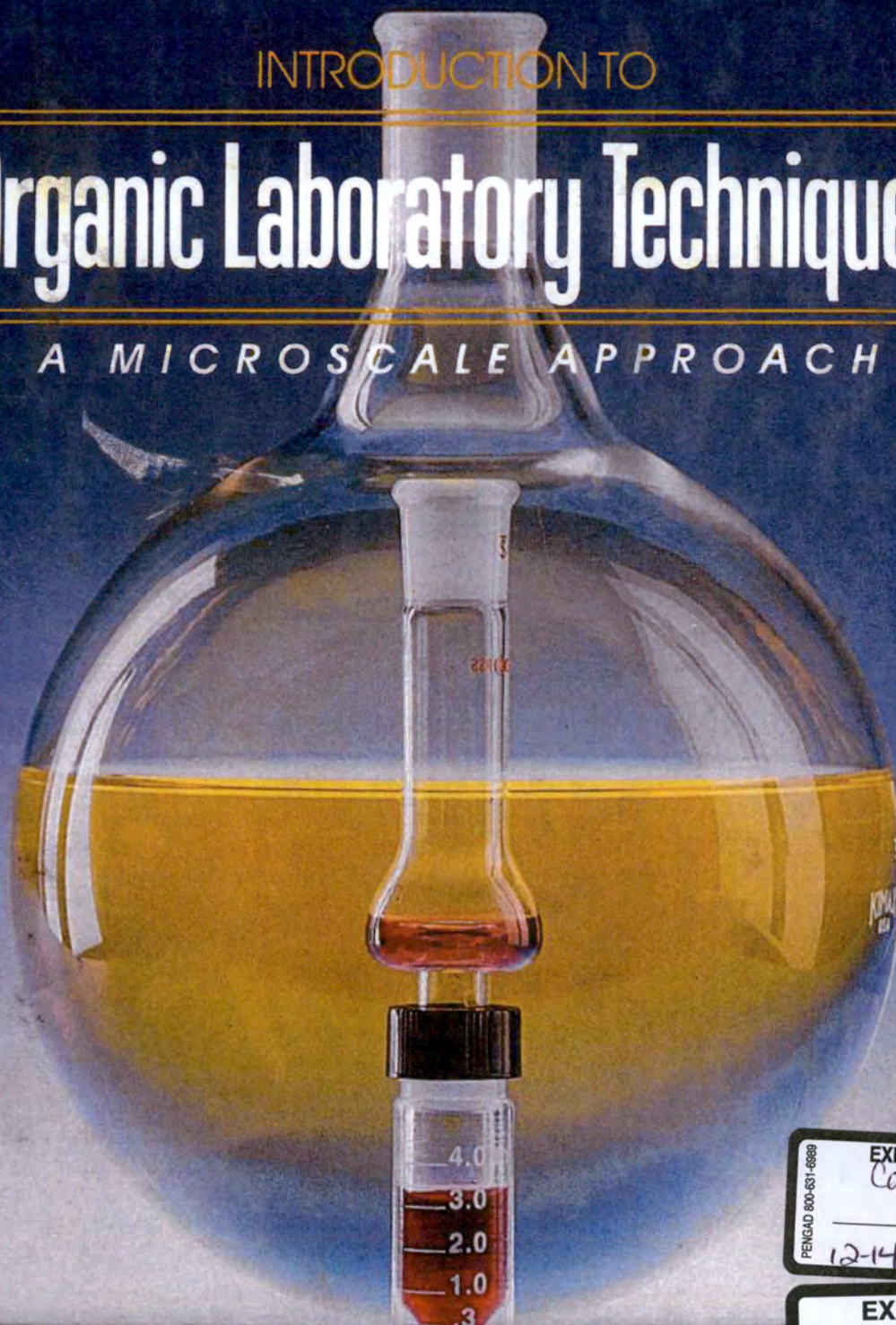


PAVIA • LAMPMAN • KRIZ • ENGEL

INTRODUCTION TO

Organic Laboratory Techniques

A MICROSCALE APPROACH



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2. Calculate the approximate boiling point of a mixture of bromobenzene and water at atmospheric pressure. A table of vapor pressures of water and bromobenzene at various temperatures is given.

TEMPERATURE (°C)	VAPOR PRESSURES (mmHg)	
	Water	Bromobenzene
93	588	110
94	611	114
95	634	118
96	657	122
97	682	127
98	707	131
99	733	136

3. Calculate the weight of nitrobenzene that codistills (bp 99 °C) with each gram of water during a steam distillation. You may need the data given in the previous problem.
4. A mixture of *p*-nitrophenol and *o*-nitrophenol can be separated by steam distillation. The *o*-nitrophenol is steam volatile while the *para* isomer is not volatile. Explain. Base your answer on the ability of the isomers to form hydrogen bonds internally.

Technique 12

COLUMN CHROMATOGRAPHY

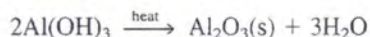
The most modern and sophisticated methods of separating mixtures that the organic chemist has available all involve **chromatography**. Chromatography is defined as the separation of a mixture of two or more different compounds or ions by distribution between two phases, one of which is stationary and the other moving. Various types of chromatography are possible, depending on the nature of the two phases involved: **solid-liquid** (column, thin-layer, and paper), **liquid-liquid**, (high performance liquid chromatography), and **gas-liquid** (vapor-phase) chromatographic methods are common.

All chromatography works on much the same principle as solvent extraction (Technique 7). Basically, the methods depend on differential solubilities or adsorptivities of the substances to be separated relative to the two phases between which they are to be partitioned. In this chapter, column chromatography, a solid-liquid method is considered. A section of this chapter is also devoted to a brief examination of high performance liquid chromatography. Thin-layer chromatography is examined in Technique 13; gas chromatography, a gas-liquid method, is discussed in Technique 14.

12.1 ADSORBENTS

Column chromatography is a technique based on both adsorptivity and solubility. It is a solid-liquid phase-partitioning technique. The solid may be almost any material that does not dissolve in the associated liquid phase; those solids most commonly used are silica gel, $\text{SiO}_2 \cdot x\text{H}_2\text{O}$, also called silicic acid, and alumina, $\text{Al}_2\text{O}_3 \cdot x\text{H}_2\text{O}$. These compounds are used in their powdered or finely ground (usually 200- to 400-mesh) forms.

Most alumina used for chromatography is prepared from the impure ore bauxite, $\text{Al}_2\text{O}_3 \cdot x\text{H}_2\text{O} + \text{Fe}_2\text{O}_3$. The bauxite is dissolved in hot sodium hydroxide and filtered to remove the insoluble iron oxides; the alumina in the ore forms the soluble amphoteric hydroxide $\text{Al}(\text{OH})_4^-$. The hydroxide is precipitated by CO_2 (which reduces the pH) as $\text{Al}(\text{OH})_3$. When heated, the $\text{Al}(\text{OH})_3$ loses water to form pure alumina, Al_2O_3 .



Alumina prepared in this way is called **basic alumina**, because it still contains some hydroxides. Basic alumina cannot be used for chromatography of compounds that are base-sensitive. Therefore, it is washed with acid to neutralize the base, giving **acid-washed alumina**. This material is unsatisfactory unless it has been washed with enough water to remove **all** the acid; on being so washed, it becomes the best chromatographic material, called **neutral alumina**. If a compound is acid-sensitive, either basic or neutral alumina must be used. One should be careful to ascertain what type of alumina is being used for chromatography. Silica gel is not available in any form other than that suitable for chromatography.

12.2 INTERACTIONS

If powdered or finely ground alumina (or silica gel) is added to a solution containing an organic compound, some of the organic compound will **adsorb** onto or adhere to the fine particles of alumina. Many kinds of intermolecular forces cause organic molecules to bind to alumina. These forces vary in strength according to their type. Non-polar compounds bind to the alumina using only van der Waals forces. These are weak forces, and nonpolar molecules do not bind strongly unless they have extremely high molecular weights. The most important interactions are those typical of polar organic compounds. Either these forces are of the dipole-dipole type or they involve some direct interaction (coordination, hydrogen-bonding, or salt formation). These types of interactions are illustrated in Figure 12-1, which for convenience shows only a portion of the alumina structure. Similar interactions occur with silica gel. The strengths of such interactions vary in the approximate order:

**Salt formation > coordination > hydrogen-bonding >
dipole-dipole > van der Waals**

Strength of interaction varies among compounds. For instance, a strongly basic amine would bind more strongly than a weakly basic one (by coordination). In fact, strong bases and strong acids often interact so strongly that they **dissolve** alumina to some extent. One can use the following rule of thumb:

The more polar the functional group, the stronger the bond to alumina (or silica gel).

