ –ethyl acetate–methanol (3:1:1)	Multiple development also done	81
Ink	Pigment Red 5 and solvent dyes	Silica gel	Benzene–methanol–ethyl acetate	Location by spectrophotometry	83
	Nitrophenylenediamine, aminophenol, resorcinol	Silica gel 60	Benzene–ethyl acetate (1:1)	Located with Ehrlich reagent and by heating at	84

70°C for 30 min

	<i>p</i> -Phenylenediamine, toluene-2,5-diamine, and 4-methoxy- <i>m</i> -phenylenediamine	Silica gel 60	Ethyl acetate		85
Colored coated tablets	Tartrazine and coccine, as well as other dyes	Carboxymethyl cellulose MN300	2% aq. NaCl–10% aq. NH ₃ (93:5)	Extraction method reported	86
Miscellaneous	Eight azine dyes and five oxazine dyes	Silica gel	Benzene–CHCl ₃ –ethanol (plus 0.5 mL of HCl) and some other solvents reported	Methanolic 5% H ₂ SO ₄ as spray reagent	87
	(a) 29 acid dyes	Poly amide plates	(a) Acetone–methanol–H ₂ O–aq. 25% NH ₃ (16:16:18:3)	—	88
	(b) Six different 1:2 metal complex dyes	Poly amide plates	(b) Ethyl acetate–ethanol–H ₂ O–25% NH ₃ (16:16:18:1)	—	88
	Reactive and direct dyes	Silica gel G	Phenol–water (4:1)	—	89
	Direct dyes	Silica gel 60	Butanol–aq. 25% NH ₃ –DMF (8:7:4) or phenol–acetone–aq. 25% NH ₃	—	89a
	Fluorescein, eosin, benzoflavine, etc.	pH gradient TLC plates from silica gel 60 H F ₂₅₄	CHCl ₃ –methanol or ethanol (19:1; 9:1; 17:3)	Located at 365 nm	90, 91

Source	Dyes obtained	Stationary phase (adsorbent)	Mobile phase(s) [solvent system(s)]	Other techniques/ visualization, etc.	Ref.

Azo dyes	Silufol UV plates	CHCl ₃ –ethyl acetate (4:1) CHCl ₃ –methanol (5:1; 19:1)	—	92
Dyes used in medical laboratories	Silufol R	Propanol–formic acid (2:1)	—	93
Azobenzene, Dimethyl Yellow, Sudan G, etc.	Silica gels of various types	Benzene	—	94
Commercial triarylmethane dyes (commonly used as analytical reagents and indicators)	Silica gel	(a) Ethyl acetate–aq. 25% NH ₃ –propan-2-ol (I) (5:3:3) for chrome azurols (b) 1,4-Dioxane–aq. 25% NH ₃ –I (2:1:1) for Phenol Red and some other systems	—	95
Cationic dyes (Methylene Blue, thiomine, azure dyes)	Silufol silica gel	Butanol–anhyd. acetic acid– ethanol–H ₂ O (5:1:2:2)	Quantitative analysis	96
Romanian and Cottestren dyes	Silica gel RG, Camag silica gel (gypsum as binder)	Sixteen mobile phases including toluene–acetone (20:1) reported	15 µg of dye could be chromatographed	97
Disperse dye (C.I. Direct Blue 139 from mixture)	Silica gel 60	First direction: water—saturated ethyl methyl ketone Second direction: aq. NH ₃ – 50% methanol	Two-dimensional TLC and detection at 254 nm	98
Azo dyes	Anasil G or Kiesel-gel 60 F ₂₅₄	Adsorbent layer located between radioluminescence sources and scanning spectrophotometer records spectra at $\lambda_{\max}$ value of each component		99
Various substituted azobenzenes	Silica SI-60/silica gel	Solvent composition effects on R_f values studied	Chromatographic data interpreted in terms of mechanism	100
Commercial acid dyes of monoazo-, diazoanthraquinone, and triphenylmethane types	Octadecylsilyl-bonded silica gel	(a) Aq. methanol (58–80%) or (b) Aq. 60% acetone	—	101

Eriochrome Cyanine (R), Chrome Azurol (S), and Eriochrome Azurol (B)	Silica gel SG ₄₁ (Whatman)	Butanol–anhyd. acetic acid–H ₂ O (7:1:3 or 7:1:5)	Absorption spectra and extraction method also given	103
Eight mixtures of a reactive dye (4:1) (monochloro-1,3,5-triazene) and a direct dye	Silica gel	(a) Butyl acetate–pyridine–H ₂ O (6:9:5) (b) Propanol–isobutyl alcohol–ethyl acetate–H ₂ O (4:2:1:3)	Adulteration of reactive dyes with cheaper direct dyes can be detected	104
Reactive dyes	Kiesel-gel G	(a) Butanol–H ₂ O–dimethylformamide (160:60:19) (b) Butanol–anhyd. dimethylformamide–anhyd. acetic acid (60:28:5:5) (c) Butanol–ethanol–H ₂ O (8:4:3)	Method is rapid and suitable for quality control	105
Forty-two reactive dyes, 10 acid dyes, 20 metal complex dyes, 23 basic dyes, and 27 disperse dyes	Silica gel 60	Mobile phases are reported for each class of dye	Two-dimensional development also reported	106
Rhodamine S and 6 G, eosin K, and eosin	Silufol	3 mL toluene+5 drops <i>o</i> -cresol+3 mL alcohol+ 0.5 mL aq. 20% NH ₃	Separation done in mixture	107
Amaranth (in Hibiscus)	Silica gel	(a) 4% soln. of sodium citrate in 5% aq. NH ₃ , (b) CH ₃ COOH–HCOOH–C ₂ H ₅ OH–H ₂ O(1:1:1:7), or (c) butanol–pyridine–H ₂ O (6:4:3)	Dye content evaluated by determining the anthrocyanin-to-dye ratio	108

Source	Dyes obtained	Stationary phase (adsorbent)	Mobile phase(s) [solvent system(s)]	Other techniques/ visualization, etc.	Ref.
	Rhodamine B, fluorescein sodium, eosin, erythrosine, Rose Bengal, and Phloxine P	Silufol	Chlorobenzene–ethylacetate–acetic acid (90:10:3)	Detected under 254 nm radiation	109
	Brilliant Green, Patent Blue, Brilliant Blue	Silica gel G	Eight solvent systems reported	Detected 0.2 µg of dye	110

FCF (isolated from cosmetics)					
Basic dyes	Kiesel-gel G	Eight solvent systems based on mixtures of CHCl_3 , alcohol or ketones, weak acid, water, and pyridine studied	Two-dimensional development also reported	111, 111a	
Acid Green 5 in mixture	Cellulose	Butyl acetate–dimethylformamide– H_2O (10:5:1)	Spectrophotometry reported	112	
Water-soluble food colors	Silica gel	Ten solvent systems reported	—	113	
Ponceau 6R, C.I. Acid Red 44, and C.I. Acid Red 41 (extracted from cakes and pastries)	Microcrystalline cellulose powder and silica gel G	Seven solvent systems reported	Recoveries of dyes were 95–98%	114	
4-Benzylidene-1-pyrimidin-2-ylpyrazolin-5-one dyes	Silica gel	Acetic acid–propan-2-ol–ethyl acetate (in various compositions)	—	114a	
Cottestren dyes	Zeolite NaX (0.5–5 μm)	Nitrobenzene–benzene–heptane (4:2:1)	—	114b	
Vat dyes	Silica gel	Nine different nonreducing solvent systems	Comparison with reversed-phase TLC reported	114c	

Table 2 R_f Values of Some Dyes Extracted from Water—Soluble Dyestuffs

Dye	R_f value	ν_{max} in solution (nm)	Recovery (%)
Naphthol Yellow S	0.65	426	93
Tartrazine	0.20	423	88
Ponceau 3R	0.33	499	90
Amaranth	0.10	522	89
Rhodamine B	0.45	552	84
Ery thro sine B	0.95	522	91
Indigo Carmine	0.00	603	77
Patent Blue V	0.75	634	92

Mobile phase: ethanol–*n*-butanol–water (9:1:2, v/v).

Source: Adapted from Ref. 72.

Na₂SO₄–methanol–ethyl methyl ketone (1:1:1). The dye spots on the plate were treated with 1,4-dithiothreitol–1,4-dithioerythritol (3:1) as matrix to enable recording of the FAB-MS spectrum. R_f values were obtained with each of the two mobile phases and the characteristic molecular ion species by mass-to-charge ratio (m/e) were observed by FAB-MS, in which lower detection limits ranged from 0.03 to 5 $\mu\text{g/spot}$ for 26 dyes.

Gerasimov et al. (102d) identified a set of nine synthetic food dyes with TLC by examining the color images of the chromatograms by computer processing. The process includes scanning the chromatographic plates processing the images with appropriate programs.

In another study (102e), Gerasimov proposed a procedure for the qualitative and quantitative determination of dyes after thin-layer chromatography using an example of five synthetic food colors: Brilliant Blue, tartrazine, Sunset Yellow, Ponceau 4R, and azorubine. The procedure was applicable even when the dyes were incompletely separated on the chromatograms.

Table 3 Eluotropic Series of High-Boiling Solvents and R_f Values for Indigo

Solvent	R_f for indigo	Solvent	R_f for indigo
1-Chloro- <i>n</i> -dodecane	0.06	Di- <i>n</i> -Propyl phthalate	0.73
1-Bromonaphthalene	0.12	Di- <i>n</i> -butyl adipate	0.75
1-Chloronaphthalene	0.15	Diethyl phthalate	0.81
2-Ethyl-naphthalene	0.16	Dimethyl phthalate	0.84
1-Methylnaphthalene	0.32	<i>n</i> -Butyl benzoate	0.95
Diphenyl ether	0.32	Ethyl anthranilate	0.97
Diisooctyl adipate	0.43	Nitrobenzene	1.00
Diisobutyl phthalate	0.58	2-Methylbenzothiazole	1.00
Benzyl benzoate	0.58	<i>N</i> -Methyl-2-pyrrolidone	1.00
Di- <i>n</i> -butyl phthalate	0.63	2-Ethyl- <i>n</i> -hexanol	1.00
Benzophenone	0.70	Quinoline	1.00

Source: Adapted from Ref. 102.

C. Cosmetic Dyes

Silica gel 60 plates were used to identify lip cosmetics (57). The R_f values of some cosmetic dyes obtained from lipsticks are recorded in Table 4 along with their colors. A mixture of 15 mL of ethyl acetate, 3 mL of methanol, and 3 mL of ammonium hydroxide–water (3:7) solvent (a) and dichloromethane solvent (b) were used as the

mobile phases. The shiny surface from the rounded end of the lipstick was removed with tissue, and the lipstick was weighed. The TLC plate was activated, and 10–20 mg of lipstick was applied directly to the plate. The plate was developed in two separate steps: oil-soluble, unsulfonated colors (D&C Orange 17 and D&C Red 36) were separated using dichloromethane, and other colors were separated using solvent (b).

Mikami and coworkers (57a) analyzed coal tar dyes used in the cosmetics and food industries by TLC. The dyes were spotted on reversed-phase RP-18 F₂₅₄ S plates, and the plates were developed in four solvent systems: acetonitrile-methanol-5% sodium sulfate solution (3:3:10), methyl ethyl ketone-methanol-aqueous 5% sodium sulfate (1:1:1), acetonitrile-methanol-aqueous 5% sodium sulfate (1:1:1), and acetonitrile-CH₂Cl₂-aqueous 5% sodium sulfate (10:1:5). The visible absorption spectra of the dyes were measured by scanning densitometry at 370–700 nm.

D. Acid and 1:1 and 1:2 Metal Complex Dyes

Acid and metal complex dyes belong to different groups of chemical substances. Thirty-eight commercial dyes of these classes were studied on silica gel TLC plates (116). The best results for the separation of acid dyes are shown in Table 5. The data on 1:1 metal complex dyes are recorded in Table 6, and those for 1:2 complex dyes in Table 7. The solvent systems used are given in each table. It was observed that well-shaped spots without tailing were obtained for acid dyes and 1:2 metal complex dyes. The separation of 1:1 metal complex dyes was also clear, but the spots were diffuse and showed tailing. The best solvent systems were S₁ and S₂ for the acid dyes, S₂ and S₄ for the 1:1 metal complex dyes, and S₁ and S₃ for the 1:2 metal complex dyes.

E. Cyanine Dyes

Precoated 100 μ m silica gel plates from Eastman Kodak and precoated 250 μ m silica gel sheets from EM Labs were used to separate some cyanine dyes (117). The R_f values along with mobile

Table 4 TLC of Organic Colors in Lip Cosmetics³

Type of color	Name of dye	Color of spot	R_f
Oil- soluble	D&C Red 36	Orange	0.9
Unsulfonated	D&C Orange 17	Orange	0.8
Other	FD&C Red 3	Pink fluorescence ^b	0.25
	D&C Red 21	Pink fluorescence ^b	0.22
	D&C Orange 5	Orange fluorescence ^b	0.14
		Red fluorescence ^b	0.22
	D&C Red 19	Pink fluorescence ^b	0.57
	D&C Red 7 (ca)	Red	0.24
	D&C Orange 4	Orange	0.35

D&C Red 9 (Ba)	Orange	0.41
FD&C Violet 1	Blue	0.19
D&C Blue 9	Pink (weak)	0.29

^aDichloromethane is the mobile phase for D&C Orange 17 and D&C Red 36; for the others a mixture of 15 mL ethyl acetate, 3 mL methanol, and 3 mL ammonium hydroxide-water (3:7) was used.

^bFluorescence under UV light (254 nm).

Source: Adapted from Ref. 57.

Table 5 R_f Values of Color Components of Acid Dyes During TLC on Kiesel-Gel G^a

Dye no.	Dye name	Producer	Color of component	Solvent system ^b				
				S ₁	S ₂	S ₃	S ₄	S ₇
	Polar Brilliantrot GEN	Ciba-Geigy (Basel, Switzerland)	Pink	0.23	—	0.35	0.52	0.57d
			Scarlet	0.31	0.69	0.51	0.58	0.82d
2	Polar Brilliantblau RAW	Ciba-Geigy (Basel, Switzerland)	Blue	0.21	0.65	0.32	0.53	0.52d
3	Xylenechtgrau P	Sandoz (Basel, Switzerland)	Pink	0.55	0.90	0.77	0.76	0.89
			Light blue	0.62	0.96	0.86	0.85	0.95
			Violet	0.45	0.80	0.67	0.71	—
			Marine blue	0.24	0.65	0.40	0.55	0.45
			Blue	0.15t	0.51	0.27t	0.59	—
4	Ponceau 2RL	Ciech (Warsaw, Poland)	Orange	0.41	0.77	0.65	0.70	0.87
			Red	0.04	0.56	0.07	0.37	0.15
5	Acid Black 10BS	Ciech (Warsaw, Poland)	Light blue	0.25	0.67	0.03	0.53	0.50
			Blue	0.09	0.48	—	0.19	0.00
6	Acid Brown GOL	Ciech (Warsaw, Poland)	Yellow	0.44	0.82	0.73	0.75	0.87
			Orange	0.37	0.75	0.65	0.68	0.80
			Violet	0.07	0.60	0.15	0.42	0.25
7	Acid PinkM ^c	Sojuzchimexport (Moscow, USSR)	Pink	0.09	0.52t	0.05t	0.50	0.28dt

			Orange	0.03t	—	—	0.36t	0.10dt
8	Acid Fast red 100%	Sojuzchimexport (Moscow, USSR)	Red	0.03	0.58	0.07t	0.40	0.15d
9	Acid Blue RN	Sojuzchimexport (Moscow, USSR)	Blue	0.26	0.75	0.50	0.64	0.70d
10	Acid Black ATT	Sojuzchimexport (Moscow, USSR)	Orange	0.40	0.77	0.70	0.63	0.83
			Blue	0.22	0.68	0.38	0.50	0.50
Time (min) for 12 cm migration of solvent front				80	80	30	40	110

^aThe solvent systems used were as follows: S₁, ethyl acetate–glacial acetic acid–water (3:1:2); S₂, *n*-butanol–ethyl methyl ketone–glacial acetic acid–pyridine–water (15:15:4:2:10); S₃, methyl acetate–glacial acetic acid–water (30:3:20)+5% of NaCl (with respect to H₂O); S₄, methyl acetate–glacial acetic acid–water (30:5:20)+5% of NaCl (with respect to H₂O); S₇, *n*-butanol–glacial acetic acid–water (15:4:5).

^bd=diffuse spot; t=tailing spot.

^cInsoluble residue at the origin.

Source: Adapted from Ref. 116.

phases tried are recorded in Table 8. The two silica gel coatings tended in some cases to give different results when used with the same solvents. However, reproducibility of chromatograms on the same manufacturer's coatings was good.

R_f and R_m values for seven styryl cyanine dyes were reported by Patnaik et al. (117a) for silica gel plates using H₂O–propanol–acetic acid in various proportions as mobile phases.

F. Leather Dyes

The data on R_f values of some dyes used in the leather industry (75, 76) are recorded in Tables 9A–9D. The leather dyes are divided in four groups: acid dyes, direct dyes, basic dyes, and premetallized dyes.

Table 6 R_f Values of Color Components of 1:1 Metal Complex Dyes During TLC on Kiesel-Gel G^a

Dye no.	Dye name	Color index no.	Color of component	Solvent system ^b					
				S ₁	S ₂	S ₃	S ₄	S ₅	S ₆
11	Palatinechtgelb EIN ^c	19010	Lemon yellow	—	0.31t	—	—	0.23	0.13t
			Yellow	0.00	0.05	0.14	0.25dt	0.03	0.25dt
12	Palatinechtgelb 3 GN ^c	14006	Light yellow	0.38	—	—	0.57dt	—	—
			Yellow	0.08t	0.51dt	0.36dt	—	0.50dt	0.37t

13	Palatinechtorange RN ^c	18740	Orange	0.42t	0.78dt	0.86t	0.76dt	0.77t	0.75dt
			Pink-orange	0.12dt	—	0.38dt	—	—	0.25dt
14	Palatinechtrot GREN ^c	18800	Orange- yellow	—	0.54dt	—	—	0.67dt	0.57dt
			Orange	0.25t	—	0.74t	0.55t	0.53t	0.41t
15	Palatinechtrosa BN ^c	18810	Pink-red	0.13d	—	—	—	0.48dt	0.46dt
			Pink	0.05t	0.48dt	0.31t	0.55t	0.37t	0.23t
16	Palatinechtviolet 3RN ^c	16055	Violet	0.33	0.65t	0.77t	0.70t	0.67t	0.58t
17	Palatinechtgrun BLN ^c	13425	Green	0.39	0.62t	0.82	0.64	0.65t	0.55t
18	Palatinechtblau GGN ^c		Blue	0.21	0.58	0.66	0.60t	0.60t	0.53t
19	Palatinechtbraun RN	14251	Brown	0.60d	0.83d	—	0.86	0.80	0.72d
20	Palatinechtbordeaux RN ^c	19351	Red	0.57dt	0.80d	—	0.82	0.85	0.82dt
Time (min) for 12 cm migration of solvent front				80	80	30	40	100	100

^aThe solvent systems were as follows: S₁, S₂, S₃, S₄, and S₇ as in Table 5; S₅, *n*-butanol–glacial acetic acid–water (15:4:5) +4% (w/v) sodium dodecyl sulfate (with respect to water).

^bd=diffuse spot; t=tailing spot.

Insoluble residue at origin.

Source: Adapted from Ref. 116.

G. Disperse Dyes

Disperse dyes are being used increasingly in the synthetic fiber industry, particularly with polyester fibers. Eighteen disperse dyes produced by different companies have been separated using macropolyamide sheets (118). The best solvent systems proved to be the following: S₁, benzene–light petroleum–methanol–glacial acetic acid (3:9.5:1:0.1); S₂, light petroleum–chloroform–methanol–glacial acetic acid (6:1:0.3:0.2); S₃, chloroform–*n*-hexane–methanol–glacial acetic acid (5:30:2:0.1); S₄, light petroleum–benzene–methanol (5:11:2). Some monomeric and polymeric disperse dyes have been synthesized for hydrophobic fibers by Maradiya and Patel (118a). The purity of the dyes was examined by TLC.

H. Dyes Used in Country Liquors

The addition of synthetic food colors to country liquors (e.g., Scotch from Scotland) is a common practice followed by all manufacturers. This poses a great problem for the differentiation of the genuine country liquors from imitation ones. However, a test of the genuineness of a colored country liquor can be based on the study of dyes in a specific

brand of country liquor. The separation and identification of dyes in Indian country liquors (55) have been achieved on silica gel G thin-layer plates (250 μm thickness) using *n*-butanol–glacial acetic acid–ethanol–water (10:2:0.5:5) as the mobile phase. The R_f values along with the color of the main dyes present in Indian country liquors are reported in Table 10.

IV. TLC OF DYES ON IMPREGNATED PLATES

A survey of the literature on the TLC separation of synthetic dyes indicated that little attention has been paid to the separation of dyes on impregnated plates. Only a few papers have appeared

Table 7 R_f Values of Color Components of 1:2 Metal Complex Dyes During TLC on Kiesel-Gel G^a

Dye no.	Dye name	Producer	Color of component	Solvent system ^b			
				S ₁	S ₃	S ₆	S ₇
21	Ortolangelb G	BASF (Ludwigshafen, GFR)	Lemon yellow	0.60	0.81	0.69t	0.88
22	Irgalanbrillantrot BL	Ciba-Geigy (Basel, Switzerland)	Red	0.34	0.50	0.24t	0.83
			Orange-red	0.48	0.67	0.53	—
23	Irgalanbraun 3BL	Ciba-Geigy (Basel, Switzerland)	Orange	0.68	0.96	0.78	0.91
			Gray-blue	0.63	0.91	0.71	0.88
24	Irgalangrau BRL	Ciba-Geigy (Basel, Switzerland)	Gray-blue	0.50	0.80	0.68	0.81
25	Isolanorange GGL	Bayer (Leverkusen, GFR)	Orange	0.63	0.86	0.67	0.89
26	Wofalanrubin RL	Wolfen (Bitterfeld, GDR)	Ruby	0.74	0.97	0.78	0.93
27	Wofalanoliv BL	Wolfen (Bitterfeld, GDR)	Blue-green	0.83	0.99	0.82	0.95
			Yellow-green	0.77	0.95	0.75	0.90
28	Grigio stenolana RL	ACNA (Milan, Italy)	Pink	0.75	0.94	0.86	0.96
			Pink	0.80	1.00	—	—
			Marine blue	0.65	0.90	0.71	0.87
29	Bordo stenolana 2BL	ACNA (Milan, Italy)	Pink	0.68	0.87	—	—

			Bordeaux	0.74	0.97	—	—
			Bordeaux	0.78	0.99	—	—
30	Bruno stenolana 2GL	ACNA (Milan, Italy)	Gray	0.61	0.88	—	—
			Orange	0.66	0.92	—	—
Time (min) for 12 cm migration of solvent front				80	30	65	110

^aThe solvent systems used were as follows: S₁, S₃, and S₇ as in Table 6; S₆, chloroform–isoamyl alcohol–ethyl methyl ketone–methanol–pyridine (6:5:3:2:2).

^bt=tailing spot.

Source: Adapted from Ref. 116.

in the area. Two types of impregnated layers have been used to separate the synthetic dyes: metal ion—impregnated plates and ion exchanger—impregnated plates.

A. Metal Ion—Impregnated Plates

Dyes are reported to form complexes with metal salts (119), and complexation is reported to improve the separation in many cases (120–123). Hence various metal salts have been tried by various workers to improve the separation of synthetic dyes. The separation of 42 synthetic dyes on 5% cadmium acetate–impregnated silica gel G layers was reported by Srivastava et al. (124). The dyes were divided in two groups, A and B. The group A dyes were well separated using butanol–benzene–ethyl acetate (40:35:25), and those of group B were well separated using *n*-butanol–water–formic acid (35:10:5) as mobile phase. There was complete disappearance of tailing, spots were compact, and the dyes were self-visualized. The comparison with TLC on plain silica gel indicated that tailing was much greater in the case of plain silica gel plates than on impregnated plates. It was therefore concluded that the chromatographic behavior of dyes on silica gel G plates is greatly influenced by the impregnation of plates with metal salts.

Srivastava et al. (125) separated 30 synthetic dyes on silica gel plates impregnated with ammonium molybdate and copper sulfate using BuOH–AcOH–H₂O (25:5:10) and BuOH–50%

Table 8 R_f Values for Cyanine Dyes in Selected Solvents^a

Dye ^b	Dye no.	$R_f \times 100$			
		I	II	III	IV
2,2'-Indo-4,5,4',5'-dibenzocarbocyanine	ICG	86	—	—	—
2,2'-Thiacarbocyanine	156	—	22	—	85
4,4'-Quinocarbocyanine	171	5	24	—	86
2,2'-Quinocarbocyanine	179	5	51	—	92
2,2'-Indocarbocyanine	1405	21	—	—	—

2,2'-Thiacarbocyanine	1407	51	—	—	—
2,2'-Oxathiacarbocyanine	1518	—	38	—	25
Merocyanine:2-thia derivatives	1901	70	73	18	92
2,2'-Thia-4,5,4',5'-dibenzocarbocyanine	1978	66	30	—	U
Miscellaneous <i>meso</i> -cyanines	2062	79	57	23	83

^aTLC on silica gel (EM Labs). I, methanol 100%; II, *n*-butanol–acetic acid–water (20:10:50); III, *n*-butanol–ethanol–water (90:10:10); IV, chloroform–methanol (80:20). U indicates that the TLC system was unsatisfactory for the compounds; dash (—) indicates that the compound was not tested with that TLC system.

Structures are given in Ref. 117.

Source: Adapted from Ref. 117.

NH₃–dioxane (25:5:10) as mobile phases. No tailing of spots was observed on ammonium molybdate- and copper sulfate–impregnated plates using these mobile phases. The data on hR_f values are recorded in Table 11. The hR_f values were not altered when dye mixtures were applied.

Twenty-five synthetic dyes were separated by Gupta (126) on 5% zinc sulfate–impregnated silica gel G thin-layer plates using *n*-butanol–benzene–ethyl acetate (40:35:25) as the mobile phase. The data on plain silica gel and on impregnated plates are recorded in Table 12. A difference of ± 3 units in hR_f values was taken as the criterion for satisfactory separation. Some of the typical separations that depend on hR_f values have also been achieved. It was observed that the hR_f values

Table 9A R_f Values of Some Acid Leather Dyes on Silica Gel G Plates

Dye	Solvent system ^a				
	1	2	3	4	5
1. Acid Leather Orange	0.70	0.84	0.61	0.93	0.82
2. Naphthalene Leather Orange G 132	0.68	0.82	0.72	0.89	0.82
3. Polar Brilliant Blue GAW	0.40	0.59	0.46	0.64	0.82
4. Solar Turquoise Blue FBL	0.00	0.00	0.00	0.62	0.57
5. Derma Brown G	0.00	0.00	0.00	0.00	0.00
6. Xylene Fast Green B	0.40	0.67	0.43	0.81	0.72
7. Polar Red 3B	0.50	0.58	0.40	0.63	0.72
8. Ranomil Brilliant Red 3 BN	0.39	0.67	0.41	0.73	0.73
9. Sandopal Blue	0.48	0.59	0.41	0.61	0.78
10. Derma Brilliant Red 3B	0.40	0.69	0.44	0.73	0.77

^a1 *n*-Butanol–acetic acid–water (4:1:5); 2 isopropanol–ammonia (0.9:1)–water (7:1:1); 3

isopropanol–ammonia (0.91)–water (10:1:1); 4, isopropanol–ammonia (0.91) (4:1) 5, *n*-propanol–ammonia (0.91) (6:3).

Source: Adapted from Refs. 75 and 76.

Table 9B R_f Values of Some Direct Leather Dyes on Silica Gel G Plates

Dye	Solvent system ^a		
	1	2	3
1. Eriochrome Blue Black	0.18	0.59	0.52
2. Chlorozol Orange Brown XS	0.29	0.00	0.58
3. Chlorozol Orange Brown	0.29	0.00	0.55
4. Chlorozol Green BNS	0.24	0.00	0.00

^a1, *n*-Butanol–ethanol (95%)–ammonia (0.91) (9:2:3); 2, isopropanol–ammonia (0.91)–water (10:1:1); 3, isopropanol–ammonia (0.91) (4:1).

Source: Adapted from Refs. 75 and 76.

did not change when mixtures of dyes were applied. Further, decrease in hR_f values on impregnated plates suggested that the complexation between dyes and the metal ion is an important factor in influencing the chromatographic behavior of dyes on metal salt–impregnated layers. Gupta et al. also reported the separation of twelve dyes on 1.5% NiCl_2 -impregnated layers using acetone–acetic acid–benzene (9:6:6) as mobile phase. A comparison of hR_f values on plain and impregnated layers clearly indicated better separation on NiCl_2 -impregnated silica gel layers (126a).

A method for separation of the dyes Green S, Fast Green FCF, Brilliant Blue FCF, and Blue VRS was developed using silica gel plates impregnated with 1.25% starch, 5% CdCl_2 , or 5% NiSO_4 with aqueous 80% phenol as mobile phase and was described by Prasad et al. (127). Separation of the dyes was good, especially for Green S and Brilliant Blue FCF, which are difficult to separate by normal chromatographic techniques.

B. Ion Exchanger-Impregnated Plates

Separation of dyes on anion and cation exchanger-impregnated plates by TLC was reported by Lepri et al. (128). The data on R_f values on different exchanger-impregnated plates are recorded in Tables 13 and 14. Table 13 shows that most of the dyes contain one or more sulfonic acids and/or carboxyl groups; the dyes should therefore behave like anions. Owing to the anionic character of most dyes, their chromatographic behavior can be studied in the absence of the ion-

Table 9C R_f Values of Some Basic Leather Dyes on Silica Gel G Plates

Dye	Solvent system ^a		
	1	2	3
1. Malachite Green	0.86	0.84	0.98
2. Methylene Blue G	0.54	0.26	0.65
3. Fast Rubine 4 BN	0.48	0.19	0.62
4. Auromaine O	0.93	0.63	0.95

^a1, *n*-Butanol–ethanol (95%)–water (8:1:3); 2, chloroform– isopropanol–water (1:3:1); 3, *n*-butanol–acetic acid–water (2:1:5).

Source: Adapted from Refs. 75 and 76.

Table 9D R_f Values of Some Premetallized Leather Dyes on Silica Gel G Plates

Dye	Solvent system ^a		
	1	2	3
1. Metalan Dark Blue	0.40	0.60	0.54
2. Neolan Violet Brown BY	0.24	0.61	0.45
3. Metalan Red S-BR	0.72	0.79	0.88
4. Dermalight Yellow	0.24	0.62	0.93
5. Metalan Bordeaux S-B	0.23	0.57	0.51
6. Metalan Brown S-GL	0.22	0.43	0.44
7. Irgalan Red 4GL	0.70	0.83	0.89
8. Cibalan Olive 3BL	0.21	0.10	0.87
9. Matalan Brown S-RL	0.65	0.78	0.87
10. Metalan Yellow 5-RL	0.67	0.69	0.55
11. Dermalight Brown GRL	0.69	0.57	0.84
12. Ranolan Orange RL	0.68	0.63	0.89
13. Dermallight Yellow 2 RL	0.68	0.74	0.87
14. Dermalight Orange RLN	0.33	0.56	0.65
15. Dermalight Green 5GL	0.34	0.56	0.64
16. Sandopal Gray WSI	0.69	0.57	0.88

^a1, *n*-Butanol–ethanol (95%)–water (8:1:3); 2, chloroform– isopropanol–water (1:3:1); 3, *n*-butanol–acetic acid–water (2:1:5).

Source: Adapted from Refs. 75 and 76.

Table 10 R_f Values of Some Synthetic Dyes, on Silica Gel G Plates, Extracted from Indian Country Liquors^a

Identified dye	Color of spot	R_f value
Tartrazine	Dark yellow	0.12
Red 10B	Dark pink	0.26
Sessol Orange G	Dark orange	0.36
Carmoisine	Dark pink	0.41
Amaranth	Dark pink	0.15
Indigo Carmine	Dark blue	0.34
Acid red	Red	0.65
Crocine Scarlet	Red	0.52
Basic Red	Red	0.26
Matanil Yellow	Yellow	0.68
Rhodamine B	Pink	0.69
Malachite Green	Blue	0.53

^aMobile phase: *n*-butanol–glacial acetic acid– ethanol–water (10:2:0.5:5).

Source: Adapted from Ref. 55.