A similar rule holds for solubility. Polar solvents dissolve polar compounds more effectively than nonpolar solvents; nonpolar compounds are dissolved best by nonpolar solvents. Thus, the extent to which any given solvent can wash an adsorbed compound from alumina depends almost directly on the relative polarity of the solvent. For example, while a ketone adsorbed on alumina might not be removed by hexane, it might be removed completely by chloroform. For any adsorbed material, a kind of **distribution** equilibrium can be envisioned between the adsorbent material and the solvent. This is illustrated in Figure 12-2.

The distribution equilibrium is **dynamic**, with molecules constantly **adsorbing** from the solution and **desorbing** into it. The average number of molecules remaining adsorbed on the solid particles at equilibrium depends both on the particular molecule (RX) involved and the dissolving power of the solvent with which the adsorbent must compete.

12.3 PRINCIPLE OF COLUMN CHROMATOGRAPHIC SEPARATION

The dynamic equilibrium mentioned above, and the variations in the extent to which different compounds adsorb on alumina or silica gel, underlie a versatile and ingenious

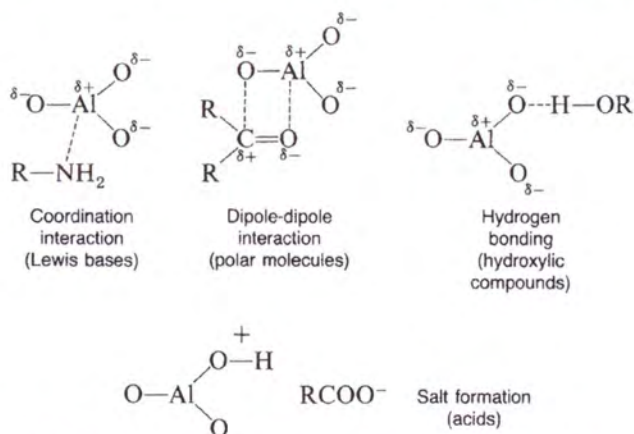


FIGURE 12-1. Possible interactions of organic compounds with alumina

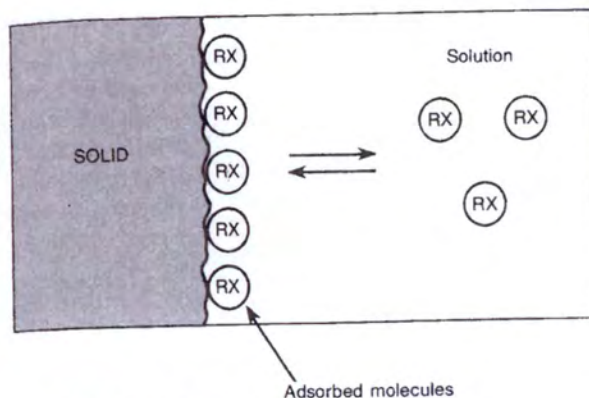


FIGURE 12-2. Dynamic adsorption equilibrium

method for **separating** mixtures of organic compounds. In this method, the mixture of compounds to be separated is introduced onto the top of a cylindrical glass column (Figure 12-3) **packed** or filled with fine alumina particles (stationary solid phase). The adsorbent is continuously washed by a flow of solvent (moving phase) passing through the column.

Initially, the components of the mixture adsorb onto the alumina particles at the top of the column. The continuous flow of solvent through the column **elutes**, or

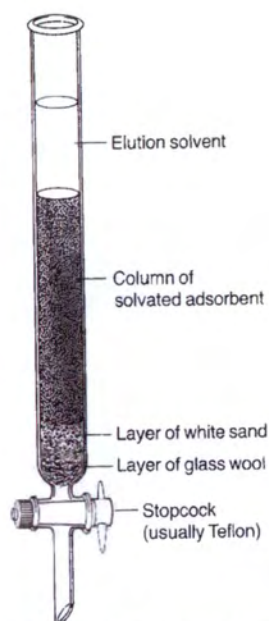


FIGURE 12-3. Chromatographic column

washes, the solutes off the alumina and sweeps them down the column. The solutes (or materials to be separated) are called **eluates** or **elutants**; and the solvents are called **eluent**s. As the solutes pass down the column to fresh alumina, new equilibria are established between the adsorbent, the solutes, and the solvent. The constant equilibration means that different compounds will move down the column at differing rates depending on their relative affinity for the adsorbent on one hand, and for the solvent on the other. Since the number of alumina particles is large, since they are closely packed, and since fresh solvent is being added continuously, the number of equilibrations between adsorbent and solvent that the solutes experience is enormous.

As the components of the mixture are separated, they begin to form moving bands (or zones), each band containing a single component. If the column is long enough and the various other parameters (column diameter, adsorbent, solvent, and flow rate) are correctly chosen, the bands separate from one another, leaving gaps of pure solvent in between. As each band (solvent and solute) passes out the bottom of the column, it can be collected before the next band arrives. If the parameters mentioned are poorly chosen, the various bands either overlap or coincide, in which case either a poor separation or no separation at all is the result. A successful chromatographic separation is illustrated in Figure 12-4.

12.4 PARAMETERS AFFECTING SEPARATION

The versatility of column chromatography results from the many factors that can be adjusted. These include:

1. Adsorbent chosen
2. Polarity of the column or solvents chosen
3. Size of the column (both length and diameter) relative to the amount of material to be chromatographed
4. Rate of elution (or flow)

By careful choice of conditions, almost any mixture can be separated. This technique has even been used to separate optical isomers. An optically active solid-phase adsorbent is used to separate the enantiomers.

Two fundamental choices for anyone attempting a chromatographic separation are the kind of adsorbent and the solvent system. In general, nonpolar compounds pass through the column faster than polar compounds since they have a smaller affinity for the adsorbent. If the adsorbent chosen binds all the solute molecules (both polar and nonpolar) strongly, they will not move down the column. On the other hand, if too polar a solvent is chosen, all the solutes (polar and nonpolar) may simply be washed through the column, with no separation taking place. The adsorbent and the solvent should be chosen so that neither is favored excessively in the equilibrium competition for solute molecules.¹

¹Often the chemist uses thin-layer chromatograph (TLC), which is described in Technique 13, to arrive at the best choices of solvents and adsorbents for the best separation. The TLC experimentation can be done quickly and with extremely small amounts (microgram quantities) of the mixture to be separated. This brings great savings of time and materials. Technique 13 describes this use of TLC.