Table 11 hR_f Values of Dyes on Impregnated and Plain Silica Gel Plates^a

Dye	hR_f		
	Plain ^b	Imp. ^c	Imp. ^d
1. Rosaniline HCl	84	57	85
2. Chrysoidine	83	69	84
3. Malachite Green	65	49	65
4. Methyl Red	88	78	80
5. Crystal Violet	72	61	73
6. Fuchsine Basic	83	62	70
7. Auramine O	73	52	82
8. Bromophenol Blue	90	90	92
9. Eosine bluish	98	98	98

10.	Bromocresol Purple	84LT	85	87
11.	Congo Red	60	59	72
12.	Titan Yellow	66	67	65
13.	Aluminon	75LT	82	66
14.	Alizarin	45ST	33	60
15.	Magneson	99	99	99
16.	Orange G	33	43	54
17.	Bromocresol Green	38	87	90
18.	Phenol Red	73	72	75
19.	Thymol Blue	85	84	86
20.	Genitan Violet	73	52	75
21.	Nevilline Brilliant Pink	97	95	96
22.	Aniline Blue	88	67	80
23.	Dichlorofluorescein	98	98	97
24.	Xylidine Ponceau	30	29	32
25.	B enzopurpurine	62	55	60
26.	Methylene Blue	42	40	43
27.	Nigrosin	00	00	00
28.	Fuchsine Acid	11	11	09
29.	Light Green	43	28	47
30.	Alizarin Blue	24LT	25	26

^aMobile phase: *n*-butanol–AcOH–H₂O (20:5:10).

^bST=slight tailing; LT=large tailing.

^cImpregnated with ammonium molybdate.

^dImpregnated with copper sulfate.

Source: Adapted from Ref. 125.

exchange process on cation exchanger—impregnated layers. Besides exchangers, cationic and anionic detergents are also used as impregnants to compare the behavior of the dyes on such layers and on anion and cation exchanger layers.

Seventeen ionic food dyes were separated by Van Peteghem and Bijl (32) by ion-pair adsorption TLC on silica gel plates. Cetyltrimethylammonium bromide (CTMA) was selected as the counterion for both isolation and separation. The thin-layer plates were impregnated with the counterion, which was also present in the eluent. The R_f values of some sulfonated dyes separated by the above technique using methanol-acetone (9:1)+1% glacial acetic acid and 0.1 M CTMA, or methanol-acetone (1:1) and 0.1 M CTMA as

mobile phase are recorded in Table 15. The orange dyes can be developed in any of the solvent systems, but they were not separated. The

Table 12 hR_f Values of Selected Dyes on Impregnated and Plain Silica Gel Plates^a

Dye	Color	hR_f	
		On plain silica gel ^b	On silica gel with ZnAc ₂
Rosaline hydrochloride	Reddish pink	70ST	38
Methyl Red	Red	82T	72
Crystal Violet	Violet	40T	21
Orange G	Light orange	20ST	17
Auramine O	Lenion yellow	52T	26
Bromophenol Blue	Violet	44ST	34
Bromothymol Blue	Dark yellow	83T	74
Phenol Red	Orange red	56	46
Thymol Blue	Orange yellow	76T	67
Acridine Orange	Yellow	50	35
Cadion 2B	Brown	95T	94
Rhodamine B	Pink	56T	04
Eosine Yellowish	Light orange	50T	42
Naviline Yellow	Pale yellow	97	92
Naviline Brilliant Pink	Violet	96T	90
Methyl Violet	Violet	56T	44
Bromocresol Purple	Brown	74ST	70
Alizarine Blue	Light pink	35	30
Bismarck Brown	Orange	76T	53
Eriochrome Black	Purple	32T	15
Benzopurpurine 4B	Light orange	52T	48
Nigrosin	Purple	0	0
Fuchsin Acid	Pink	22ST	19
Diamond Blue	Blue	78ST	71
Dichlorofluorescein	Orange	54ST	12

^aSolvent system: *n*-butanol–benzene–ethyl acetate (40:35:25).

^bST=slight tailing; T=tailing.

Source: Adapted from Ref. 126.

method requires only limited sample treatment and is very easy to apply. TLC development is must faster than for conventional methods with butanol- and/or water-containing eluents. The high separation efficiency, due to sharpness of the spots, offers a highly reliable means of identification.

V. OPTLC, HPTLC, AND REVERSED-PHASE TLC OF DYES

Besides separation of dyes by TLC, separations by reversed-phase TLC, by high-performance TLC (HPTLC), and by overpressured TLC (OPTLC) are very important owing to improved separation and shorter time requirements.

A. OPTLC of Dyes

The problem of optimization of separation techniques in thin-layer chromatography has been tackled both in theoretical treatments (129–133) and in the development of instrumentation (134–136). In overpressured TLC (137) a pressurized ultramicrochamber is used. The essential feature of this chamber system is that, in contrast to the rigid glass plate used in the earlier ultramicro-

Table 13 R_f Values of Water-Soluble Food Dyes on Layers of (a) DEAE-Cellulose, (b) PAB-Cellulose, (c) Chitosan, and (d) Microcrystalline Cellulose

Name of dye	Dye no.	0.6 M NH_4Cl in H_2O methanol (7:3, v/v)	0.1 M NaH_2PO_4	H_2O –ethanolamine (14:1, v,v)		Amount (μg)
		(a)	(b)	(c)	(d)	
Tartrazine	E 102	0.10	0.47	0.89	0.90	1.0
Chrysoin	E 103	0.21	0.38	0.68	0.72	1.0
Quinoline yellow	E 104	0.50 ^a	0.04 ^c	0.35 ^b	0.40 ^b	1.0
		0.11 ^b	0.19 ^c	0.45 ^b	0.51 ^b	
				0.69 ^a	0.76 ^a	
Acid yellow	E 105	0.25	0.65	0.75	0.79	1.0
Sunset yellow	E 110	0.33	0.78	0.81	0.87	1.0
Orange GGN	E 111	0.33	0.78	0.81	0.87	1.0
Azorubine	E 122	0.03	0.11	0.44	0.54	1.0
Amaranth	E 123	0.10	0.20	0.59	0.63	1.0

Cochineal red	E 124	0.10	0.57	0.74	0.78	1.5
Scarlet GN	E 125	0.20	0.45	0.91	0.91	1.5
Ponceau 6R	E 126	0.10	0.23	0.54	0.58	1.0
Erythrosine	E 127	0.03	0.05	0.27	0.37	1.0
Patent blue	E 131	0.81	E.S. ^d	0.89	0.89	0.5
Indigo carmine	E 132	0.08	E.S. ^d	E.S. ^d	E.S. ^d	2.0
Brilliant black BN	E 151	0.00	0.01	(0.43) ^e	0.57	1.5
Black 7984	E 152	0.00	0.01	(0.43) ^e	0.57	1.5

^aMain spot.^bSecondary spot.^cTwo spots of similar intensity.^dE.S.=elongated spot.^eParentheses indicate streaking.*Source:* Adapted from Ref. 128.

chamber (138, 139), the sorbent layer is completely covered by an elastic membrane under external pressure so that vapor phase above the layer is eliminated. Solvent is admitted into the chamber under overpressure by means of a pump system. This technique essentially combines the advantages of traditional TLC and modern high-performance TLC (HPTLC), leading to improved separation efficiency.

For OPTLC and HPTLC, precoated silica glass plates (conventional TLC plates) with indicator (silica gel 60 F₂₅₄) and without indicator, and HPTLC silica gel 60 F₂₅₄ aluminum sheets are used. The resolution and retention behavior of some dyes have been studied by OPTLC (137, 137a). Empore sheets have also been used for OPTLC of dyes (137b).

Ligor and Buszewski (137c) developed thin-layer chromatographic and overpressured thinlayer chromatographic methods for the determination of pigments in plant leaves containing anthocyanins and chlorophylls. They optimized mobile and stationary phases and other chromatographic conditions for the separation of pigments for plant leaves.

B. HPTLC of Dyes

The synthetic organic colors in lipsticks have been characterized by HPTLC (57). By combining the results from TLC and HPTLC it was possible to determine the organic colors in lip cosmetics. Some of the colors had identical R_f values on TLC, but they could be identified with the aid of

Table 14 R_f Values of Water-Soluble Food Dyes on Layers of (a) Dowex 50-X4(H^+), (b) Rexyn 102(H^+), (c) Humic Acid, (d) Silanized Silica Gel, and (e) Silanized Silica Gel Impregnated with 4% DBS^a

Dye no.	Mobile phase					Amount (μ g)
	0.1 M HCl in H ₂ O–CH ₃ OH (7:3)			H ₂ O–CH ₃ OH– acetic acid (64.3:30:5.7)		
	(a)	(b)	(c)	(d)	(e)	
E 102	0.65	0.62	0.66	0.95	0.95	1.0
E 103	0.40	0.29	0.29	0.57	0.73	1.0
E 104	0.22 ^c	0.28 ^c	0.28 ^d	0.72 ^c	0.81 ^c	1.5
	0.42 ^b	0.38 ^d	0.44 ^b	0.87 ^b	0.88 ^b	
	0.65 ^c		0.68 ^c	0.97 ^c	0.97 ^c	
E 105	0.82	0.75	0.75	0.93	0.88	1.0
E 110	0.93	0.75	0.78	0.80	0.85	1.0
E 111	0.93	0.75	0.78	0.82	0.85	1.0
E 122	0.23	0.18	0.29	0.55	0.77	1.0
E 123	0.42	0.48	0.55	0.95	0.95	1.0
E 124	0.82	0.83	0.77	0.95	0.95	1.0
E 125	0.70	0.55	0.73	0.69	0.83	1.0
E 126	0.51	0.30	0.37	0.54	0.74	1.0
E 127	0.00	0.00	0.00	0.01	0.01	1.0
E 131	E.S. ^e	0.59	0.97	0.19	0.27	1.0
E 132	E.S. ^e	0.39	(0.55) ^f	0.95	0.95	1.5
E 151	(0.06) ^f	(0.13) ^f	(0.25) ^f	0.94	0.93	1.0
E 152	(0.06) ^f	(0.13) ^f	(0.25) ^f	0.95	0.93	1.0

^aDBS=triethanolamine-dodecylbenzenesulfonate.

^bMain spot.

^cSecondary spot.

^dTwo spots of same intensity.

^eE. S.=elongated spot.

^fParantheses indicate streaking.

Source: Adapted from Ref. 128.

their retention times in HPTLC. Rosaline hydrochloride, Malachite Green, Brilliant Green, Methyl Violet, Gentian Violet, Ethyl Violet, and Victoria Blue were screened by HPTLC on 10×10 cm Whatman HPTLC plates with the use of a LAMMA-1000 laser microprobe (140). The dyes from alcoholic products were analyzed by HPTLC with the use of Whatman HPTLC plates with butanol–butan-2-ol–acetonitrile–tetrahydrofuran–ethyl methyl ketone–aqueous 0.5% NaCl–aqueous NH₃ (10:10:25:15:20:18:2) or propanol–acetonitrile–tetrahydrofuran–ethyl methyl ketone–ethyl acetate–aqueous 0.5% NaCl–aqueous NH₃ (20:15:25:10:10:18:2) as developing solvent (45).

Wimmer and Hauck (141) reported the separation of synthetic dyes on HPTLC plates using precoated Kiesel-gel 60 sheets. The conditions for optimum separation and comparison with TLC are reported (141). Several yellow, red, and blue dyes have been separated by TLC on silica gel and HPTLC on RC-2, RP-8, and RP-18 (Merck) with methanol–H₂O (17:3) as mobile phase (30).

Eleven water-soluble dyes were separated by HPTLC on silica gel 60 with methyl ketone–methanol–aqueous 28% NH₃ (8:4:1) as the developing solvent. Dyes that were insoluble in aqueous concentrated NH₃ were separated on layers of cellulose with butanol–ethyl methyl ketone–

Table 15 R_f Values of Some Sulfonated Dyes Separated by Ion Pair Thin-Layer Chromatography on Impregnated Silica Gel Layers^a

Dye	Color	R_f values in solvent	
		A	B
Tartrazine	Yellow	0.14	0.07
Chrysoin S	Yellow	0.48	0.33
Quinoline Yellow	Yellow	0.33	0.28
Acid Yellow	Yellow	0.37	0.31
Sunset Yellow FCF	Orange	0.32	0.25
Orange GGN	Orange	0.32	0.25
Azorubin	Red	0.28	0.25
Amaranth	Red	0.26	0.19
Cochineal Red A	Red	0.07	0.03
Scarlet GN	Red	0.46	0.40
Ponceau 6R	Red	0.17	0.07
Erythrosine	Red	0.34	0.20
Patent Blue V	Blue	0.36	0.34
Indigo Carmine	Blue	0.38	0.29
Green S	Green	0.44	0.39

Brilliant Black BN	Black	0.12	0.09
Black 7948	Black	0.16	0.12

^aSolvents: A, methanol-acetone (9:1)+1 % glacial acetic acid, 0.1 M CTMA (cetyltrimethylammonium bromide); B, methanol-acetone (1:1), 0.1 M CTMA.
Source: Adapted from Ref. 32.

aqueous 1% $\text{NH}_3\text{-H}_2\text{O}$ (4:2:1:1) as developing solvent (142). The advantages of the method are shorter analysis time, more data measurement per plate, high resolution, and an improved signal-to-noise ratio compared with those of TLC plates. The theory of this technique is well discussed by Jupille and Glunz (143) along with some data on the separation of dyes.

Wall (143 a) reported quantitative methods for determination of biologically important dyes using silica gel 60, silica gel G RP-8 F_{254} , or silica gel $\text{NH}_2 F_{254}$ plates by loading them with 100 ng of sample. Separation of six thiazine dyes has also been reported on silica, cyano, and C-18 plates using as the mobile phase THF- H_2O -methanol (16:3:1) (143b). Identification and determination of cationic dyes from acrylic fiber were reported on silica gel G-60 plates using ethyl methyl ketone- CH_2Cl_2 -formic acid (8:6:1) as mobile phase (143c).

High-performance thin-layer chromatography (HPTLC) on silica gel plates with two successive mobile phases has been used for analysis of seven amine azo dye isomers prohibited under a German ban (143d). Dichloromethane was used for the first development, and the second was done with toluene-tetrahydrofuran (1:1 v/v), to a distance of 8.5 cm. The spots were visualized at a wavelength of 254 nm. The limit of determination (x) and the correlation coefficient in the range $x\text{--}5x$ were reported for each amine. To increase the sensitivity of detection, the UV spectrum was acquired for each amine, and the wavelength of maximum absorbance (Δ_{max}) was used to establish the limit of determination. Synthetic mixtures and dye samples were resolved and quantified.

Flodberg and Roeraade (143e) made a device for high-pressure thin-layer chromatography. The HPTLC chamber consists of two flat stainless steel blocks mounted in a hydraulic press. The upper chamber is solid, and the lower chamber is filled with water and covered with a membrane. The mobile phase inlet is connected to a membrane, and the TLC plate is placed upside-down against the membrane. During pressurization, a stainless steel frame is pressed against the membrane and a flat urethane rubber seal. The device was used to separate the dyes on a TLC plate coated with 3 μm spherical particles with a mobile phase of CH_2Cl_2 , a flow rate of 1 mL/min, a mobile phase pressure drop of 140 bar, and a water cushion pressure of 170 bar.

C. Reversed-Phase TLC of Dyes

The separation of 26 basic dyes (suitable for dyeing acrylic fibers, silk, and tannin) was studied with the use of reversed-phase TLC plates, with aqueous 70–90% acetone as developing solvent. Development was 11.5–14.5 cm and took 30–90 min. Good separation of 10 of the dyes was attained with aqueous 70% acetone (144).

The separation of 34 commercial acid dyes of the monoazo- and diazoanthraquinone and triphenylmethane types was studied by reversed-phase TLC (RP-TLC) by application

of the dyes onto layers of octadecylsilyl-bonded silica gel for TLC, using aqueous methanol or aqueous 60% acetone as developing solvent (101).

Thirteen dyes were separated by RP-TLC on C-18 modified silica gel using methanol–acetonitrile–aqueous 5% Na₂SO₄ (3:3:10) and then methanol–ethyl methyl ketone–aqueous 5% Na₂SO₄ (1:1:1) as mobile phases in the same direction. Separation was optimum between pH 6.0 and 7.0 (145). Four basic dyes were separated on pretreated silica gel plates using HClO₄ or benzenesulfonic acid as counterion and aqueous 50% or 60% ethanol as mobile phase (146). Polyacrylonitrile plates were used for the separation of 10 basic and common food dyes along with other dyes using diethylamine–anhydrous acetic acid–H₂O (4:1:15) as mobile phase (147).

Cserhati and Forgacs (147a) reviewed the use of various liquid chromatographic techniques such as adsorption and RP-TLC and HPLC principally for the separation and quantitative determination of terpenoid-based color substances in food and food products. They also delineated future trends in the separation and identification of pigments in food and food products.

Ozeki et al. (147b) investigated a TLC method for the analysis of Gardenia Yellow in food using crocetin as an indicator. Gardenia Yellow was extracted from food samples with hydrous methanol. After evaporating the extract, the remaining residue was dissolved in water at pH 11. The resultant mixture was stirred at 50°C for 30 min. The purified mixture was then subjected to the TLC analysis. The TLC conditions were an RP-18 F₂₅₄ S plate and acetonitrile–tetrahydrofuran–oxalic acid (7:8:7) as mobile phase. The visible absorption spectra were measured using scanning densitometry. This method has been used on 37 commercial foods and is useful for the rapid analysis of Gardenia Yellow in foods.

Csiklusnadi et al. (147c) determined that the multistep gradient elution technique developed for the RP-TLC separation of pigments can be used as a pilot method for the rational design of gradient elution in RP-HPTLC for the separation of the same pigments.

Sulfonated dyes have been separated by ion-pair RP-TLC on RP-18 layers predeveloped with methanolic tetrabutylammonium bromide, using aqueous 80% methanol as mobile phase (148), and basic dyes have been separated on GDX-201-silica gel (1:1) layers using aqueous 70% methylcyanide containing 10 mM HClO₄ and 10 mM β-naphthylsulfonic acid (149).

Reversed-phase TLC coupled with fast atom bombardment spectrometry has been used for identification and separation of 27 food dyes consisting of 12 permitted for use in food and 15 unlawful dyes in Japan using C-18 modified silica gel as adsorbent and two mobile phases. The method was successfully applied for the identification of unlawful dyes in imported foods (150). The *R_f* values along with mobile phases used for these dyes are recorded in Table 16.

Miniaturization is a general trend in modern analytical methods, with the main aims being to increase sensitivity, shorten analysis times, and reduce the quantity of consumables per analysis (151). In planar chromatography, a first step in this direction was the development of modern high-performance TLC (HPTLC). Compared with conventional TLC, the layers are characterized by smaller absorbent material particle sizes and a slight reduction in layer thickness. A major final step in the direction of miniaturized planar chromatography is the use of new ultrathin layers. In contrast with those of TLC and HPTLC plates, these layers are not based on granular adsorbents but

have a monolithic structure based on the silica gel matrix. This means that there are no longer

Table 16 Reversed-Phase TLC of Food Dyes on C-18 Modified Silica Gel Plates

Dye	R_f	
	Solvent system A^a	Solvent system B^b
Ponceau 3R (R-1)	0.16	0.86
Amaranth (R-2) ^c	0.71	1.00
Erythrosine (R-3) ^c	0.00	0.40
Ponceau SX (R-4)	0.21	0.89
Oil Red (R-5)	0.00	0.06
Allura Red AC (R-40) ^c	0.34	1.00
Ponceau R (R-101)	0.22	0.89
New Coccine (R-102) ^c	0.58	1.00
Eosine (R-1 03)	0.00	0.42
Phloxine (R-104) ^c	0.00	0.15
Rose Bengal (R-105) ^c	0.00	0.19
Acid Red (R-106) ^c	0.04	0.77
Azo Rubine (R-AZ)	0.19	0.88
Orange I (0-1)	0.15	0.75
Orange RN (O-RN)	0.05	0.61
Oil Orange SS (O-SS)	0.00	0.09
Napthol Yellow S (Y-1)	0.42	0.90
Yellow AB (Y-2)	0.00	0.16
Yellow OB (Y-3)	0.00	0.13
Tartrazine (Y-4) ^c	0.79	1.00
Sunset Yellow FCF (Y-5) ^c	0.45	1.00
Guinea Green B (G-1)	0.03	0.73
Fast Green FCF (G-3) ^c	0.17	1.00
Wool Green S (G-S)	0.22	0.90
Brilliant Blue FCF (B-1) ^c	0.14	1:00
Indigo Carmine (B-2) ^c	0.66	1.00

Acid Violet 6B (V-1)	0.01	0.67

^aSolvent system A=methanol–acetonitrile-5% sodium sulfate solution (3:3:10).

^bSolvent system B=methanol–methyl ethyl ketone-5% sodium sulfate solution (1:1:1).

^cPermitted dyes for use in foods in Japan.

Source: Adapted from Ref. 150.

separate particles, as in TLC and HPTLC. Moreover, a binder is not needed to fix the layer onto the glass plate. To be suitable for chromatographic purposes, the monolithic silica gel of the new ultrathin layers has meso- and macropores, with fine capillaries penetrating the layer. The layer thickness of approximately 10 μm is considerably less than that of conventional TLC and HPTLC layers. The exciting properties of these new ultrathin silica gel plates, especially their selectivity and separation efficiency, were demonstrated by separations of steroids, pesticides, and some dyestuffs.

Berezkin and Mardanov (152) proposed a version of thin-layer chromatography with forced flow of the mobile phase in microchannels packed with a sorbent. The dependence of retention parameters R_f and the number of theoretical plates in the course of the elution of hydrophilic dyes (Naphthol Red and Methyl Red) on the eluent (propan-2-ol) pressure and developing time were studied. Forced-flow TLC would be useful as a pilot microtechnique.

VI. CONCLUSIONS

Color chemistry is more than the synthesis of brilliant dyes for textiles. Today colorants are also used in information storage devices, lasers, liquid crystal displays, solar energy conversion systems, and food additives. Moreover, analytical techniques such as affinity chromatography and histological staining depend on dyes. Considering the scope of chromatographic investigations in the synthetic dyestuff field, insufficient work has been done. Although a number of papers have appeared on the TLC of food dyes, little work has been reported on the separation of other types of synthetic dyes. TLC of dyes on untreated plates sometimes gives diffuse spots and tailing. This may be overcome by performing TLC on treated plates, where the separation efficiency is increased due to sharpness of the spots. OPTLC, HPTLC, and reversed-phase TLC give still better results owing to the advantages of shorter analysis time, more data measurement per plate, high resolution and improved signal-to-noise ratio. However, little work has been done on the separation of dyes by HPTLC, OPTLC, reversed-phase TLC, or TLC on treated (impregnated) plates. Because all these techniques give better separation along with other advantages, they must be explored further.

Dyes can be divided into two classes depending on their solubility, i.e., water-soluble and insoluble. One difficulty in dealing with many dyes is that the choice of solvents is very limited. Hence this area also needs further exploration. However, substances that are virtually insoluble at room temperature can often be analyzed successfully by TLC at elevated temperatures if the samples to be analyzed exhibit sufficient thermal stability.

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Toxins (Natural)

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I. INTRODUCTION

Natural toxins are the toxins produced mainly by some microorganisms, microscopic plants and animals, and some higher plants and animals. These toxins vary widely in their structures, toxicity, distribution, and diversity. In the course of their evolution, humans must have learned by trial and error to avoid plants and animals that cause acute toxicity. However, through various dietary sources some of these toxins can enter the human body and cause long-term defects. Toxins such as hemagglutinin produced by castor beans and kidney beans cause short-term effects, whereas safrole produced by black pepper and pyrrolizidine alkaloids produced by plants in the family Compositae are carcinogenic. Various carcinogenic toxins such as mycotoxins are produced by many species of fungi.

Poisonous animals whose tissues are toxic when eaten by humans are restricted almost entirely to the marine world. More than 1000 species of marine organisms are either toxic or poisonous. Shellfish poisoning [paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), diarrhetic shellfish poisoning (DSP), and amnesic shellfish poisoning (ASP)] caused by the consumption of shellfish contaminated with these toxins as well as fish poisoning (ichthyotoxism) caused by the consumption of toxic fish species are common toxicities. Thin-layer chromatography (TLC) has been used for the analysis of various natural compounds, and this chapter briefly discusses the application of TLC in the separation and detection of some natural toxins in food.

II. NATURAL TOXINS IN FOOD AND FEED

A. Mycotoxins

Grains, cereals, and cereal products are exposed to the growth and development of a great number of molds that synthesize biologically active toxins known as mycotoxins. There are two major types of mycotoxins that have direct or indirect harmful effects on human health. They are aflatoxins (G_1 , B_1 , and M_1) and ochratoxins (A, B, and C) (1).

High-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), and enzyme-linked immunosorbent assay (ELISA) are the main analytical methods for mycotoxins. TLC has been widely used since the early 1960s for the qualitative and quantitative analysis of mycotoxins.

1. Aflatoxins

Aflatoxins B₁, B₂, G₂, and G₁ are the four naturally occurring aflatoxins that have acute toxicity and carcinogenic effects. These toxic metabolites are produced mainly by *Aspergillus flavus* and the closely related *Aspergillus parasiticus*. Within animal systems these toxins can produce sec-ondary metabolites such as aflatoxins M₁, M₂, P₁, and Q₁ and aflatoxicol, whereas aflatoxins B₂α, G₂α, and D₁ are produced spontaneously in chemical environments.

A variety of aflatoxins have been detected in foods. Aflatoxins in peanuts can be extracted by blending the nuts with 77% methanol and dissolving the purified toxins in toluene–acetonitrile (98:2) for spotting on TLC plates (2). Aflatoxins in corn are extracted with 85% methanol, whereas chloroform–water (5:2) is used to extract them from coconut, copra, and copra meal. Activated silica plates are commonly used for the analysis of aflatoxins with various solvent systems. An excellent review including 540 references on the extraction, separation, and detection of various mycotoxins and their metabolites is given in Ref. 3. Separated toxins can be detected under UV light. Some of the commonly used solvent systems and adsorbents are listed in Table 1.

2. Sterigmatocystin

Sterigmatocystin toxins are isolated from *A. versicolor*, *A. nidulans*, and *A. bipolaris*. The toxins are extracted with an azeotropic mixture of chloroform and methanol for 16 h (3). Various extraction methods used by a number of authors are presented in Ref. 3. Some common solvent systems and adsorbents used for the separation of sterygmatocystin metabolites are listed in Table 2. The separated toxins can be detected under longwave UV light. Sterygmatoctystin can be seen as fluorescent brick red spots, whereas 0-methyl sterygmatoctystin can be seen as pale blue spots (31). When these plates are sprayed with aluminum chloride solution followed by heating at 120°C for a few minutes, the red color of sterygmatoctystin is changed to yellow, whereas the blue fluorescent color of *O*-methyl sterygmatoctystin in changed to yellow-green.

3. Versicolorins

Versicolorins are derivatives of sterygmatoctystin. The main ones are versicolorin A, B, and C; averufin; and norsolorinic acid. They can be extracted from *A. parasiticus* using methanol or acetonitrile with some water (32). Common solvents and adsorbents for the separation of these mycotoxins are given in Table 3. The separated toxins can be detected by their distinctive colors under UV light.

4. Trichothecenes

Trichothecenes are a group of toxins that consists of over 125 structurally closely related compounds produced mainly by various fungi; some are produced by higher plants. Various silica gel TLC methods have been used to identify and quantify these toxins. Some of the solvent systems are shown in Table 4.

T-2 toxin ($4\beta,15$ -diacetoxy- $8\alpha,3$ -methylbutyryloxy); 3α :-hydroxy-12,13-epoxytrichothec-9-ene) is a naturally occurring toxic substance in a variety of agricultural commodities including grains and beans. A widely spread fungus on a variety of plants and soil, *Fusarium sporotrichioides*, is known to produce T-2 toxin in these foodstuffs. T-2 toxin can cause abdominal pain, diarrhea, abortion, dermal necrosis, chills, and inhibition of protein synthesis in various mammals, including humans. TLC has been used to detect this toxin in a number of processed cereals (38). They can be detected under UV light with detection limits of 50 ng per spot.

5. Small Lactones

Small lactones are mycotoxins with either a five- or six-membered cyclic lactone ring in their structure. Some commonly found compounds are penicillic acid, mycophenolic acid, butenolide, patulin, citreoviridin, and ascladiol.

a. Penicillic Acid. Penicillic acid is produced by the fungus *Penicillium cyclopium*. It can be extracted from food items such as peas, rice, oats, coconut, and cheese and even from sausages contaminated with this fungus (3). Although it can be easily extracted with chloroform-methanol (9:1) from corn and with dichloromethane-methanol (1:1) from peas, rice, and oats, there are many other solvent systems for the extraction. TLC has been used for the separation of penicillic acid; some of the common solvent systems are given in Ref. 3. Silica gel is commonly used as the adsorbent. Oxalic acid-treated silica gel plates are developed in chloroform-methanol (98:2) or chloroform-acetone (9:1) (26) whereas Silufol plates are developed in toluene-ethyl acetate-

Table 1 Adsorbents and Solvent Systems Used in TLC for Mycotoxins

Compound	Commodity	Adsorbent	Solvent system ^a	Detection	Ref.
Aflatoxins	Rice starch	Silica gel	C:M (98:2)		4, 5
B ₁ , B ₂ , G ₁ , G ₂	Authentic standards	Silica gel	CA (9:1)	UV, dual-wavelength TLC scanner	6
	Peptone media	Silica gel	C:A:W (93:7:3)	Fluorodensitometer	7, 8
B ₁ , G ₁	<i>A. flavus</i> culture	Silica gel 7G	T:EA:FA (6:3:1)	Fluorimetry	9
B ₁ , B ₂	Peanut meal, peanut butter		C:A:W (88:12:1.5) C:A:B (90:10:10)	Spectrometry (>0.5 ng/spot) >26 ng/g	10

B ₁ , B ₂ , G ₁ , G ₂	Corn, peanut butter, raw peanuts	Silica gel	DEE		11
		Silica gel	DEE, C:A:IP: (85:15:15)		
Aflatoxicols Q ₁ , H ₁ , M ₁ , B _{2a}		Silica gel	DEE, C:A:IP (85:15:15), B:Et (40:4) or C:M (90:1)		48
B ₁ , B ₂ , G ₁ , G ₂		Silica gel L, Silufol sheets	B:DEE:H (1:1:1) or C:B:A (9:1:1) or C:B:EA:A (10:4:4:3)	UV (365 nm), 0.2– 0.5 ng/spot	13
Aflatoxins	Maize	Silica gel	T:IAA:M (90:32:2)	TFAA or 25% H ₂ SO ₄	14
B ₁		Silica gel G- 25	C:A:W (90:10:1.5), MCl:A:W (90:1: (1.5)		15
D ₁	Corn	Silica gel 60 Silica gel 7G	MCl:M (95:5)	UV	16
B ₁ , B _{2a} , G ₁ , G _{2a}	Human urine	Silica gel G- HR	C:A:IP (85:10:5)		17
Metabolites of B ₁	Rat liver	Silica gel	C:A (9:1)	Fluorescence UV	18
M ₁	Milk powder	Silica gel G- HR	DEE:M:W (95:4:1), T:EA:FA (50:45:5), T:EA:C:FA (40:50:10:5)	H ₂ SO ₄ –H ₂ O (1:3)	19
M ₁	Milk	Kieselgel 60, aluminum foil	T:F:EA (6:1:3), C:A:IP (87:10:3)	UV light	20
M ₁	Milk and diary products	Silica gel	H:A (9:1)+C:A:IP, (85:10:5)	Spray HNO ₃ /fluorescence at 365 nm	21
M ₁	Milk and milk products	Silufol sheets	DEE (100%)+C:A:H (35:50:15)+ DEE:H:M:W (80:10:8:2)	360 nm	22

^aA=acetone, B=benzene, C=chloroform, DEE=diethyl ether, Et=ethanol, EA =ethyl acetate, FA=formic acid, I=isopropanol, IAA=isoamyl alcohol, M=methanol, MCl= methylene chloride, H=hexane, T=toluene, TFAA=trifluoroacetic acid, W=water.