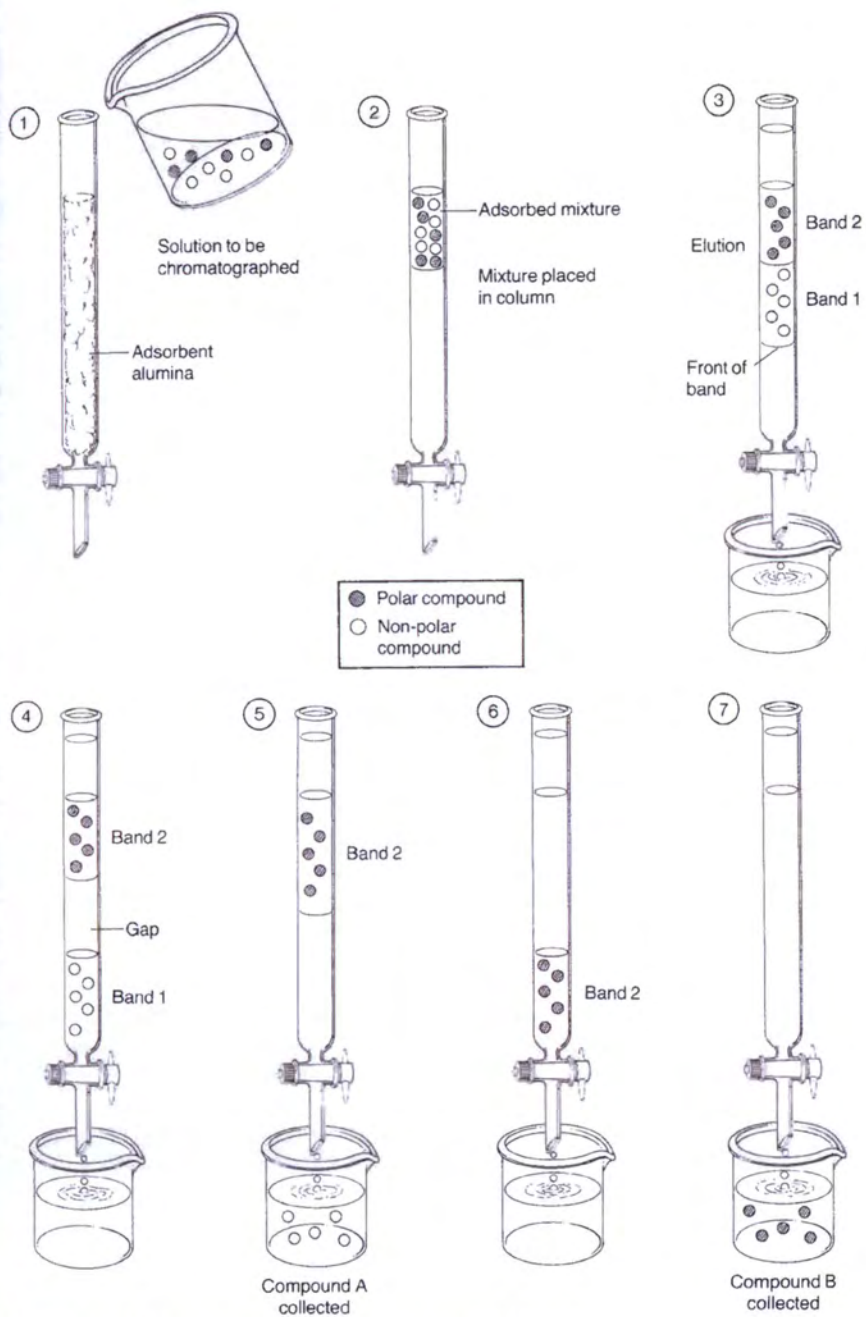


FIGURE 12-4. Sequence of steps in a chromatographic separation

A. Adsorbents

In Table 12-1 various kinds of adsorbents (solid phases) used in column chromatography are listed. The choice of adsorbent often depends on the types of compounds to be separated. Cellulose, starch, and sugars are used for polyfunctional plant and animal materials (natural products) very sensitive to acid-base interactions. Magnesium silicate is often used for separating acetylated sugars, steroids, and essential oils. Silica gel and Florisil are relatively mild toward most compounds and are widely used for a variety of functional groups—hydrocarbons, alcohols, ketones, esters, acids, azo compounds, amines. Alumina is the most widely used adsorbent and is obtained in the three forms mentioned in Section 12.1: acidic, basic, and neutral. The pH of acidic or acid-washed alumina is approximately 4. This adsorbent is particularly useful for separating acidic materials such as carboxylic acids and amino acids. Basic alumina has a pH of 10 and is useful in separating amines. Neutral alumina can be used to separate a variety of non-acidic and non-basic materials.

The approximate strength of the various adsorbents listed in Table 12-1 is also given. The order is only approximate, and therefore it may vary. For instance, the strength, or separating abilities, of alumina and silica gel largely depend on the amount of water present. Water binds very tightly to either adsorbent, taking up sites on the particles that could otherwise be used for equilibration with solute molecules. If one adds water to the adsorbent it is said to have been **deactivated**. Anhydrous alumina or silica gel are said to be highly **activated**. High activity is usually avoided with these adsorbents. Use of the highly active forms of either alumina or silica gel, or of the acidic or basic forms of alumina, can often lead to molecular rearrangement or decomposition in certain types of solute compounds.

The chemist can select the degree of activity which is appropriate to carry out a particular separation. To accomplish this, highly activated alumina is mixed thoroughly with a precisely measured quantity of water. The water partially hydrates the alumina and thus reduces its activity. By carefully determining the amount of water required, the chemist can have available an entire spectrum of possible activities.

TABLE 12-1. Solid Adsorbents for Column Chromatography

Paper	
Cellulose	
Starch	
Sugars	
Magnesium silicate	
Calcium sulfate	
Silicic acid	
Silica gel	
Florisil	
Magnesium oxide	
Aluminum oxide (Alumina)*	
Activated charcoal (Norit)	

Increasing strength of
binding interactions
toward polar compounds

*Basic, acid-washed, and neutral.

chromatography
grounds to be
and animal
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B. Solvents

In Table 12-2 some common chromatographic solvents are listed along with their relative ability to dissolve polar compounds. Sometimes a single solvent can be found that will separate all the components of a mixture. Sometimes a mixture of solvents can be found that will achieve separation. More often, one must start elution with a nonpolar solvent to remove relatively nonpolar compounds from the column and then gradually increase the solvent polarity to force compounds of greater polarity to come down the column, or elute. The approximate order in which various classes of compounds elute by this procedure is given in Table 12-3. In general, nonpolar compounds travel through the column faster (elute first), and polar compounds travel more slowly (elute last). However, molecular weight is also a factor in determining order of elution. A nonpolar compound of high molecular weight travels more slowly than a nonpolar compound of low molecular weight, and it may even be passed by some polar compounds.

TABLE 12-2. Solvents (Eluents) for Chromatography

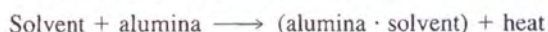
Petroleum ether	Increasing polarity and "solvent power" toward polar functional groups
Cyclohexane	
Carbon tetrachloride*	
Toluene	
Chloroform*	
Methylene chloride	
Diethyl ether	
Ethyl acetate	
Acetone	
Pyridine	
Ethanol	
Methanol	
Water	
Acetic acid	

*Suspected carcinogens.

TABLE 12-3. Elution Sequence for Compounds

Hydrocarbons	Fastest (will elute with nonpolar solvent)
Olefins	Order of elution
Ethers	
Halocarbons	
Aromatics	
Ketones	
Aldehydes	
Esters	
Alcohols	
Amines	
Acids, strong	
bases	Slowest (need a polar solvent)

When the polarity of the solvent has to be changed during a chromatographic separation, some precautions must be taken. Rapid changes from one solvent to another are to be avoided (especially when silica gel or alumina is involved). Usually, small percentages of a new solvent are mixed slowly into the one in use until the percentage reaches the desired level. If this is not done, the column packing often "cracks" as a result of the heat liberated when alumina or silica gel is mixed with a solvent. The solvent solvates the adsorbent, and the formation of a weak bond generates heat.



Often enough heat is generated locally to evaporate the solvent. The formation of vapor creates bubbles, which force a separation of the column packing; this is called **cracking**. A cracked column does not give a good separation since it has discontinuities in the **packing**. The way in which a column is packed or filled with adsorbent is also very important in preventing cracking.

That the solvent itself has a tendency to adsorb on the alumina is an important factor in how compounds move down the column. The solvent can displace the adsorbed compound if it is more polar than the compound, and hence can move it down the column. Thus, a more polar solvent not only dissolves more compound, but also is effective in removing the compound from the alumina since it displaces the compound from its site of adsorption.

Certain solvents should be avoided with alumina or silica gel, especially with the acidic, basic, and highly active forms. For instance, with any of these adsorbents, acetone dimerizes *via* an aldol condensation to give diacetone alcohol. Mixtures of esters transesterify (exchange their alcoholic portions) when ethyl acetate or an alcohol is the eluent. Finally, the most active solvents (pyridine, methanol, water, and acetic acid) dissolve and elute some of the adsorbent itself. Generally, try to avoid going to solvents more polar than diethyl ether or methylene chloride in the eluent series (Table 12-2).

C. Column Size and Adsorbent Quantity

The column size and the amount of adsorbent must also be selected correctly to separate a given amount of sample well. As a rule of thumb, the amount of adsorbent should be 25 to 30 times, by weight, the amount of material to be separated by chromatography. Furthermore, the column should have a height-to-diameter ratio of about 8:1. Some typical relations of this sort are given in Table 12-4.

TABLE 12-4. Size of Column and Amount of Adsorbent for Typical Sample Sizes

AMOUNT OF SAMPLE (g)	AMOUNT OF ADSORBENT (g)	COLUMN DIAMETER (mm)	COLUMN HEIGHT (mm)
0.01	0.3	3.5	30
0.10	3.0	7.5	60
1.00	30.0	16.0	130
10.00	300.0	35.0	280

Note, as a caution, that the difficulty of the separation is also a factor in determining the size and length of the column to be used and in the amount of adsorbent needed. Compounds that do not separate easily may require larger columns and more adsorbent than specified in Table 12-4. For easily separated compounds, a smaller column and less adsorbent may suffice.