Table 2 Solvent Systems Used for the Separation of Sterygmatoxystins by TLC

Metabolite	Adsorbent	Solvent system ^a	Ref.
Sterygmatoxystin, 5-methoxysterygmatoxystin, <i>o</i> -methylsterygmatoxystin	Silica gel G	DEE:M:W (96:3:1), T:EA:AA (50:30:4)	23
		B:DMK (10:0.2), B:M (10:0.2), CTCI:DMK (10:0.2), CTCI:M (10:0.5), C:M (10:0.5)	24
Sterygmatoxystin, 5-methoxysterygmatoxystin	Silica gel	T:M (99:1)	25
		C:M (97:3), C:DMK:H (7:2:1), C:DMK (9:1), B:C:DMK (9:8:3)	26
Sterygmatoxystin	Silufol	B:M:AA (24:2:1), T:EA:FA (6:3:1), B:Et (95:5), C:M (4:1), C:MIBK (4:1), C:DMK (9:1), C:AA:DEE (17:1:3), nBut:AA:W (4:1:4)	27
Sterygmatoxystin, <i>o</i> -methylsterygmatoxystin	Silica gel 60	DEE:M:W (96:3:1), T:EA:DMK (60:25:15), T:EA:AA (50:30:4), C: DMK (10:0.5), C:M (10:0.5), CTCI:M (10:2)	28
	Adsorbosil-1	DEE:M:W (96:3:1)	29
Mixture of aflatoxins, Sterygmatoxystin, anthraquinones	Alumina-backed silica gel 60	1. C:A (80:15) to separate aflatoxins	30
		2. DEE:M:W (96:3:1) to separate anthraquinone and Sterygmatoxystin	
		3. T:EA:A:AA (60:25:15:2) to separate anthraquinone	
Mix of Sterygmatoxystin, <i>o</i> -methyl Sterygmatoxystin, aflatoxins B, and DMK (10:0.5), C:M (10:0.5), CTCI:M (10:2) –G ₁	Silica gel	DEE:M:W (96:3:1), T:EA:AA (50:30:4), T:EA:DMK (60:25:15), C: DMK (10:0.5), C:M (10:0.5), CTCI:M (10:2)	28

^aA=acetone, AA=acetic acid, B=benzene, C=chloroform, CTCI=carbon tetrachloride, DMK=dimethyl ketone, DEE=diethyl ether, DMK= dimethyl ketone, Et=ethanol, EA=ethyl acetate, FA=formic acid, H=hexane, I=isopropanol, IAA=isoamyl alcohol, M=methanol, MIBK=methyl isobutyl ketone, MCl=methylene chloride, nBut=n-butane, T=toluene, TFAA=trifluoroacetic acid, W=water.

Table 3 Solvent Systems and Adsorbents for the Separation of Versicolorins by TLC

Metabolite	Adsorbent	Solvent system ^a	Detection (UV)	Ref.
Versicolorin A	Adsorbosil-1	B:AA (95:5)	Visible light (yellow-orange)	3, 30
Versicolorin B	Silica gel	B:AA (95:5)	Yellow	
Versicolorin C	Silica gel	B:AA (95:5)	yellow	
Averufin	Silica gel	C:DMK:HAA (97:2:1)	Red	
Norsolorinic acid	Adsorbosil-1	C:DMK:H (85:5:20)	Orange-red	
Versiconal hemiacetal acetate	Silica AR TLC-7G	T:EA(27:12)	Orange-red	

^aAA=acetic acid, B=benzene, C=chloroform, DMK=dimethyl ketone, EA=ethyl acetate, H=hexane, T=toluene.

90% formic acid (6:3:1), benzene–methanol–acetic acid (24:2:1), or benzene-ethanol (95:5) for the separation of these toxins (27).

b. Mycophenolic Acid. Mycophenolic acid is mainly produced by the fungus *Penicillium roqueforti*, and it can be extracted from blue cheese. It is separated on silica gel TLC plates using benzene–ethyl acetate–formic acid (66:33:1), diethyl ether–hexane–90% formic acid (60:20:0.4), chloroform–dimethyl ketone (93:7:1) (39), or benzene–methanol–acetic acid (24:2:1) (27). Silufol plates are also used as the adsorbent, and the toxins are eluted by TLC using benzene– ethanol (95:5), chloroform–methanol (4:1), toluene–ethyl acetate–90% formic acid (6:3:1), or chloroform–acetic acid–water (4:1:4) (27). The gray spots of toxins on TLC plates can be detected under visible light after spraying with *p*-anisaldehyde.

Table 4 Solvent Systems and Adsorbents for the Separation of Trichothecenes by TLC

Metabolite ^a	Adsorbent	Solvent system ^b	Detection	Ref.
3-Acetyldeoxynivalenol	Merck silica gel F ₂₅₄	DEE: A (9:1)	20% sulfuric acid	33
Dis-tricothecene esters	Silica	T:EA:A(7:2:1)	Fluoresce under long-wave UV	34
Dis-T2 toxin	Silica			
Dis-HT-2 toxin	Silica			
Dis-DAS toxin	Silica			
Dis-iso-HT-2 toxin				
Dis-T-2 triol	Siliac			

Dis-T-2 tetraol				
Neosolaniol mono-acetate	Silica gel GH-R	C:A (97:3) or T:EA:FA (5:4:1)	50% ethanolic sulfuric acid	35
T2 toxins (T2-triol)		C:M (95:5), C:EA:M (140:5:5), EA:H (3:2)		36
Verrucaric-A	Alumina silica	C:M (98:2), B:THF (85:15)		37
Roridin	Alumina silica	C:M (98:2), B:THF (85:15)		

^aDis=diphenylindenone sulfonyl.

^bA=acetone, B=benzene, C=chloroform, DEE=diethyl ether, E=ethanol, EA=ethyl acetate, FA formic acid, H=hexane, M=methanol, T=toluene, THF=tetrahydrofuran.

c. Butenolide. Butenolide has been extracted from moldy grains contaminated with the fungus *Fusarium nivale* (40). It is eluted on silica gel plates using chloroform–methanol (93:7) or toluene–ethyl acetate–formic acid (6:3:1) as solvent (41). It is also eluted on Silufol plates using chloroform–methanol (4:1), toluene–ethyl acetate–formic acid (6:3:1), or butanol–acetic acid–water (4:1:4). The separated toxin can be seen as gray spots under visible light after spraying with *p*-anisaldehyde or as yellow spots after spraying with 2,4-dinitrophenylhydrazine followed by heating at 100°C for 2–3 min (40).

d. Patulin. Patulin is a secondary metabolite produced by several species of molds, including *Penicillium expansus*. This mycotoxin has strong antibacterial activity against many bacteria. It is carcinogenic, mutagenic, and highly toxic to animal cells. Although apples and pears are good sources, patulin is commonly found in apple juice (43). However, it has also been found in some cereals, legumes, and sunflower seeds (44).

Silica gel is commonly used for the separation of patulin by TLC, with various solvent systems (see Table 5). After the separation on TLC plates, patulin is detected by spraying phenylhydrazinium chloride (50), 3-methyl-2-benzothiazolinone hydrazone (MBTH)–hydrochloric acid (51, 52), or 4% phenylhydrazine hydrochloride followed by heating for 2–3 min (53). Patulin is clearly seen as yellow spots. Patulin in apple products is also determined by a rapid TLC scanning method using plastic-backed TLC plates precoated with silica gel containing fluorescent indicator UV₂₅₄ (54). The plates are developed in toluene–ethyl acetate–formic acid (6:3:1), and after drying in air the plates are sprayed with 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH) followed by heating at 130°C for 15 min. Fluorescent spots are measured by scanning densitometry.

e. Citreoviridin. Citreoviridin mycotoxin is produced by *P. charlesii* and is extracted from mold-contaminated rice (55) and pecans (56). It is separated on silica gel TLC plates with chloroform–methanol–dimethyl ketone (45:3:2), chloroform–methanol (9:1), or ethyl acetate–toluene (1:1) (57). Solvent systems commonly used for the separation of other mycotoxins, such as ethyl acetate–toluene (1:1), chloroform–methanol (9:1), and chloroform–methanol–dimethyl ketone (45:3:2) (57), are also used for the separation of Citreoviridin on silica gel plates. Ethyl acetate–toluene (3:1) is used on Kiesel-gel G 1500 (58), whereas Silufol plates are developed in benzene–

Table 5 Solvent Systems for the Separation of Patulin by TLC

Adsorbent	Solvent system ^a	Ref.
Silica gel	Methanol (100%), C:M (1:1), chloroform (100%), E:W (4:1), T:EA:FA (6:3:1), B:P:W (2:2:1), B:M:AA (24:2:1)	45, 46
Silica gel 60	C:DMK (90:10), C:M (95:5), T:AA:FA (90%) (50:40:10), Pen:EA (96:4), DIPE:P:E:Pyr (84:12:4:0.8)	39
Silica gel F ₂₅₄	B:C:DMK (45:40:15), C:M (97:3), C:DMK:P (85:15:20), C:DMK:H (7:2:1), C:DMK (9:1)	26
Silica gel G-HR	T:EA:FA (5:4:1)	46
Silical gel K-5	T:EA:FA(95%) (5:4:1)	47
Kiesel-gel 60 F	DCM:EA (95:45)	48
Kiesel-gel 60 G	T:EA:FA (85%) (50:40:10)	49
Silufol	B:M:AA (24:2:1), T:EA:FA (90%) (6:3:1), B:E (95:5), C:M (4:1), C:MIBK (4:1), C:DMK (9:1), C:AA:DEE (17:1:3), But:AA:W(4:1:4)	27

^aB=benzene, But=butanol, C=chloroform, DCM=dichloromethane, DEE=diethyl ether, DIPE=diisopropyl ether, DMK=dimethyl ketone, E=ethanol, EA=ethyl acetate, FA=formic acid, H=hexane, M=methanol, MIBK=methyl isobutyl ketone, P=propanol, Pen=pentane, Pyr=pyridine, T=toluene, W=water.

methanol–acetic acid (24:2:1), chloroform–acetic acid–diethyl ether (17:1:3), or chloroform–methanol (4:1) (27) for the separation of citreoviridin toxins.

6. Macrocyclic Lactones

a. Zearalenone. Zearalenone and its related mycotoxins are produced by *Fusarium* species. They are often found together with trichothecenes and have been extracted from foodstuffs such as barley, oats, wheat, sorghum, and corn colonized by the fungus. Silica gel is the adsorbent most commonly used for TLC to separate these toxins. Zearalenone toxins spotted on silica gel TLC plates are separated by various solvent systems, including toluene–ethanol (1:1), toluene–chloroform–acetone (3:15:2) (59), toluene–ethyl acetate–chloroform (2:1:1), diethyl ether–cyclohexane (3:1) (60), chloroform–methanol (97:3), ethyl acetate–hexane (1:1), and benzene–chloroform–acetone (45:45:15) (26). Zearalenone toxins on silica gel G are eluted with benzene–methanol–acetic acid (24:2:1), benzene–ethanol (95:5), or chloroform–acetone (9:1) (27). These toxins can be visualized as pink spots after the plates are sprayed with 0.7% aqueous Fast Violet B salt solution followed by spraying with a buffer solution (pH 9) and drying (61). Charred spots are seen when the plates are sprayed with concentrated sulfuric acid followed by heating for 10 min at 100°C. When the plates are sprayed with 50% methanolic sulfuric acid followed by heating for 15 min at 120°C, the separated Zearalenone toxins can be visualized initially as yellow spots, which then turn to brown (62).

b. Cytochalasans. Cytochalasans are the secondary metabolites of many varieties of fungi. Methods for the isolation, separation, purification, and detection of these toxins have been reviewed (63). Cytochalasins, zygospurin, aspochalasins, deoxaphomin, proxyphomins, and protophomin are the common Cytochalasans.

Cytochalasins. Silica gel is commonly used for the separation of cytochalasins by TLC. Solvent systems such as chloroform–methanol–formic acid (95:5:5), chloroform–diethylamine (90:10), chloroform–methanol (95:5), diisopropyl ether–ethyl acetate (90:10), cyclohexane–ethyl acetate–diethylamine (60:30:10), benzene–methanol (70:30), and *n*-butanol–formic acid–water (80:10:10) can be successfully used for the separation of cytochalasins on silica gel plates (64). Different R_f values are obtained by these solvent systems. Cytochalasins C and D are not well separated when chloroform–methanol (95:5), chloroform–diethylamine (90:10), or cyclohexane–ethyl acetate–diethylamine (60:30:10) is used as the solvent system to separate them on silica gel G TLC plates. However, they can be visualized by their different colors as they fluoresce under UV light after spraying with sulfuric acid followed by heating. Spots of cytochalsin C can be seen as dull orange, whereas cytochalasin D can be seen as yellow (64). Cytochalasins A, B, and C are separated on precoated sulfo-sheets in addition to silica gel plates using various solvent systems (27), whereas cytochalasins H and J are separated on silica gel containing 15% gypsum (65).

Zygosporin. Zygosporin toxins have been extracted from *Zygosporium masonii*, and TLC can be used for their separation on silica gel using chloroform–methanol (9:1) as the solvent system (3).

Aspochalasins. Four isomers of aspochalasins (A, B, C, and D) have been isolated from *Aspergillus parasiticus*. These toxins are separated using chloroform–methyl acetate (4:1) on Kiesel-gel 60 (66). They are detected by heating after spraying with 50% sulfuric acid. Toluene–ethyl acetate–formic acid (5:4:1) is used as the solvent system to separate chaetoglobosins K and L on silica gels.

Deoxaphomin, proxyphomins, and protophomin are separated on preparative TLC plates using chloroform–acetone (3:1) (67). Silica gel F₂₅₄ is used to separate chaetoglobosins A, B, C, D, and E using benzene–ethyl acetate (1:1) and benzene–chloroform–methanol (10:10:3) as solvent systems. Separated toxins are detected under UV irradiation after spraying with Ehrlich's reagent followed by heating (68).

7. Ochratoxin

Ochratoxins A, B, and C are toxic products of seven molds of the genus *Penicillium* and six molds of the genus *Aspergillus* growing on various kinds of cereals. These ochratoxins can persist in the foodstuff even after the destruction of the molds. Thermal processing can reduce these toxins by 20%, but boiling does not destroy them (69).

Ochratoxin A is a typical nephrotoxin for both animals and humans (69). TLC has been used to determine the ochratoxin A levels in 92 samples of wheat and 52 samples of corn from the surroundings of Slavonski Brod, Slovenia (70). The occurrence of ochratoxin A has been reported in barley, corn, pig serum, swine tissues, pig kidneys, sausages, human serum, and kidney as well as roasted coffee (3).

Ochratoxins can be detected under longwave UV light. Ochratoxin A can be seen as green fluorescent spots, whereas ochratoxins B can be visualized as blue fluorescent

spots. However, the colors of the spots can be changed to purple-blue when they are exposed to ammonia or sprayed with sodium hydroxide. Ochratoxin A can be seen as blue-green spots on acidic plates (71). Various solvent systems are used to separate ochratoxins using TLC plates, and some of them are listed in Table 6.

8. Rubratoxin

Rubratoxins A and B have been extracted from moldy rice and corn. Silica gel is commonly used for the separation of these toxins by TLC. Because they tend to oxidize rapidly, plates should be developed under nitrogen (3, 72). Rubratoxins are also separated on silica gel F₂₅₄ plates using chloroform–methanol–glacial acetic acid (80:20:2) (73). Silica gel HF₂₅₄₊₃₆₆ plates are developed in ethyl acetate–acetic acid (85:15). Rubratoxins are normally detected after heating at 200°C for 10 min followed by exposure to ammonia for another 10 min. Although they produce green fluorescence after heating, exposure to ammonia or spraying reagents increases the fluorescence intensity. Under longwave UV light, these toxins are seen as blue spots (74). The plates can also be sprayed with 2',7'-dichlorofluorescein to increase the color intensity.

9. Hydroxyanthraquinones

Rugulosin, emodin, and luteoskyrin are the most important hydroxyanthraquinones. Silica gel, either alone or impregnated with oxalic acid, is commonly used to analyze these toxins by TLC. The plates are developed in chloroform–methanol (97:3), benzene–ethyl acetate–acetic acid (45:55:1), or carbon tetrachloride–chloroform (90:10) (75). Silica gel 7GF plates are developed in toluene–ethyl acetate–formic acid (5:4:1) and chloroform–acetone (83:7) (76). Silica gel plates impregnated with oxalic acid are developed in acetone–hexane–water (6:3:1.5) (77). Table 7 shows various solvent systems used for the separation of hydroxyanthraquinone metabolites by TLC.

Table 6 Solvent Systems for the Separation of Ochratoxins by TLC

Metabolite	Adsorbent	Solvent system ^a	Ref.
Ochratoxins A and B	Oxalic acid–treated silica gel	C:A(9:1), C:M (98:2)	26
Ochratoxins A and B	Rice starch	T:AA(20:0.15)	5
Ochratoxins A and B	Silica gel	B:AA(3:1)	71
Ochratoxins A	Silica gel	T:EA:AA (5:4:1), B:AA (3:1)	71
Ochratoxin B and 4-hydroxy-ochratoxin A	Silica gel	C:AA(4:1)	71
Ochratoxin C	Silica gel	B:AA(25:1)	72
Ochratoxins A, B, and C	Silica gel G	B:E (95:5), C:MIBK (4:1), C:A (9:1), B:M:AA (24:2:1)	27

^aA=acetone, B=benzene, C=chloroform, E=ethanol, EA=ethyl acetate, M=methanol, MIBK=methyl isobutyl ketone, T=toluene.

Table 7 Solvent Systems for the Separation of Hydroxyanthraquinones by TLC

Hydroxyanthra-quinone metabolite	Solvent system ^a	Detection
Aurantioskyrin	B:A(4:1)	Magnesium acetate
Auroskyrin	B:A(20:1)	Magnesium acetate (yellow)
Catenarin	B: A (20:1), B:A(4:1)	Magnesium acetate
Chrysophanol	B:H (1:1), B:A (4:1), A:H:W (5:5:3.5)	Magnesium acetate (yellow)
Deoxyluteoskyrin	B:A(4:1)	Magnesium acetate (yellow)
Deoxyrubroskyrin	B:A(4:1)	
Dianhydrorugulosin	B:H (1:1)	Magnesium acetate (yellow)
Dicatenarin	B:A(4:1)	Magnesium acetate
Emodin	B:A (20:1), B:A (4:1), A:H:W (5:5:3.5)	Magnesium acetate (yellow)
Iridoskyrin	B:H (1:1), A:H:W (5:5:3.5)	Magnesium acetate
Islandicin	B:H (1:1), B:A (4:1), A:H:W (5:5:3.5)	Magnesium acetate
Luteoskyrin	B:A (4:1), A:H:W (5:5:3.5)	Magnesium acetate (yellow)
Punikoskyrin	B:A(20:1)	Magnesium acetate
Rhodoislandin A	B: A (20:1)	Magnesium acetate
Rhodoislandin B	B: A (20:1)	Magnesium acetate
Roseoskyrin	B:H(1:1)	Magnesium acetate
Rubroskyrin	B:A (4:1), A:H:W (5:5:3.5)	Magnesium acetate
Skyrin	A:H:W (5:5:3.5), B:A (20:1)	Magnesium acetate
4 α -Oxyluteoskyriin	B:A(4:1)	Magnesium acetate

^aA=acetone, B=benzene, H=hexane, T=toluene, W=water.

Source: Ref. 3.

10. *epi*-Polythiopiperazine-3,6-Diones

epi-Polythiopiperazine-3,6-diones are secondary metabolites of mycotoxins such as sporidesmins that cause facial eczema in some grazing animals (3) and some antibiotics such as gliotoxins produced by some fungi. Gliotoxins are produced by *Aspergillus fumigatus* (78) and are separated on silica gel 60 plates by using a methylene chloride–methanol (97:3) solvent system. They are detected by spraying the plates with 5% silver nitrate in 90% ethanol (79). Silica gel F₂₅₄ plates are used to analyze sporidesmins H and J by developing in benzene–ethyl acetate (4:1) (80), and either 5% silver nitrate or chromic acid is sprayed to detect them (79).

11. Tremorgenic Mycotoxins

Tremorgenic mycotoxins have a common indole moiety in their structures. Silica gels and modified silica gels are commonly used to analyze these toxins by TLC using a variety of solvent systems (Table 8).

12. *Alternaria* Toxins

Mycotoxins known as *Alternaria* toxins have been extracted from rice, maize, tomatoes, and barley contaminated with the fungus *Alternaria alternata* (90). They include altenuene, alternariol, alternariol methyl ether, and altertoxin I and II. Keisel-gel 60 F₂₅₄ plates are used for the separation of these toxins using toluene–ethyl acetate–formic acid (6:3:1) and chloroform–ethanol–ethyl acetate (90:5:5) as solvent systems. The separated toxins are detected by quenching of fluorescence under UV light or by spraying with 20% ethanolic aluminum chloride. Altertoxins produce a characteristic yellow fluorescence, whereas alternariol, alternariol methyl ether, and altenuene produce violet-blue fluorescence (91).

13. Citrinin

Thin-layer chromatography can be used for qualitative and quantitative analysis of citrinin in various food commodities. Although silica gel and Silufol plates are most commonly used as the

Table 8 Solvent Systems for the Separation of Tremorgenic Mycotoxins by TLC

Toxin	Adsorbent	Solvent system ^a	Detection	Ref.
Paspaline and paspalicine	Kiesel-gel	Chloroform	50% ethanolic sulfuric acid and heat, blue spots	81
Paxilline			50% ethanolic sulfuric acid and heat, blue spots	82
Fumitremorgin A	Silica gel GH-R	C:A (97:3) T:EA:FA	50% ethanolic	83

		(5:4:1)	H ₂ SO ₄ +heat, bright orange	
Verruculogen	Keisel-gel/silica gel F ₂₅₄	T:EA:FA (5:4:1)		82, 84
Penitrem A, B, C, D, E, and F	Silica gel	H:EA (70:30), DCM:A (85:15), B:A (85:15)	50% ethanolic H ₂ SO ₄ +heat, blue spots	85–87
Janthitrem A and B	Silica gel F ₂₅₄	T:EA:A (3:2:1)	Blue fluorescence under longwave UV	88
Territrems A and B	Silica gel	B:EA (1:1), T:EA 65% FA (5:4:1)	Blue fluorescence	89

^aA=acetone, B=benzene, C=chloroform, DCM=dichloromethane, EA=ethyl acetate, FA=formic acid, T=toluene.

adsorbents, the best results can be obtained if these plates are impregnated with oxalic acid (92). Silica gel F₂₅₄ plates are used to separate citrinin with chloroform–methanol (75:25) as the solvent system (93). A benzene–methanol–acetic acid (10:2:1) system is used to elute citrinin on Kiesel-gel plates (94). Separated toxin can be detected as yellow fluorescent spots under UV light (94).

14. α -Cyclopiazonic Acid

α -Cyclopiazonic acid has been extracted from cornmeal, beans, pecans, and macaroni infested with *Aspergillus* and *Penicillium* species (95). Solvent systems such as chloroform–methanol (98:2), chloroform–acetone (9:1), and chloroform–methyl isobutyl ketone (4:1) are used to separate this toxin on silica gel impregnated with either oxalic acid or tartaric acid (3). Ehrlich's reagent is commonly used for the detection. Spraying with concentrated sulfuric acid followed by heating is also used (96).

15. Roquefortine

Roquefortines are secondary metabolites of the fungus *Penicillium roquefortine* and have been isolated from blue cheese (13). These toxins are eluted on silica gel TLC plates using basic solvent systems such as chloroform–methanol–25% ammonia solution (70:10:0.5) or acidic solvent systems such as toluene–ethyl acetate–formic acid (5:4:1) (97). Separated toxins can be detected as yellow spots after spraying with 50% sulfuric acid (98), whereas they turn into blue-gray spots followed by heating. They also can be visualized as green fluorescent spots under longwave UV light followed by exposure of the plates to shortwave UV light for about 30 s (97).

16. Xanthomegnin, Viomellein, Vioxanthin

Xanthomegnin, viomellein, and vioxanthin are toxic metabolites of a variety of fungi including *Penicillium* and *Aspergillus*. They are eluted on silica gel using benzene–methanol–acetic acid (18:1:1) or toluene–ethyl acetate–formic acid (6:3:1) (9).

Xanthomegnin can be detected as yellow-orange spots after allowing the plates to stand for about 6 h, whereas viomellein spots can change from yellow-green to yellow-brown (99).

17. Naphtho- γ -Pyrone

Naphtho- γ -pyrones have been extracted from the mycelia of the fungus *Aspergillus niger* and from food commodities such as rice, corn, and cottonseed infested with this fungus (3). Benzene–ethyl acetate–formic acid (10:4:1) is used as the solvent to elute these toxins on LHP-KF plates by HPTLC (100). They can be detected as yellow, violet, or orange spots under longwave UV light, whereas derivatives of naphtho- γ -pyrones such as flavasperone; fonsecin monomethyl ether; rubrofusarin; aurasperone A, B, C, and D; and isoaurasperone A can produce different colors after spraying with Gibbs reagent.

18 Fusarin

Commonly found fusarin C is mutagenic. The fusarins are mainly produced by *Fusarium moniliforme* and are extracted with water and methylene chloride–2-propanol (1:1). After spotting on silica gel TLC plates, they are separated using chloroform–methanol (9:1) or chloroform–isopropanol (9:1) as solvent. The separated toxins are detected under UV light as bright yellow spots (101, 102).

19. Cyclosporin

Cyclosporin toxin can be separated on silica gel plates by developing in butanol–acetic acid–water (4:1:1) (108). Separated toxins can be detected as orange-brown spots after treatment with ninhydrin solution.

20. Fumonisin

Fumonisin are the secondary metabolic products of mycotoxins produced by the fungus *Fusarium proliferatum*. They persist in harvested and stored grains and grow well when moisture levels become favorable. They cause diseases in crops and also have a carcinogenic effect associated with toxigenic effects such as leukoencephalomalacia in animals (104). Because fumonisins do not have characteristic chromophores for absorption in the UV-visible range, it is necessary to perform pre- or postchromatographic derivatization to produce colored or fluorescent compounds. They are then visualized on TLC plates using ninhydrin, *p*-anisaldehyde, *o*-phthalaldehyde, and fluorescamine (105). Fumonisin B₁, B₂, and B₃ are easily detected at microgram levels by TLC plates coated with either normal-phase silica gel or reversed-phased C-18 adsorbent (chemically modified silica gel) (106). TLC combined with overpressured layer chromatography (OPLC) is also used to separate all fumonisins of the B series (107).

21. Ergovaline

Ergovaline is a mycotoxin produced by *Neotyphodium coenophialum* growing in the seeds of members of Gramineae family. Fescue cytotoxicosis and rye grass staggers in animals are caused by the poisoning of these animals with *Neotyphodium*-infested forage. HPTLC is used as a rapid technique to detect these toxins in the seeds of endophyte-infested perennial rye grass and tall fescue (108). Toxin samples and standard ergovaline solutions are sprayed automatically with high purity pressurized nitrogen gas on a glass HPTLC plate, coated with silica gel 60 with a film thickness of 0.25 mm. The plates are developed in chloroform and ethanol (17.5:2.5) for 20 min. After drying, the plates are read using fluorimetry with an excitation wavelength of 318 nm and an emission wavelength of 400 nm. Ergonovine, ergotamine, ergocryptine, ergocristine, and ergocornine are well separated and detected with detection limits of 0.5 µg/g.

B. Cyanobacterial Toxins

Freshwater and marine cyanobacteria species are known to produce a wide array of toxins and their metabolites. Two-dimensional (2-D) TLC is used for the chemical and ecological evaluation of cyanobacterial crude extracts from the filamentous cyanobacteria *Lyngbya majuscula*, *S. calcicola*, and *Microcoleus* species (109). Antillatoxin, aplysiatoxin, debromoaplysiatoxin, barbamide, curacin A, carbamin A and B, grandadiene, grandamide, kalkipyron, and lyngbyatoxin are some of the toxic metabolites produced by *Lyngbya*. Majusculamide A and B; malyngamide A, B, H, I, J, K, and L; malyngolide; microcolin A and B; and yapaoamide are produced by *L. majuscula*. Toxic metabolites of *Hormothamnion enteromorphoides*, *Symploca hydroides*, *Synechocystis* species, and mixed marine cyanobacterial assemblages include nakienone A, B, and C; nakitriol; hormothamnin A; dolastatin 10 and 12; symplostatin 1; lyngbystatin 1; and majusculamide. The structures of these compounds are given in Ref. 109. Silica gel 60 TLC sheets with UV₂₅₄ fluorescent indicator are used for the detection of most of these toxic metabolites. Nonpigmented UV₂₅₄-fluorescing compounds can be seen directly. The sheets are sprayed with a light, even coating of sulfuric acid in ethanol (1:19) and gently heated with either a high temperature air gun or a hot plate to detect nonfluorescing compounds by acid charring.

C. Plant Toxins

1. Linamarin

Linamarin is a toxic natural cyanogenic glucoside in the tubers and leaves of cassava (*Manihot esculenta* Crantz), which is the major staple food in sub-Saharan Africa. Cassava leaves are also used as a major vegetable in other parts of Africa and some parts of Asia. The tubers and leaves contain the cyanogenic glucosides linamarin and lotaustralin, and various analytical techniques are used to determine these toxins in the tubers.

A simple, rapid, and accurate high-performance thin-layer chromatographic method is used for direct quantitative analysis of linamarin in cassava (110). Silica gel 60 F₂₅₄ HPTLC plates are used as the adsorbent. They are developed in ethyl acetate-acetone-water (40:50:10) and ethyl acetate-formic acid-water (60:10:10), and the toxin spots are visualized by dipping the plates into a chamber containing aniline (2%) and orthophosphoric acid (15%) in acetone. Scanning densitometry is used for quantification.

2. Amygdalin

Amygdalin is a toxin present in the seeds of bitter apricot (*Prunus armeniaca*) and other *Prunus* species (111, 112). The mechanism of the enzymatic degradation of these toxins by β -glycosidase (amydalase and linamarase) extracted from the yeast *Endomyces fibuliger* has been studied using TLC. Analyses of this toxin are performed on silica gel 60 F₂₅₄ sheets (64271: Merck, Darmstadt, Germany), and the plates are developed in ethyl acetate-acetone-chloroform-methanol (5:3.75:1.5:1.25) (113). Visualization of the reaction products of the spots is done using a 1% aqueous solution of β -glycosidase as the source of enzyme.

3. Glycoalkaloids

a. Solanine and Chaconine. The toxic steroidal glycoalkaloids such as α -solanine and α -chaconine are naturally occurring compounds found in plants of the Solanaceae family including potatoes, tomatoes, and sweet pepper. The concentration of these compounds is increased by mechanical injuries as well as by fungal or viral attacks. Some cultivars may contain high amounts of these glycoalkaloids. High-performance thin-layer chromatography is used to determine the two main toxic glycoalkaloids, solanine and chaconine, in potatoes (114). Silica gel 60 HPTLC plates are also used as the adsorbent. The plates are developed in chloroform-methanol-aqueous ammonium hydroxide (70:30:5). Separated toxins are detected using six different detection reagents—molybdophosphoric acid, paraformaldehyde reagent, Ce(IV) sulfate reagent, Dragendorff's reagent, and concentrated sulfuric acid in ethanol—and the detected spots are quantified by densitometry. The fluorescence enables the detection of 10 ng of each glycoalkaloid.

b. Other Solanine Derivatives. Thin-layer chromatography is used for the detection of spirosolane, solanidane, solasodine, dehydrotomatine, α -tomatine, α -solanine, α -chaconine, dehydrotomatidine, and tomatodine, which are also glycoalkaloid toxins produced by potatoes and tomatoes (115). Normal-phase TLC on precoated silica gel 60 F₂₅₄ sheets is used for the analysis, and the plates are developed in a chamber saturated with 95% ethanol or methanol-chloroform (2:1). The toxins are detected after spraying with aqueous cobalt(II) thiocyanate solution or with methanolic (95%) sulfuric acid (1:1) followed by heating. However, cobalt solution does not produce any color differences among the spots of different alkaloids, and the color of the spots usually disappears in 10 min.

4. Sweet Potato Toxins

Toxic metabolites are produced by sweet potatoes (*Ipomea batatas*) as a result of stress in response to damage by insects, mold growth, mechanical injury, or some chemicals (116). Ipomeamarone and ipomeanine are hepatotoxic, whereas 4-ipomeanol, 1-ipomeanol, ipomeanine, and 1,4-ipomeadol cause damage to the lungs (117). After the extraction of these toxins from sweet potatoes with ether, they are partially purified on a silica column and eluted with various amounts of ethyl acetate and hexane. TLC is done for these toxins on silica gel plates using benzene–methanol (9:1) as the solvent system (118). The separated toxins are detected after the plates are sprayed with Ehrlich's reagent followed by gentle heating to develop the spots. Ipomeamarone and 1,4-ipomeadiol are seen as pink-orange spots, whereas 1-ipomeanol and 4-ipomeanol are visualized as purple spots with different R_f values. Ipomeanine is seen as a light gray spot.