D. Flow Rate

The rate at which solvent flows through the column also is significant in the effectiveness of a separation. In general, the time the mixture to be separated remains on the column is directly proportional to the extent of equilibration between stationary and moving phases. Thus, similar compounds eventually separate if they remain on the column long enough. The time a material remains on the column depends on the flow rate of the solvent. If the flow is too slow, however, the dissolved substances in the mixture may diffuse faster than the rate at which they move down the column. Then the bands grow wider and more diffuse, and the separation becomes poor.

12.5 PACKING THE COLUMN: TYPICAL PROBLEMS

The most critical operation in column chromatography is packing (filling) the column with adsorbent. The **column packing** must be evenly packed and free of irregularities, air bubbles, and gaps. As a compound travels down the column, it moves in an advancing zone, or **band**. It is important that the leading edge, or **front**, of this band be horizontal, or perpendicular to the long axis of the column. If two bands are close together and do not have horizontal band fronts, it is impossible to collect one band while completely excluding the other. The leading edge of the second band begins to elute before the first band has finished eluting. This condition can be seen in Figure 12-5. There are two main reasons for this problem. First, if the top surface edge of the adsorbent packing is not level, non-horizontal bands result. Second, bands may also be non-horizontal if the column is not held in an exactly vertical position in both planes (front-to-back and side-to-side). When you are preparing a column, both of these factors must be watched carefully.

Another phenomenon, called **streaming**, or **channeling**, occurs when part of the band front advances ahead of the major part of the band. Channeling occurs if there are any cracks or irregularities in the adsorbent surface or any irregularities caused by air bubbles in the packing. A part of the advancing front moves ahead of the rest of the band by flowing through the channel. Two examples of channeling are shown in Figure 12-6.

12.6 PACKING THE COLUMN: METHODS

The following methods are used to avoid problems resulting from uneven packing and column irregularities. These procedures should be followed carefully in preparing a

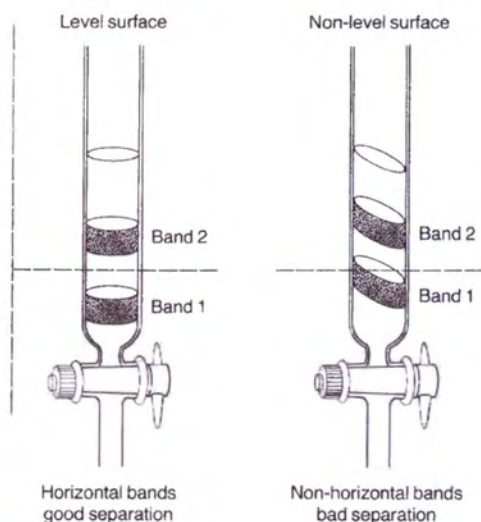


FIGURE 12-5. Comparison of horizontal and non-horizontal band fronts

chromatography column. Failure to pay close attention to the preparation of the column may well affect the quality of the separation.

Preparation of a column involves two distinct stages. In the first stage, a support base on which the packing will rest is prepared. This must be done so that the packing, a finely divided material, does not wash out the bottom of the column. In the second stage, the column of adsorbent is deposited on top of the supporting base. Several alternatives for the second process are detailed on pp 709-711.

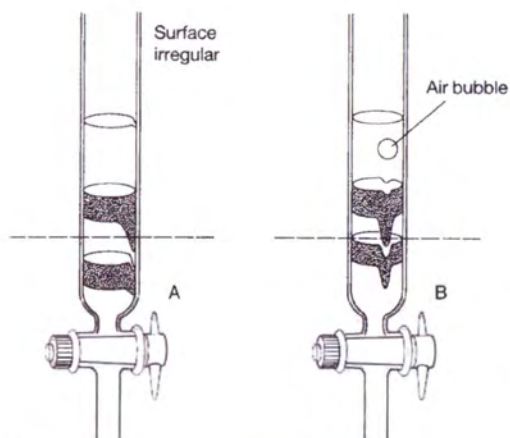


FIGURE 12-6. Channeling complications

A. Preparing the Support Base

Microscale

For microscale applications, select a Pasteur pipet (5 $\frac{3}{4}$ -inch) and clamp it upright in a vertical position. In order to reduce the amount of solvent which is needed to fill the column, it is convenient to break off most of the tip of the pipet. Place a small ball of cotton into the pipet and tamp it into position using a glass rod or a piece of wire. The correct position of the cotton is shown in Figure 12-7. A microscale chromatography column is packed by the "dry pack method," described in Part B of this section.

An alternative apparatus for microscale column chromatography is a commercial column, such as the one shown in Figure 12-8. This type of column is made of glass, and it has a solvent-resistant plastic stopcock at the bottom. The stopcock assembly contains a filter disc to support the adsorbent column. An optional upper fitting, also made of solvent-resistant plastic, serves as a solvent reservoir. The column shown in Figure 12-8 is equipped with the solvent reservoir. This type of column is available in a variety of lengths, ranging from 100 mm to 300 mm.

Standard Scale

For large-scale applications, clamp a chromatography column upright in a vertical position. The column (Figure 12-3) is a piece of cylindrical glass tubing with a stop-

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FIGURE 12-7. Microscale chromatography column

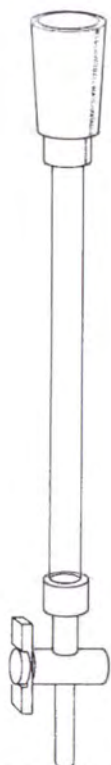


FIGURE 12-8. Commercial microscale chromatography column. (The column is shown equipped with an optional solvent reservoir.)

cock attached at one end. The stopcock usually has a Teflon plug since stopcock grease (used on glass plugs) dissolves in many of the organic solvents used as eluents. Stopcock grease in the eluent will contaminate the eluates.

Instead of a stopcock, a piece of flexible tubing may be attached to the bottom of the column, with a screw clamp used to stop or regulate the flow (Figure 12-9). When a screw clamp is used, care must be taken that the tubing used is not dissolved by the solvents that will pass through the column during the experiment. Rubber, for instance, dissolves in chloroform, benzene, methylene chloride, toluene, or tetrahydrofuran (THF). Tygon tubing dissolves (actually, the plasticizer is removed) in many different solvents, including benzene, methylene chloride, chloroform, ether, ethyl acetate, toluene, and THF. Polyethylene tubing is the best choice for use at the end of a column, since it is inert with most solvents.

Next, the column is partially filled with a quantity of solvent, usually a non-polar solvent like hexane, and a support for the finely divided adsorbent is prepared in the following way. A loose plug of glass wool is tamped down into the bottom of the column with a long glass rod until all entrapped air is forced out as bubbles. Take care



FIGURE 12-9. Tubing with screw clamp to regulate solvent flow on a chromatography column

not to plug the column totally by tamping the glass wool too hard. A small layer of clean white sand is formed on top of the glass wool by pouring sand into the column. The column is tapped to level the surface of the sand. Any sand adhering to the side of the column is washed down with a small quantity of solvent. The sand forms a base that supports the column of adsorbent and prevents it from washing through the stopcock. The column is packed in one of two ways: by the "slurry method" or by the "dry pack method."

B. Depositing the Adsorbent

Slurry Method

The slurry method is not recommended as a microscale method for use with Pasteur pipets. On a very small scale, it is too difficult to pack the column with the slurry without losing the solvent before the packing has been completed. Microscale columns should be packed by the "dry pack" method.

In the slurry method, the adsorbent is packed into the column as a slurry. A **slurry** is a mixture of a solvent and an undissolved solid. The slurry is prepared in a separate container by adding the solid adsorbent, a little at a time, to a quantity of the solvent. This order of addition (adsorbent to solvent) should be followed strictly since the adsorbent solvates and liberates heat. If the solvent is added to the adsorbent, it may boil away almost as fast as it is added due to heat evolved. This will be especially true if ether or another low-boiling solvent is used. When this happens, the final mixture will be uneven and lumpy. Enough adsorbent is added to the solvent, with swirling, to form a thick, but flowing, slurry. The slurry should be swirled until it is homogeneous and relatively free of entrapped air bubbles.

For a standard-sized column, the procedure is as follows. When the slurry has been prepared, the column is filled about half-full with solvent, and the stopcock is opened to allow solvent to drain slowly into a large beaker. The slurry is mixed by swirling and is then poured in portions into the top of the draining column (a wide-necked funnel may be useful here). Be sure to swirl the slurry thoroughly before each addition to the column. The column is tapped constantly and **gently** on the side, during the pouring operation, with the fingers or with a pencil fitted with a rubber stopper. A short piece of large-diameter pressure tubing may also be used for tapping. The tapping promotes even settling and mixing and gives an evenly packed column free of air bubbles. Tapping is continued until all the material has settled, showing a well-defined level at the top of the column. Solvent from the collecting beaker may be re-added to the slurry if it becomes too thick to be poured into the column at one time. In fact, the collected solvent should be cycled through the column several times to ensure that settling is complete and that the column is firmly packed. The downward flow of solvent tends to compact the adsorbent. One should take care never to let the column run dry during packing.