5. Gossypol

Gossypol is a highly toxic polyphenolic binaphthylaldehyde found in cottonseed. Although ruminants can tolerate this toxin better than nonruminants, it can be toxic to many other animals, insects, and microorganisms (117). TLC is done on silica gel plates with benzene–dioxane–acetic acid (91:10:4) as the solvent system. Gossypol content is determined by the measurement of absorbance at 483 nm after reaction with aniline, and the toxin is seen as a blue fluorescent spot ($R_f=0.87$) (118). Silica gel plates are also developed in hexane–ethyl acetate–acetic acid (199:199:2), and dianilinogossypol is visualized as a yellow spot ($R_f=0.68$) (120).

6. Pyrrolizidine Alkaloids

Pyrrolizidine alkaloids are found in over 200 plants around the world, and over 150 different ones have been reported. Some of these are plant species in the genera *Crotalaria*, *Senecio*, *Heliotropium*, *Trichodesmo*, *Echium*, and *Amsinckia* (117). The toxicity of the individual alkaloids varies widely, and some of these toxins can enter the human body through foods such as grains, milk, meat, honey, and herbal teas. Plants such as *Senecio jacobaea* that contain these toxic alkaloids are lethal to cattle and horses.

Thin-layer chromatography has been used for the analysis of pyrrolizidine alkaloids (121). Plant toxins are extracted and then spotted on silica gel TLC plates. The plates are then developed in two different solvent systems, most of which are listed in Table 9. The separated toxins are detected by spraying the plates initially with *o*-chloranil in benzene to oxidize the toxin to pyrrols and then with *p*-dimethylaminobenzaldehyde and boron trifluoride etherate in ethanol. Deep purple derivatives are formed after the toxins react with *p*-dimethylaminobenzaldehyde. Because alkaloids that contain the N-oxide part do not react with *o*-chloranil, plates with these compounds must be sprayed with acetic anhydride prior to the derivatization with *p*-dimethylaminobenzaldehyde. The purple spots that represent pyrrolizidine toxins are individually identified by comparing the R_f values with those of their corresponding standards.

D. Marine Toxins

1. Paralytic Shellfish Poison

Although HPLC and capillary electrophoresis either alone or combined with mass spectrometry have been used for the determination of paralytic shellfish poisoning (PSP) toxins, TLC can also be used for the separation and detection of major PSP toxins, including saxitoxin; neosaxitoxin; gonyautoxins I, II, III, and IV; B₁ toxins; B₂ toxins; C₁, C₂, C₃, and C₄ toxins; and their decarbamoyl derivatives. Toxins extracted from algal cells or shellfish are developed on silica gel TLC plates using pyridine–ethyl acetate–water–acetic acid (75:25:30:15) for 1.5 h (122). Plates are then sprayed with 1% H₂O₂ solution followed by heating at 100°C for 30 min, and the separated toxins are visualized as fluorescent spots. Toxin-spotted plates are also developed in *n*-butanol–acetic acid–water (2:1:1) (123) and are detected with H₂O₂. Separated toxins are detected by spraying the Fast Blue reagent on the plates (124).

Table 9 Solvent Systems for the Analysis of Pyrrolizidine Toxins by TLC^a

Alkaloid	Detection limit (μg)	R_f	
		Mobile phase 1	Mobile phase 2
Anacrotine	0.5	0.40	0.61
Integerrimine	2.0	0.62	0.82
Jacobine	1.0	0.37	0.79
Jacoline	0.5	0.29	0.52
Jaconine	2.0	0.61	0.79
Monocrotaline	1.0	0.38	0.63
Monocrotaline N-oxide	—	0.17	0.05
Platyphylline	25.0	0.46	0.78
Retronecine	<0.15	0.05	0.21
Retorsine	2.0	0.35	0.54
Riddelliine	2.0	0.34	0.54
Riddelliine N-oxide	—	0.19	0.04
Senecione	1.0	0.62	0.82
Senecione N-oxide	—	0.39	0.15
Seneciophylline	2.0	0.61	0.82
Spectabiline	2.0	0.37	0.68

^aMobile phase 1: chloroform–methanol–17% ammonium hydroxide (165:31:4); mobile phase 2:

chloroform–acetone–ethanol–ammonium hydroxide (5:3:1:1).

Source: Adapted from Ref. 117.

During the last three decades, TLC techniques have evolved considerably. One obvious advancement is the use of silica- or alumina-coated quartz rods (Chromarods) instead of TLC plates. After the sample is spotted, these rods are developed in a solvent system just as TLC plates are for elution of the compounds of interest. The compounds separate along the cylindrical rod as bands, and these bands are pyrolyzed over the flame. Various charged ions produced in the flame are detected by the flame ionization detector, which is functionally quite similar to the flame ionization detection (FID) technology in gas chromatography. Compounds with N, P, or halogen atoms give greater response with flame thermionic detection (FTID) than with FID. Although the TLC-FID technique has been used for the analysis of lipids and amino acids, it has rarely been used for the analysis of natural toxins. However, major PSP toxins can be separated on Chromarods-SIII after development in chloroform–methanol–water–acetic acid (30:50:8:2) (125). Separated toxin bands are detected by a flame thermionic detector, which is more sensitive than the FID for nitrogenous compounds such as PSP toxins. Some of the advantages of Iatroscan with FID/FTID analyses over classical TLC are that it is simple, inexpensive, rapid, and more accurate for quantitative analyses. Unlike conventional TLC, a sample size as small as 0.2 μ L can be used in the Iatroscan TLC-FID or FTID system. As many as 60 samples can be detected in 1 h.

2. Tetrodotoxin

Tetrodotoxin is a strong neurotoxin that is the main cause of puffer fish poisoning. The toxin is produced by a number of puffer fish species as well as some seaweeds and marine bacteria. In addition to other analytical methods, TLC has been used for the identification of tetrodotoxin and its derivatives produced by the *Vibrio* strain in the intestine of the puffer fish, *Fugu vermicularis radiatus* (126). TLC is performed on silica gel LHP-K linear high-performance TLC plates using pyridine–ethyl acetate–acetic acid–water (15:5:3:4) as the solvent system. Separated toxins are visualized as pink spots after spraying the plates with Weber reagent or as yellow fluorescent spots under UV light (365 nm) after spraying with 10% KOH followed by heating.

Silica gel 60 F₂₅₄ precoated plates are also used as the adsorbent to separate and identify tetrodotoxins in the horseshoe crab, *Carcinoscorpius rotundicauda*, using the same solvent system (127). The toxins are detected after spraying the plates with 10% potassium hydroxide followed by 1% H₂O₂. The toxins are visualized as yellow fluorescent spots under UV light (365 nm). TLC is also performed on Chromarods-SIII with flame ionization detection using the Iatroscan for the analysis of tetrodotoxins in fish tissues (128).

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Glossary

- Absorption** Retention of a solute by partitioning into a liquid or liquidlike stationary phase coated on or bonded to a surface.
- Accuracy** The agreement between an experimental result (a single measurement or the mean of several replicate measurements) and the true or theoretical value.
- Activation** The process of heating an adsorbent layer to drive off moisture and convert it to its most attractive or retentive state.
- Activity** The relative strength of the surface of a sorbent such as silica gel or alumina in adsorption chromatography. Activity is reduced by adding water or another polar modifier that is attracted by hydrogen bonding to the active sites.
- Activity grades** (Brockmann activity grades) A standard grading system for the activity (adsorptivity) of alumina based on deactivation with water. Grade I is anhydrous alumina and has the highest activity. Grades II, III, IV, and V contain 3%, 6%, 10%, and 15% (by weight) water, respectively.
- Additive** A substance added to the mobile phase to improve solute separation or detection.
- Adsorption** The attraction between the surface atoms of a solid and an external molecule by intermolecular forces such as hydrogen bonds, London forces, electrostatic forces, and charge transfer forces. The adsorbent is the stationary phase for adsorption TLC.
- Alumina layer** An aluminum oxide layer that is available with basic, neutral, or acid modifications and is used in normal-phase adsorption TLC.
- Amino layer** A propylamino layer used in normal bonded phase TLC or as a weak anion exchanger.
- Analyte** A solute that is to be identified or, more often, quantitatively determined by thin-layer chromatography or other method.
- Analytical TLC** Thin-layer chromatography performed on 100–250 μm layers for the purpose of separation, identification, or quantification of substances.
- Anion exchange** The mode of TLC that uses a layer with a structure, such as a bonded amino group, that can separate anions.
- Anticircular TLC** Development of a layer from an outer circle of initial zones toward the center.
- Argentation TLC** Thin-layer chromatography using a layer, usually silica gel, impregnated with silver nitrate for the purpose of improving the separation of certain compounds.
- Ascending development** The usual mode of thin-layer chromatography development in which the mobile phase moves upward by capillary action of the sorbent, as opposed to circular, descending, or horizontal development.

- Azeotrope (azeotropic mixture)** A liquid mixture of two or more substances that behaves like a single substance in that the vapor produced by partial evaporation of the liquid has the same composition as the liquid.
- Band** Chromatographic zone, usually in the shape of a horizontal line rather than a round spot.
- Binary mobile phase** A mobile phase with two components such as solvents, acids, bases, or buffers.
- Binder** Any chemical added to a sorbent to improve the stability or hardness of the layer.
- Bonded phase** A stationary phase chemically bonded to (as opposed to mechanically deposited on) a support material.
- Capacity factor (k')** A measure of sample retention by a layer: $k' = (1 - R_f)/R_f$.
- Cation exchange** The mode of TLC that uses a layer with a structure, such as a sulfonic acid functional group, that can separate cations.
- Centrifugal layer chromatography** Analytical or preparative chromatography in which solvent is driven through the layer by centrifugal force in circular, anticircular, or linear modes.
- Chamber** The tank, jar, or vessel of other type in which thin-layer chromatographic development is carried out.
- Chamber saturation** Equilibration of the chamber or tank with mobile phase vapor before the plate is developed.
- Chiral layers** Layers that can separate enantiomeric mixtures.
- Chlorosilane** A chemical reagent used to prepare siloxane bonded phases such as C-18 used in bonded-phase TLC.
- Chromatogram** The series of zones on or in the layer after development, or the densitometric scan of the zones.
- Chromatographic solvent** Solvent or mixture of solvents used as the mobile phase.
- Chromatographic system** The combination of the solvent, sorbent, and the sample mixture. The interactions of the chromatographic system determine the selectivity of the separation.
- Chromatography** A method of analysis in which the flow of mobile phase promotes the separation of substances by differential migration from a narrow initial zone in a porous, sorptive, stationary medium.
- Chromatostrips** An early term used for narrow thin-layer plates.
- Chromogenic** A reagent or reaction causing a solute to become colored.
- Cochromatography** Development of a mixture prepared by adding a known standard to a sample thought to consist of or contain that substance. Formation of two zones from the mixture indicates the nonequivalence of the standard and sample, whereas the inability to separate the mixture is one piece of evidence for confirmation of identity.
- Column chromatography** Chromatography carried out by passing a liquid (or gaseous) mobile phase through a stationary phase packed in a column (*see* Open-column chromatography and HPLC).
- Confirmation** An ancillary qualitative analysis that proves the identity of a constituent in a sample with greater certainty than the original TLC analysis, usually by means of some type of on-line or off-line spectrometry. Quantitative results can also be confirmed by reanalysis of the sample using an independent method.

- Conjugate** The combined form in which drugs or pesticides can be found in plant or human samples, e.g., glucuronide or sulfate. Conjugates usually require hydrolysis by acid, base, or enzymes to free the analyte prior to TLC analysis.
- Continuous development** Development of a layer for a greater distance than the actual length; also called overrun development.
- Deactivation** Adding moisture to an adsorbent layer to lower its retention of polar solutes.
- Demixing** Separation of mobile phase components during development, leading to the formation of one or more solvent fronts along the layer.
- Densitometry** Quantification of a zone directly on the layer with an instrument that measures color, absorption of ultraviolet light, fluorescence, or radioactivity.
- Deproteinization** A sample preparation procedure involving removal of protein, e.g., by precipitation with a reagent.
- Derivatization** Reaction of solutes before chromatography or directly on the layer for the purpose of facilitating separation or detection.
- Desalting** A sample preparation procedure involving removal of salts by some procedure such as ion exchange or dialysis.
- Descending chromatography** Chromatography in which the mobile phase moves downward through the layer.
- Destructive detection** A detection method that irreversibly changes the chemical nature of the solute, e.g., sulfuric acid charring.
- Detection** The process of locating a separated substance on a chromatogram, whether by physical methods, chemical methods, or biological methods (visualization).
- Development** The movement of the mobile phase through the layer to form the chromatogram. This term does *not* mean detection of the zones.
- Development solvent** The mobile phase.
- Diatomaceous earth** A naturally occurring fine white powder formed from the skeletons of microscopic marine organisms (kieselguhr).
- Diethylaminoethyl (DEAE)** A weak anion-exchange layer material.
- Diol** A hydrophilic phase having two—OH groups on adjacent carbon atoms of an aliphatic chain. The layer can function with a normal- or reversed-phase mechanism.
- Displacement TLC** Mode of TLC in which the mobile phase contains a “displacer component” that has a higher affinity for the stationary phase than any of the solutes to be separated. This is in contrast to the usual elution mode of TLC, in which the mobile phase has a weaker affinity than the analytes for the sorbent. In displacement chromatography, development leads to formation of a “displacement train” of zones, all moving with the same velocity in reverse order of affinity for the sorbent and having higher concentration than in linear elution chromatography. Displacement TLC has been used to scout for optimum displacers and determine solute distribution for displacement column HPLC, which is used mostly for preparative separations. (J Bariska, T Csermely, S Furst, H Kalasz, M Bhatori. Displacement thin layer chromatography. *J Liq Chromatogr Relat Technol* 23:531–549, 2000.)
- Distribution constant (*K*)** See Partition Coefficient.
- Drop chromatography** Application of a drop of mixture solution to a layer, followed by applications of drops of a solvent on top of the sample to develop it into concentric rings.

Efficiency The narrowness of a peak compared with the length of time the component is in contact with the stationary phase. Efficiency is measured by plate number N or height equivalent to a theoretical plate, H .

Eluent A solvent used to remove or elute a substance away from the sorbent during recovery for preparative or quantitative TLC. Also, the mobile phase used to perform column chromatography, but an improper term for the mobile phase used for development in thin-layer chromatography.

Eluotropic series A series of solvents arranged in order of increasing ability to displace a solute, i.e., increasing polarity in adsorption chromatography.

Elution The removal of a solute from a sorbent by passage of a suitable solvent. The eluent is the solvent, and the effluent or eluate is the liquid flowing from the sorbent (the eluent $\neq$ the solute). *Elution development* is defined as the development of a small amount of sample through a column or flat bed of sorbent with a liquid less strongly sorbed than the sample. The terms *elution development* and *chromatography* are generally used synonymously; the term *displacement development* is used when the developing solvent is more strongly sorbed than the sample, and the term *frontal analysis* is used when the sample is passed continuously through the sorbent.

Enrichment The concentration effect obtained during sample preparation. If a compound contained in 1 L of water is collected on a solid-phase extraction column and is subsequently eluted with 2 mL of a solvent, the enrichment factor is 500.

Flatbed (or planar) chromatography Common term for paper chromatography and TLC.

Fluorogenic A reagent or reaction causing a solute to become fluorescent.

Forced-flow planar chromatography (FFPC) Planar chromatography in which solvent migrates through the layer under the influence of external pressure (overpressured layer chromatography, OPLC) or centrifugal force (rotation planar chromatography, RPC).

Front The visible boundary at the junction of the mobile phase wetted layer and the “dry” layer.

FT spectroscopy Fourier transform. A type of spectroscopy in which the intensity or radiant power of many wavelengths are simultaneously measured as a function of time, and the resultant time-domain spectrum is converted by use of a computer to a conventional frequency-domain spectrum by Fourier analysis (a mathematical way to decompose a signal into its component wavelengths).

GC Gas chromatography, most often gas-liquid partition chromatography (GLC).

Gel filtration (gel permeation) Size-exclusion chromatography using a polymer gel layer or clean-up column. Gel filtration uses a polydextran gel, highly cross-linked polymer, silica gel, or other porous medium and an aqueous mobile phase. Biopolymers and water—soluble polymers are usually separated. Gel permeation uses a styrene-divinylbenzene type of gel and an organic mobile phase to separate organic species and polymers.

Gradient The change in layer of mobile-phase composition made to improve separations.
 H (HETP) See Plate number.

Hard layer An abrasion-resistant sorbent layer bound to the backing by an organic polymer.

Homolog A member of a homologous series, which is a related succession of compounds each containing one or more carbon atoms and two more hydrogen atoms than the one before it in the series. The alcohols methanol, ethanol, and propanol are homologs.

HPLC High-performance column liquid chromatography, performed under high pressure with narrow-bore columns containing small-diameter packings and continuous-flow detectors.

HPTLC High-performance thin-layer chromatography, performed on layers with small, uniform, densely packed sorbent particles.

hR_f $100 \times R_f$.

Hydrophilic Substances that are soluble in water or other polar solutions.

Hydrophobic Substances that are soluble in nonpolar hydrocarbon solvents and insoluble in water.

Impregnated layer A layer containing a liquid or solid chemical or mixtures of chemicals added to aid separation or detection of solutes.

In situ Occurring "in place," directly on the layer.

Ion exchange A process whereby ions of the same charge sign replace one another in a given phase. In chromatography, the term usually refers to systems in which the stationary phase is made up of an ionic polymer. This can be a synthetic resin, a cellulose or Sephadex-type exchanger, or various types of inorganic materials.

IR Infrared.

Isomer One of two or more compounds having the same molecular weight and formula but differing with respect to the arrangement or configuration of the atoms. Examples of isomers are *ortho*-(1, 2)-, *meta*-(1, 3)-, and *para*-(1, 4)-dichlorobenzene.

Kieselguhr A weakly adsorptive diatomaceous earth used in TLC mostly as a preadsorbent material and also used as a sample clean-up medium.

Ligand exchange TLC A technique in which chelating ligands are added to the mobile phase and undergo sorption onto the layer. These sorbed molecules act as chelating agents with certain solutes to enhance their separation. Chelating stationary phases in which chelating groups are chemically bonded to the sorbent can also be used.

Lipophilic *See* Hydrophobic.

Liquid chromatography The type of chromatography in which the mobile phase is a liquid and the stationary phase is a layer or column.

Liquid—liquid TLC *See* Partition TLC.

Mass spectrometry A method for structure determination based on ionization of the compound and the sorting out of ions in a mass analyzer.

Mass transfer Movement of a solute between the stationary and mobile phases.

Metabolite The product(s) resulting from one or more chemical reactions that change or break down a compound such as a drug or pesticide in a biological system (e.g., human or animal body or plant) or the environment.

Microgram (μg) 10^{-6} g or 100 ng.

Migration Travel of a solute through the layer in the direction of the mobile phase flow.

Mobile phase The moving liquid phase used for development, often called the development solvent or solvent.

Multimodal layer A layer, such as cyano- or amino-bonded silica gel, that can operate with two or more separation mechanisms depending on the choice of mobile phase.

Multimodal separation A separation involving two distinct techniques, such as GC and TLC or HPLC and TLC. TLC is normally the second of the two techniques used. The coupling of two separation techniques is also called “multidimensional chromatography.”

Multiple development Repeated development of the chromatogram in the same direction with one mobile phase or different mobile phases for the same or different distances.

N-Chamber A square or rectangular tank or chamber, or a cylinder, with (saturated) or without (unsaturated) a paper liner and covered with a top, for development of TLC or HPTLC plates.

Nanogram (ng) 10^{-9} g or $0.001 \mu\text{g}$.

nm Nanometer or 10^{-9} m.

NMR (or Nuclear magnetic resonance spectrometry) A method for structure determination of organic compounds based on the magnetic properties of different nuclei.

Nondestructive detection Detection of a substance on a chromatogram by a process that will not permanently change the chemical nature of the substances being detected.

Normal-phase chromatography Adsorption or partition TLC in which the stationary phase is polar in relation to the mobile phase.

Octadecylsilane The most popular reversed-phase sorbent in TLC. Abbreviated in bonded silica layer names as C-18.

Open-column chromatography Liquid-column chromatography performed in the classical manner in a relatively large bore, usually glass, column under gravity or low-pressure flow. Used as a clean-up method for samples prior to TLC.

Origin The location of the applied sample; also, the starting point for chromatographic development of the applied sample.

Overrun development Continuous development beyond the top edge of the layer.

Partition coefficient or ratio (K_d) The ratio of concentrations of solute in each phase: $K_d = C_s / C_m$, where C_s and C_m are the concentrations in the stationary and mobile phases, respectively.

Partition TLC Separation of solutes based on differential distribution between a stationary liquid supported on the layer and the liquid mobile phase. In normal-phase partition, the stationary liquid is more polar than the mobile phase, whereas in reversed-phase partition, the stationary liquid is the less polar. The term is also used for TLC on bonded phases in some cases.

PC Paper chromatography. Also used as an abbreviation for planar chromatography in some cases.

Phenyl layer A layer composed of a nonpolar bonded phase prepared by reaction of dimethylphenylchlorosilane or alkoxysilane with silica gel. Some literature sources indicate that it has selective affinity for aromatic compounds.

Picogram (pg) 10^{-12} g or 10^{-3} ng.

Planar (or Flatbed) chromatography Common term for thin-layer or paper chromatography.

Plate height (HETP or H) The length of the layer divided by the number of plates (N). Small plate heights result in better resolution.

Plate number (N) $N=16(d_r/W)^2$, where d_r is the distance the spot has migrated and W is its width (size in the direction of development). N can be measured using either the spots on the plate or their densitometric scans.

PMD Programmed multiple development. The repeated development of a TLC plate with the same mobile phase or different mobile phases in the same direction for gradually increasing distances, using an automated commercial instrument. Also termed *automated multiple development (AMD)*.

Polarity The effect of the combined interactions between a functional group and a layer or mobile phase, i.e., dispersion, dipole, and/or hydrogen bonding forces, is designated "polarity." In chromatographic systems, benzene is described as a more polar solvent than toluene, although only the latter has a true dipole moment, the classical requirement for polarity. In chromatographic behavior, hydrocarbons and halogens are of low polarity, esters and ketones have intermediate polarity, and alcohols and amines are highly polar. The polarity of a silica gel surface is reduced by impregnation or chemical bonding with a hydrocarbon.

Precision The agreement or lack of scatter among replicate determinations or analyses, without regard to the true answer.

Precoated plates Commercial plates or sheets sold with the layer already formed and ready for use.

Preloading Sorption of gaseous molecules by the layer prior to development with the mobile phase; also called layer conditioning or preadsorption.

Preparative TLC Thin-layer chromatography of larger quantities of material on thick layers for the purpose of isolating separated substances for further analysis or use. The term *preparative layer chromatography (PLC)* is sometimes used instead.

Qualitative analysis A TLC analysis that identifies the constituents present in a sample.

Quantitative analysis A TLC analysis that determines the weight or concentration of a constituent of a sample.

R_f The ratio of the distance of migration of the center of a zone divided by the distance of migration of the solvent front, both measured from the origin.

R_m A value used in relating chromatographic behavior to chemical structure:
 $R_m = \log(1/R_f - 1)$.

R_x The same as R_f except that the distance of solvent front migration is replaced by the distance of migration of some reference compound x .

Radial (circular) development Development of a layer in such a manner as to form circular or arc-shaped zones. Some workers differentiate circular and radial TLC by the use of "circular" for the case where one initial zone is developed into circular zones and "radial" for development of a series of initial zones, spotted in a circular pattern, into arcs.

Raman spectroscopy A technique in which a beam of intense monochromatographic radiation, such as from a laser, is focused on a sample, and scattering produces radiation that is shifted slightly in frequency by an increment of energy corresponding to a natural transition of the molecule (Raman lines). The method complements IR absorption spectroscopy for measurement of molecular vibrations.

Relative standard deviation (RSD) The standard deviation of a series of replicate analyses is divided by the mean, and the resulting quotient is multiplied by 100. Also called *coefficient of variation*.

Resolution The ability to separate two zones. The mathematical description of resolution is $R = 2(R_{f1} - R_{f2}) / (W_1 + W_2)$, where R_{f1} and R_{f2} are the R_f values of any two zones and W_1 and W_2 are their respective zone lengths (in the direction of development). Migration distances can be used instead of R_f values in this equation. Resolution is the result of the combined contributions of efficiency (zone compactness), selectivity (separation of zone centers), and capacity (average R_f value of the pair of substances to be separated).

Retention factor Another name for *capacity factor*.

Reversed-phase chromatography Liquid—liquid partition TLC in which the stationary phase is nonpolar compared to the mobile phase. The layer can be impregnated or bonded.

Rod TLC TLC carried out on sintered glass rods, usually in conjunction with a scanning flame ionization detector.

Sandwich chamber (S-chamber) A developing chamber formed from the plate itself, a spacer, and a layered or nonlayered cover plate that stands in a trough containing the mobile phase, or some other type of small-volume chamber in which vapor-phase saturation occurs quickly.

Saturated The condition of a chamber that is lined with paper and equilibrated with mobile phase vapors before chromatographic development is begun.

Secondary front An additional solvent or mobile phase front below the primary front, that occurs because the mobile phase components demix.

Selectivity The ability of a chromatographic system to produce different R_f values for the components of a mixture, i.e., to separate the zone centers.

Sensitivity The ability to detect or measure a small mass of analyte.

Separation number A measure of the separating power of a TLC system: The number of substances completely separated (resolution=1) between $R_f=0$ and $R_f=1$ by a homogeneous mobile phase (no solvent gradients in the direction of development).

Silanol An Si—OH group on the surface of silica gel.

Silica gel The most common layer material, used unmodified for adsorption TLC, as a support for partition and bonded-phase TLC, and with different pore sizes for size-exclusion TLC. It has an amorphous, porous structure with siloxane and silanol groups on the surface.

Siloxane Si—O—Si groups on the surface of silica gel.

Soft layer A sorbent layer prepared without binder or with gypsum binder (*see* Hard layer).

Solid-phase extraction (SPE) An alternative to traditional separatory funnel extraction in which an analyte is extracted from a liquid sample by use of a solid packed in a small column, cartridge, or disk. The sample is forced through the solid, which is an adsorbent or bonded phase, with the aid of vacuum or pressure; the analyte is retained on the solid, and it is subsequently eluted with a small volume of a strong solvent, usually resulting in significant enrichment. The latest format for SPE is placement of the packing (usually 20–40 μm diameter particle size) in the wells of a 96-well flow-through plate.

Solute A general term for the compounds or ions being chromatographed.

Solvent The liquid used to dissolve the sample for application to the layer. Sometimes used to refer to the *mobile phase* or to the liquid used to elute chromatographed zones from scraped layer material.

Solvent front The farthest point of movement of the mobile phase during development.

Solvent strength (ϵ^0) A measure of the polarity of a solvent for liquid—solid adsorption chromatography. It is based on the free energy of adsorption onto a standard surface. Values for common solvents range from 0.00 (pentane) to 0.95 (methanol).

Sorbent The layer material used in TLC.

Sorption A general term for the attraction between a layer and a solute, without specification of the type of physical mechanism (i.e., adsorption, partition, ion exchange) or mixed mechanism involved. *Sorbent* is a related general term referring to the layer itself.

Spectroscopy An analytical technique based on the interaction of electromagnetic radiation with matter. Also called *spectrometry*.

Spot Used synonymously with *zone*, but usually meant to indicate a round or elliptical shape.

Spot capacity Same as *separation number*.

Stationary phase The solid sorbent layer, with or without any impregnation agent, preloaded vapor molecules, or immobilized mobile phase component(s).

Stepwise development Development using a mobile phase whose composition is changed using discontinuous, stepped gradients, in contrast to continuously variable gradient elution.

Straight-phase chromatography Another name for *normal-phase chromatography*.

Streak An initial zone in the shape of a narrow horizontal line at the origin.

Streaking See Tailing.

Supercritical fluid extraction (SFE) A technique for extracting analytes from sample matrices by using a dense gas. Carbon dioxide, which becomes a supercritical fluid when used above its critical pressure (1070 psi) and temperature (31°C), is the most widely applied extraction medium for SFE because it is nontoxic and nonflammable and facilitates extractions at low temperatures in a nonoxidizing environment.

Tailing Formation of a zone with an elongated rear portion, often leading to incomplete resolution.

Throughput A term used mostly in the context of “sample throughput,” which indicates the number of samples that can be analyzed by a particular method in a given period of time. TLC has high sample throughput because multiple samples can be applied to a single plate. “Solvent throughput” is a term that designates the amount of solvent passing through the layer. For example, there is higher solvent throughput with an unsaturated N-chamber than with a saturated N-chamber because of the greater amount of solvent that passes through the layer to replace the solvent that has evaporated from it.

TLC Thin-layer chromatography.

TLG Thin-layer gel chromatography, in which separations are based mainly on solute sizes.

Trailing See Tailing.

Two-dimensional development Successive development of a chromatogram with the same solvent or different solvents in directions at a 90° angle to each other.

Unsaturated The condition of a chamber that has the mobile phase and plate added together so that equilibration with the vapors is occurring during chromatographic development.

UV Ultraviolet.

Validation The process of determining the suitability of a given TLC method for an intended application, such as qualitative identification, assays, semiquantitative limit tests, or quantitative determination of impurities in pharmaceutical analysis. The characteristics tested can include accuracy, precision, specificity, detection limit, quantification limit, linearity, and robustness.

Visualization Detection of the zones on a chromatogram.

Zone The area of distribution on the layer containing the individual solutes or mixture before, during, or after chromatography. The initial zone is the applied sample prior to development. *Band*, *zone*, and *spot* are often used more or less interchangeably, but *spot* usually denotes a round zone, and *band* a flat, horizontally elongated zone.

Directory of Manufacturers and Suppliers of Plates, Equipment, and Instruments for Thin-Layer Chromatography

The following are useful general sources of information on instrumentation, accessories, chromatoplates, chemicals, and literature for TLC.

2003 Lab Guide. American Chemical Society, 1155 16th Street, N.W., Washington, DC 20036. Also is available online at <http://pubs.acs.org/labguide>

American Laboratory Buyers' Guide Edition, February 2002, Volume 37, Number 7, International Scientific Communications, Inc., 30 Controls Drive, P.O. Box 870, Shelton, CT 06484-0870. <http://www.iscpubs.com>

LCGC 2002-2003 Buyers' Guide. Volume 20, Number 8, LCGC, 131 West 1st Street, Duluth, MN 55802. <http://www.chromatographyonline.com>

2002 Directory, Laboratory Equipment, PO Box 7500, Highlands Ranch, CO 80163-7500. <http://www.laboratoryequipment.com>

SELECTED LIST OF TLC COMPANIES

Alltech Associates Inc., 2051 Waukegan Road, Deerfield, IL 60015-1899. 800-ALLTECH. <http://www.alltechweb.com>

Analtech Inc., 75 Blue Hen Drive, Newark, DE 19713. 800-441-7540. <http://www.thinlayer.com>

Bioscan, Inc., 4590 Macarthur Boulevard, N.W., Washington, DC 20007-4226. 800-255-7226. <http://www.bioscan.com>

Bodman Industries, P.O. Box 2421, Aston, PA 19014. 800-241-8774. <http://www.bodman.com> Camag Scientific Inc., 515 Cornelius Harnett Drive, Wilmington, NC 28401-2856. 800-334-3909. <http://www.camagusa.com>

Desaga GmbH, Postfach 1280, D-69153 Wiesloch, Germany. +49-6222-9288-0. e-mail: sales@desaga-gmbh.de

EM Science, 400 S. Democrat Road, Gibbstown, NJ 08027-1239. 800-222-0342. <http://www.emscience.com> (EM Science is an affiliate of Merck KGaA, Darmstadt, Germany.)

Mallinckrodt Baker, Inc. 222 Red School Lane, Phillipsburg, NY 08865-2200. 800JTBAKER. www.jtbaker.com

Macherey-Nagel, 6 South 3rd Street, Suite 402, Easton, PA 18042. 610-559-9848. <http://www.macherey-nagel.com>

Shimadzu Scientific Instruments, Inc., 7102 Riverwood Drive, Columbia, MD 21046–2502. 800–477–1227. <http://www.shimadzu.com>

Whatman Inc., 9 Bridewell Place, Clifton, NJ 07014–1725. 973–773–5800. <http://www.whatman.com>

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