Dry Pack Method 1

In the first of the dry pack methods introduced here, the column is filled with solvent and allowed to drain **slowly**. The dry adsorbent is added, a little at a time, while the column is tapped constantly, as described above.

Pasteur Pipet. To fill a microscale column, fill the Pasteur pipet (with the cotton plug and the layer of sand, prepared as described above) about half full with solvent. Using a microspatula, add the solid adsorbent slowly to the solvent in the column. As the solid is added, tap the column **gently** with a pencil, a finger, or a glass rod. The tapping promotes even settling and mixing and gives an evenly packed column free of air bubbles. As the adsorbent is being added, solvent will flow out of the Pasteur pipet. Since the adsorbent must not be allowed to dry during the packing process, a means of controlling the solvent flow must be utilized. If a piece of small-diameter plastic tubing is available, it can be fitted over the narrow tip of the Pasteur pipet. The flow rate can then be controlled using a screw clamp. A simple approach to controlling the flow rate is to use a finger over the top of the Pasteur pipet, much as one controls the flow of liquid in a volumetric pipet. Continue adding the adsorbent slowly, with constant tapping, until the level of the adsorbent has reached the desired level. As the column is being packed, be careful not to let the column run dry. The final column should appear as shown in Figure 12-7.

Commercial Microscale Column. The procedure to fill a commercial microscale column is essentially the same as that used to fill a Pasteur pipet. The commercial column has the advantage that it is much easier to control the flow of solvent from the column during the filling process because the stopcock can be adjusted appropriately. It is not necessary to use a cotton plug or to deposit a layer of sand before adding the adsorbent. The presence of the fritted disc at the base of the column acts to prevent adsorbent from escaping from the column.

is follows. When the slurry has the solvent, and the stopcock is open, the slurry is mixed by tapping the draining column (a wide-mouthed bottle) thoroughly before each addition and **gently** on the side, during the addition. The column is fitted with a rubber stopper. A beaker is used for tapping. The tapping is done on a newly packed column free of air bubbles, showing a well-defined meniscus. The solvent beaker may be re-added to the column at one time. In fact, the solvent is added several times to ensure that the column is packed. The downward flow of solvent is never to let the column

the column is filled with solvent and, a little at a time, while the

fill the Pasteur pipet (with the solvent above) about half full with solvent. It is slowly added to the solvent in the column with a pencil, a finger, or a glass rod. An evenly packed column, solvent will flow out of the column and dry during the packing process. If a piece of small-diameter glass is used for the narrow tip of the Pasteur pipet, a simple approach to the Pasteur pipet, much as one is adding the adsorbent slowly, is reached the desired level. As the column is run dry. The final column

procedure to fill a commercial microscale Pasteur pipet. The commercial Pasteur pipet can be adjusted appropriately. A layer of sand before adding solvent to the column acts to prevent

Macroscale Column. A plug of cotton is placed at the base of the column, and an even layer of sand is formed on top (see p 707). The column is filled about half full with solvent, and the solid adsorbent is added carefully from a beaker, while the solvent is allowed to flow slowly from the column. As the solid is added, the column is tapped as described for the "slurry method" in order to ensure that the column is packed evenly. When the column has the desired length, no more adsorbent is added. This method also gives an evenly packed column. For the same reasons as those already described, solvent should be cycled through this column (for macroscale applications) several times before each use. The same portion of solvent that has drained from the column during the packing is used to cycle through the column.

Dry Pack Method 2

Pasteur Pipet. An alternative dry pack method for microscale columns is to fill the Pasteur pipet with **dry** adsorbent, without any solvent. A plug of cotton and a thin layer of sand are positioned in the bottom of the Pasteur pipet. The desired amount of adsorbent is added slowly, with constant tapping, until the level of adsorbent has reached the desired height. Figure 12-7 can be used as a guide in order to judge the correct height of the column of adsorbent. When the column is packed, solvent is allowed to percolate through the adsorbent until the entire column is moistened. The solvent is not added until just before the column is to be used.

This method is not recommended for use with silica gel, nor for experiments where a very careful separation is required.

This method is useful when the adsorbent is alumina, but it does not give satisfactory results with silica gel. Even with alumina, poor separations can arise owing to uneven packing, air bubbles, and cracking, especially if a solvent that has a highly exothermic heat of solvation is used.

Commercial Microscale Column. The method used is similar to that described for Pasteur pipets, except that the plug of cotton and the layer of sand are not required. The flow rate of solvent through the column can be controlled using the stopcock which is part of the column assembly (see Figure 12-8).

Macroscale Column. Macroscale columns can also be packed by a dry pack method which is similar to the microscale methods described above. The disadvantages described for the microscale method also apply to the macroscale method. This method is not recommended for use with silica gel or alumina since the combination leads to uneven packing, air bubbles, and cracking, especially if a solvent that has a highly exothermic heat of solvation is used.

12.7 APPLYING THE SAMPLE TO THE COLUMN

The solvent (or solvent mixture) used to pack the column is normally the least polar elution solvent one intends to use during the chromatography. The compounds to be

chromatographed will not be highly soluble in the solvent. If they were, they would probably have a greater affinity for the solvent than for the adsorbent, and would pass right through the column without equilibrating with the stationary phase.

The first elution solvent, however, is generally not a good solvent to use in preparing the sample to be placed on the column. Since the compounds are not highly soluble in nonpolar solvent, it would take a large amount of the initial solvent to dissolve the compounds, and it would be difficult to get the mixture to form a narrow band on top of the column. A narrow band is ideal for an optimum separation of components. For the best separation, therefore, the compound is applied to the top of the column undiluted if it is a liquid, or in a **very small** amount of highly polar solvent if it is a solid. Water must not be used to dissolve the initial sample being chromatographed, as it reacts with the column packing.

In adding the sample to the column, the following procedure should be used. Lower the solvent level to the top of the adsorbent column by draining the solvent from the column. Add the sample (either a pure liquid or a solution) to form a small layer on top of the adsorbent. A Pasteur pipet is convenient for adding the sample to the column. Take care not to disturb the surface of the adsorbent. This is done best by touching the pipet to the inside of the glass column and slowly draining it so as to allow the sample to spread into a thin film, which slowly descends to cover the entire adsorbent surface. Drain the pipet close to the surface of the adsorbent. When all the sample has been added, drain this small layer of liquid into the column until the top surface of the column **just begins** to dry. Then add a small layer of the chromatographic solvent carefully with a Pasteur pipet, again with care not to disturb the surface. Drain this small layer of solvent into the column until the top surface of the column just dries. Add another small layer of fresh solvent, if necessary, and repeat the process until it is clear that the sample is strongly adsorbed on the top of the column. If the sample is colored and the fresh layer of solvent acquires some of this color, the sample has not been properly adsorbed. Once the sample has been properly applied, the level surface of the adsorbent may be protected by carefully filling the top of the column with solvent and sprinkling clean, white sand into the column so as to form a small protective layer on top of the adsorbent. For microscale applications, this layer of sand is not required.

Separations are often better if the sample is allowed to stand a short time on the column before elution. This allows a true equilibrium to be established. In columns that stand for too long, however, the adsorbent often compacts or even swells, and the flow can become annoyingly slow. Diffusion of the sample to widen the bands also becomes a problem if a column is allowed to stand over an extended period. For small-scale chromatography, using Pasteur pipets, there is no stopcock, and it is not possible to stop the flow. In this case it is not considered necessary to allow the column to stand.

12.8 ELUTION TECHNIQUES

Solvents for analytical and preparative chromatography should be pure reagents. Commercial-grade solvents often contain small amounts of residue, which remain when the solvent is evaporated. For normal work, and for relatively easy separations that take

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only small amounts of solvent, the residue usually presents few problems. For large-scale work, commercial-grade solvents may have to be redistilled before use. This is especially true for hydrocarbon solvents, which tend to have more residue than other solvent types. Most of the experiments in this laboratory manual have been designed to avoid this particular problem.

The need for pure solvents is especially acute with high performance liquid chromatography (Section 12.16). The narrow bore of the column and very small particle size of the column packing require that solvents be particularly pure and free of insoluble residue. In most cases, the solvents must be filtered through ultra-fine filters and they must be **degassed** (have dissolved gases removed) before they can be used.

One usually begins elution of the products with a nonpolar solvent, like hexane or petroleum ether. The polarity of the elution solvent can be increased gradually by adding successively greater percentages of either ether or toluene (for instance, 1%, 2%, 5%, 10%, 15%, 25%, 50%, 100%) or some other solvent of greater solvent power (polarity) than hexane. The transition from one solvent to another should not be too rapid in most solvent changes. If the two solvents to be changed differ greatly in their heats of solvation in binding to the adsorbent, enough heat can be generated to crack the column. Ether is especially troublesome in this respect, as it has both a low boiling point and a relatively high heat of solvation. Most organic compounds can be separated on silica gel or alumina using hexane-ether or hexane-toluene combinations for elution, and following these by pure methylene chloride. Solvents of greater polarity are usually avoided for the various reasons mentioned above. In microscale work, the usual procedure is to use only one solvent for the chromatography. Changing solvent polarity during the experiment is relatively rare.

The flow of solvent through the column should not be too rapid, or the solutes will not have time to equilibrate with the adsorbent as they pass down the column. If the rate of flow is too low, or stopped for a period, diffusion can become a problem—the solute band will diffuse, or spread out, in all directions. In either of these cases, separation will be poor. As a general rule (and only an approximate one), most macroscale columns are run with flow rates ranging from 5 to 50 drops of effluent per minute; a steady flow of solvent is usually avoided. Microscale columns made from Pasteur pipets do not have a means of controlling the solvent flow rate, but commercial microscale columns are equipped with stopcocks. The solvent flow rate in this type of column can be adjusted in a manner similar to that used with larger columns. To avoid diffusion of the bands, do not stop the column and do not set it aside overnight.

12.9 RESERVOIRS

For a microscale chromatography, the portion of the Pasteur pipet above the adsorbent is used as a reservoir of solvent. Fresh solvent, as needed, is added by means of another Pasteur pipet. When it is necessary to change solvent, the new solvent is also added in this manner. In some cases, the chromatography may proceed too slowly; the rate of solvent flow can be accelerated by attaching a rubber dropper bulb to the top of the Pasteur pipet column and squeezing **gently**. The additional air pressure forces the

solvent through the column more rapidly. If this technique is used, however, care must be taken to remove the rubber bulb from the column before releasing it. Otherwise, air may be drawn up through the bottom of the column, destroying the column packing.

When large quantities of solvent are used in a chromatographic separation, it is often convenient to use a solvent reservoir to forestall having to add small portions of fresh solvent continually. The simplest type of reservoir, a feature of many columns, is created by fusing the top of the column to a round-bottom flask (Figure 12-10A). If the column has a standard taper joint at its top, a reservoir can be created by joining a standard-taper separatory funnel to the column (Figure 12-10B). In this arrangement, the stopcock is left open, and no stopper is placed in the top of the separatory funnel. A third common arrangement is shown in Figure 12-10C. A separatory funnel is filled with solvent; its stopper is wetted with solvent and put **firmly** in place. The funnel is inserted into the empty filling space at the top of the chromatographic column, and the stopcock is opened. Solvent flows out of the funnel, filling the space at the top of the column until the solvent level is well above the outlet of the separatory funnel. As

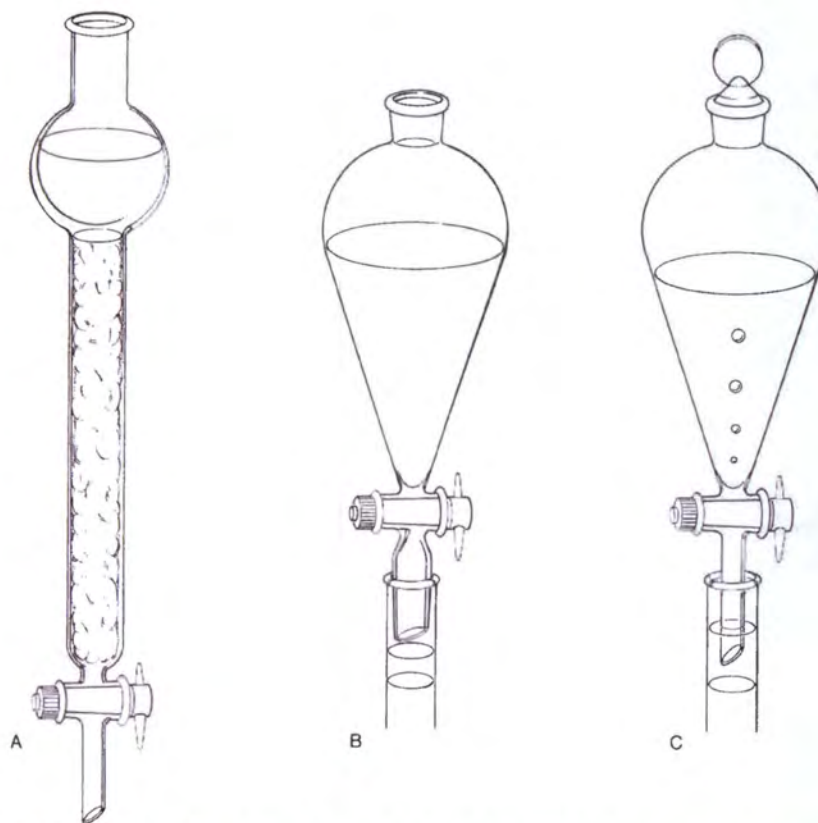


FIGURE 12-10. Various types of solvent-reservoir arrangements for chromatographic columns

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solvent drains from the column, this arrangement automatically refills the space at the top of the column by allowing air to enter through the stem of the separatory funnel. Some microscale columns, such as that shown in Figure 12-8, are equipped with a solvent reservoir which fits onto the top of the column. It functions just like the reservoirs described above.

12.10 MONITORING THE COLUMN

It is a happy instance when the compounds to be separated are colored. The separation can then be followed visually and the various bands collected separately as they elute from the column. For the majority of organic compounds, however, this lucky circumstance does not exist, and other methods must be used to determine the positions of the bands. The most common method of following a separation of colorless compounds is to collect **fractions** of constant volume in preweighed flasks, to evaporate the solvent from each fraction, and to reweigh the flask plus any residue. A plot of fraction number *versus* the weight of the residues after evaporation of solvent gives a plot like that in Figure 12-11. Clearly, Fractions 2 through 7 (Peak 1) may be combined as a single compound, and so can fractions 8 through 11 (Peak 2) and 12 through 15 (Peak 3). The size of the fractions collected (1 mL, 10 mL, 100 mL, or 500 mL) depends on the size of the column and the ease of separation.

Another common method of monitoring the column is to mix an inorganic phosphor into the adsorbent used to pack the column. When the column is illuminated with an ultraviolet light, the adsorbent treated in this way fluoresces. However, many solutes have the ability to **quench** the fluorescence of the indicator phosphor. In areas in which solutes are present, the adsorbent does not fluoresce, and a dark band is visible. In this type of column, the separation can also be followed visually.

Thin-layer chromatography is often used to monitor a column. This method is described in Technique 13 (Section 13.10, p 734). Several sophisticated instrumental

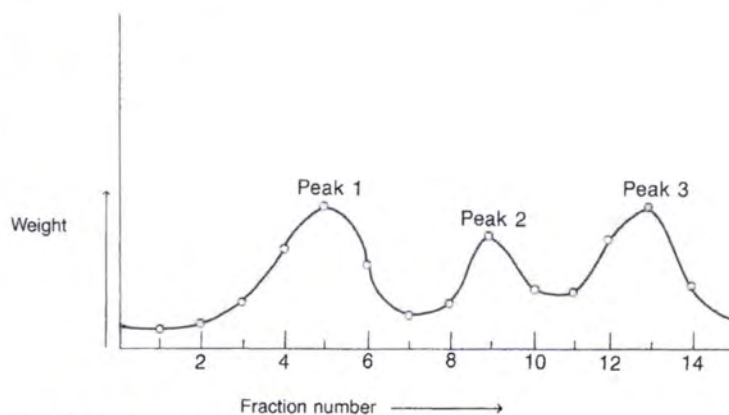


FIGURE 12-11. Typical elution graph

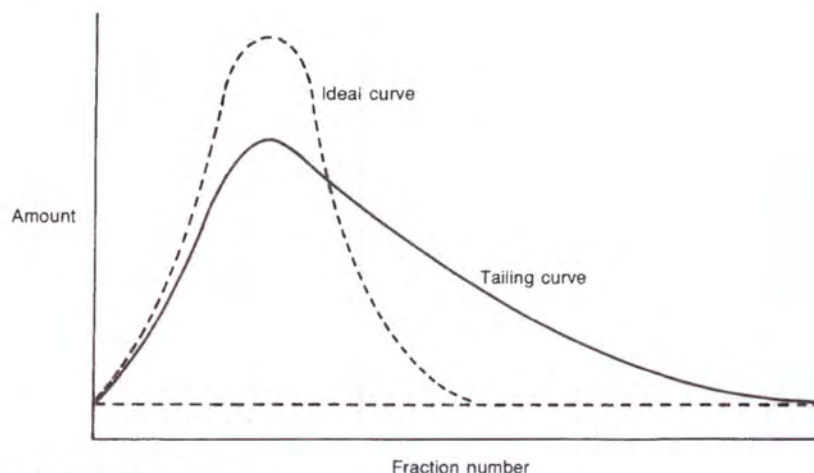


FIGURE 12-12. Elution curves: One ideal and one that "tails"

and spectroscopic methods, which we shall not detail, can also monitor a chromatographic separation.

12.11 TAILING

Often when a single solvent is used for elution, an elution curve (weight-vs.-fraction) like that shown as a solid line in Figure 12-12 is observed. An ideal elution curve is shown by dashed lines. In the non-ideal curve, the compound is said to be **tailing**. Tailing can interfere with the beginning of a curve or a peak of a second component and lead to a poor separation. One way to avoid this is to increase the polarity of the solvent constantly while eluting. In this way, at the tail of the peak, where the solvent polarity is increasing, the compound will move slightly faster than at the front and allow the tail to squeeze forward, forming a more nearly ideal band.

12.12 RECOVERING THE SEPARATED COMPOUNDS

In recovering each of the separated compounds of a chromatographic separation when they are solids, the various correct fractions are combined and evaporated. If the combined fractions contain sufficient material, they may be purified by recrystallization. If the compounds are liquids, the correct fractions are combined, and the solvent is evaporated. If sufficient material has been collected, liquid samples can be purified by distillation. The combination of chromatography-crystallization or chromatography-distillation usually yields very pure compounds. For microscale applications, the amount of sample collected is too small to allow a purification by crystallization or

distillation. The samples which are obtained after the solvent has been evaporated are considered to be sufficiently pure, and no additional purification is attempted.

12.13 DECOLORIZATION BY COLUMN CHROMATOGRAPHY

A common outcome of organic reactions is the formation of a product which is contaminated by highly-colored impurities. Very often these impurities are highly-polar, and they have a high molecular weight, as well as being colored. The purification of the desired product requires that these impurities be removed. Section 5.6 of Technique 5 (pp 589–591) details methods of decolorizing an organic product. In most cases, these methods involve the use of a form of activated charcoal, or Norit.

An alternative, which is applied conveniently in microscale experiments, is to remove the colored impurity by column chromatography. Owing to the polarity of the impurities, the colored components are strongly adsorbed on the stationary phase of the column, while the less-polar desired product passes through the column and is collected.

Microscale decolorization of a solution on a chromatography column requires that a column be prepared in a Pasteur pipet, using either alumina or silica gel as the adsorbent (Section 12.6). The sample to be decolorized is diluted to the point where crystallization within the column will not take place, and it is then passed through the column in the usual manner. The desired compound is collected as it exits the column, and the excess solvent is removed by evaporation (Technique 3, Section 3.9, p 560).

12.14 GEL CHROMATOGRAPHY

The stationary phase in gel chromatography consists of a cross-linked polymeric material. Molecules are separated according to their **size** by their ability to penetrate a sievelike structure. Molecules permeate the porous stationary phase as they move down the column. Small molecules penetrate the porous structure more easily than large ones. Thus, the large molecules move through the column faster than the smaller ones and elute first. The separation of molecules by gel chromatography is depicted in Figure 12–13. With adsorption chromatography using materials such as alumina or silica, the order is usually the reverse: Small molecules (of low molecular weight) pass through the column **faster** than large molecules (of high molecular weight) because large molecules are more strongly attracted to the polar stationary phase.

Equivalent terms used by chemists for the gel-chromatography technique are **gel filtration** (biochemistry term), **gel-permeation chromatography** (polymer chemistry term), and **molecular sieve chromatography**. **Size-exclusion chromatography** is a general term for the technique, and it is perhaps the most descriptive term for what occurs on a molecular level.

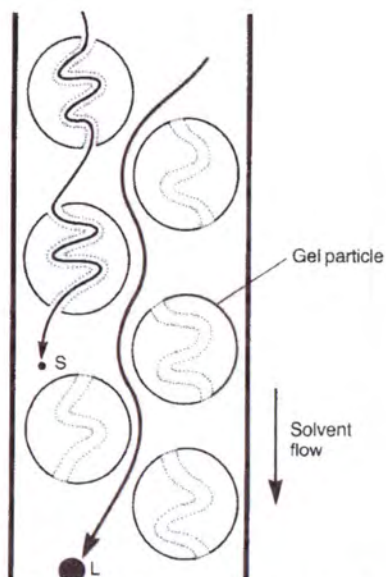
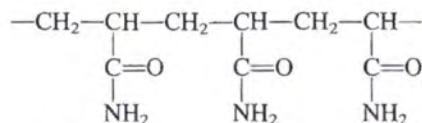


FIGURE 12-13. Gel chromatography: Comparison of the paths of large (L) and small (S) molecules through the column during the same interval of time.

Sephadex is one of the most popular materials for gel chromatography. It is widely used by biochemists for separating proteins, nucleic acids, enzymes, and carbohydrates. Most often water or aqueous solutions of buffers are used as the moving phase. Chemically, Sephadex is a polymeric carbohydrate that has been cross-linked. The degree of cross-linking determines the size of the "holes" in the polymer matrix. In addition, the hydroxyl groups on the polymer can adsorb water, which causes the material to swell. As it expands, "holes" are created in the matrix. Several different gels are available from manufacturers, each with its own set of characteristics. For example, a typical Sephadex gel, such as G-75, can separate molecules in the molecular-weight (MW) range 3,000 to 70,000. Assume for the moment that one has a four-component mixture containing compounds with molecular weights of 10,000, 20,000, 50,000, and 100,000. The 100,000-MW compound would pass through the column first, because it cannot penetrate the polymer matrix. The 50,000-, 20,000-, and 10,000-MW compounds penetrate the matrix to varying degrees and would be separated. The molecules would elute in the order given (decreasing order of molecular weights). The gel separates on the basis of molecular size and configuration, rather than molecular weight.

Sephadex LH-20 has been developed for non-aqueous solvents. Some of the hydroxyl groups have been alkylated, and thus the material can swell under both aqueous and non-aqueous conditions (it now has "organic" character). This material can be used with several organic solvents, such as alcohol, acetone, methylene chloride, and aromatic hydrocarbons.

Another type of gel is based on a polyacrylamide structure (Bio-Gel P and Poly-Sep AA). A portion of a polyacrylamide chain is shown below.



Gels of this type can also be used in water and some polar organic solvents. They tend to be more stable than Sephadex, especially under acidic conditions. Polyacrylamides can be used for many biochemical applications involving macromolecules. For separating synthetic polymers, cross-linked polystyrene beads (copolymer of styrene and divinylbenzene) find common application. Again the beads are swollen before use. Common organic solvents can be used to elute the polymers. As with other gels, the higher-molecular-weight compounds elute before the lower-molecular-weight compounds.

12.15 DRY-COLUMN CHROMATOGRAPHY

In dry-column chromatography, the entire column is packed dry without any solvent. The most common column for this method consists of a piece of flexible nylon tubing that has been sealed at one end (stapled). After some glass wool has been inserted into the bottom of the tubing, the bottom is perforated in several places so that air can be expelled. The column is then filled in portions with adsorbent, usually alumina. After each addition, the adsorbent is compacted by dropping the tubing repeatedly on a hard surface.

Instead of applying the sample as described in Section 12.7, the material to be separated is dissolved in a volatile solvent and mixed with the adsorbent. The solvent is removed from this adsorbent by evaporation. The resulting dry powder is introduced into the packed column. Solvent is allowed to percolate down the column slowly until the solvent front is about one centimeter from the end of the column. Only a single pure solvent or solvent mixture is used to perform the separation in dry-column chromatography.

In dry-column chromatography, in contrast to elution chromatography (Section 12.8), neither the solvent nor the separated materials actually leave the column. At the end of the chromatography, the plastic tubing is cut in segments to recover the separated compounds. Each portion of adsorbent together with the pure compound is swirled with a volatile solvent to remove the compound from the adsorbent. Once the adsorbent is removed by filtration, the solvent is evaporated to yield the purified compound. With colored compounds, one can easily decide where to cut the tubing. With colorless materials, an ultraviolet light (Technique 13, Section 13.7, p 732) may be used to detect compounds before slicing the tubing. Alternatively, one may use a new technique, in which the tubing is punctured at 1-cm intervals with glass pipets. By this technique, a small amount of adsorbent is removed with the pipet and analyzed by thin-layer chromatography, as in the method described in Technique 13, Section 13.10,

p 734). Using these analyses, one can decide where the column should be cut for the best separation of components.

There is a basic similarity between dry-column chromatography and thin-layer chromatography (TLC), described in Technique 13. The separation is virtually identical to that which is obtained with TLC. One can expect the same results from both methods so long as the same adsorbent and the same solvent are used in each case. Thus, the R_f value (Technique 13, Section 13.9, p 733) obtained by TLC correlates well with the value from the dry-column chromatographic method. The main advantage of this method over conventional TLC is that much larger samples can be separated.

12.16 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

If the column packing used in column chromatography is made more dense by using an adsorbent that has a smaller particle size, the separation that can be achieved is greater. The solute molecules encounter a much larger surface area on which they can be adsorbed as they pass through the column packing. At the same time, the solvent spaces between the particles are reduced in size. As a result of this tight packing, equilibrium between the liquid and solid phases can be established very rapidly with a fairly short column, and the degree of separation is markedly improved. The disadvantage of making the column packing more dense is that the solvent flow rate becomes very slow or even stops. Gravity is not strong enough to pull the solvent through a tightly packed column.

A recently developed technique can be applied to obtain much better separations with tightly packed columns. A pump is used to force the solvent through the column packing. As a result, solvent flow rate is increased, while the advantage of better separation is retained. This technique, called **high performance liquid chromatography (HPLC)**, is becoming widely applied to problems where separations by ordinary column chromatography are unsatisfactory. Because the pump often provides pressures in excess of 1000 pounds per square inch (psi), this method is also known as **high pressure liquid chromatography**. High pressures are not required, however, and satisfactory separations can be achieved with pressures as low as 100 psi.

The basic design of an HPLC instrument is shown in Figure 12-14. The instrument contains the following essential components:

1. Solvent reservoir
2. Solvent filter and degasser
3. Pump
4. Pressure gauge
5. Sample injection system
6. Column
7. Detector
8. Amplifier and electronic controls
9. Chart recorder.

There may be other variations on this simple design. Some instruments have heated ovens in order to maintain the column at a specified temperature, fraction collectors,

and microprocessor-controlled data-handling systems. Additional filters for the solvent and sample may also be included. One may find it interesting to compare this schematic diagram with that shown in Technique 14 (Figure 14-2, p 740) for a gas chromatography instrument. Many of the essential components are common to both types of instruments.

The chromatography column is generally packed with silica or alumina adsorbents. Unlike column chromatography, however, the adsorbents used for HPLC have a much smaller particle size. Typically, particle size ranges from 5 to 20 micrometers in diameter for HPLC, while it is on the order of 100 micrometers for column chromatography. The actual column may be constructed of heavy glass or even stainless steel. A strong column is required to withstand the high pressures that may be used.

A flow-through **detector** must be provided to determine when a substance has passed through the column. In most applications, the detector either detects the change in index of refraction of the liquid as its composition changes, or it detects the presence of solute by its absorption of ultraviolet light. The signal generated by the detector is amplified and treated electronically in a manner similar to that found in gas chromatographs (Technique 14, Section 14.6, p 744).

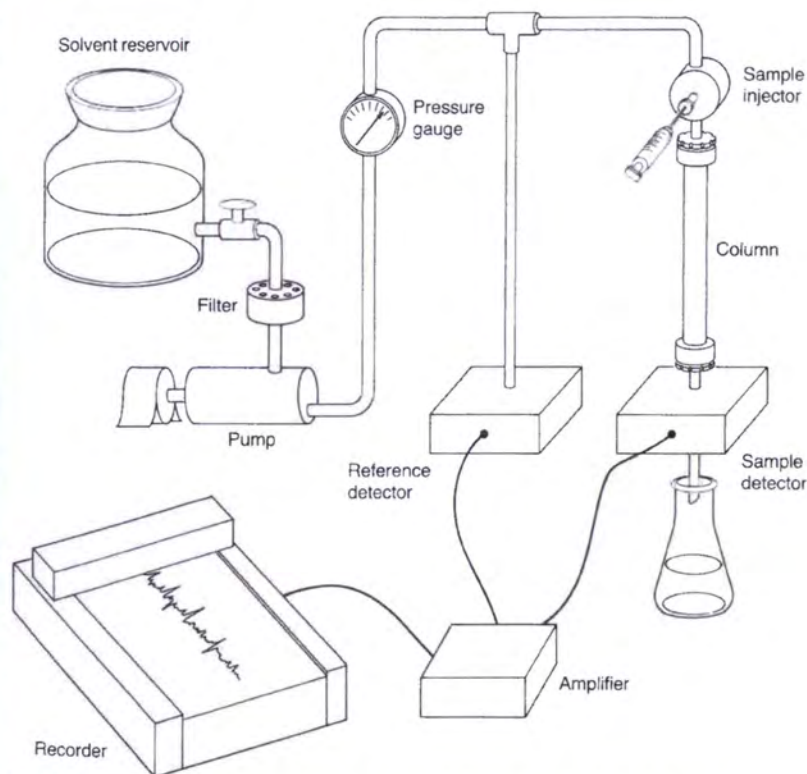


FIGURE 12-14. Schematic diagram of a high performance liquid chromatograph

A variation unique to HPLC is to use a nonpolar column packing with a polar solvent. This is called **reversed-phase chromatography**. In this method, the silica column packing is treated with alkylating agents. As a result, nonpolar alkyl groups are bonded to the silica surface, making the adsorbent nonpolar. When a polar solvent is used, polar solutes are more strongly attracted to the mobile (solvent) phase than they are to the stationary phase (adsorbent). The order of elution, therefore, is reversed, with polar solutes passing through the column faster than nonpolar solutes. In many applications, better separations can be achieved with reversed-phase chromatography than with polar adsorbents.

If a single solvent (or solvent mixture) is used for the entire separation, the chromatogram is said to be **isocratic**. Special electronic units are available, which will allow one to program changes in the solvent composition from the beginning to the end of the chromatography. These are called **gradient elution systems**. With gradient elution, the time required for a separation may be shortened considerably.

High performance liquid chromatography is an excellent analytical technique, but the separated compounds may also be isolated. The technique can be used for preparative experiments. Just as in column chromatography, the fractions can be collected into individual receiving containers as they pass through the column. The solvents can be evaporated from these fractions, allowing one to isolate separated components of the original mixture.

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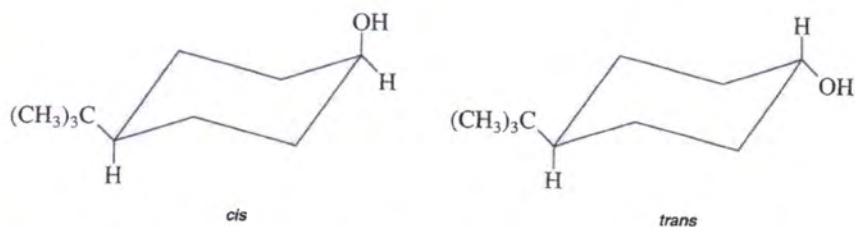
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PROBLEMS

1. A sample was placed on a chromatography column. Methylene chloride was used as the eluting solvent. No separation of the components in the sample was observed. What must have been happening during this experiment? How would you change the experiment in order to overcome this problem?
2. You are about to purify an impure sample of naphthalene by column chromatography. What solvent should you use to elute the sample?
3. Consider a sample which is a mixture composed of biphenyl, benzoic acid, and benzyl alcohol. Predict the order of elution of the components in this mixture. Assume that the chromatography uses a silica column and the solvent system is based on cyclohexane, with an increasing proportion of methylene chloride being added as a function of time.
4. An orange compound was added to the top of a chromatography column. Solvent was added immediately, with the result that the entire volume of solvent in the solvent reservoir turned orange. No separation could be obtained from the chromatography experiment. What went wrong?
5. A yellow compound, dissolved in methylene chloride, is added to a chromatography column. The elution is begun using petroleum ether as the solvent. After six liters of solvent had passed through the

column, the yellow band still had not traveled down the column appreciably. What should be done to make this experiment work better?

6. You have 0.50 g of a mixture which you wish to purify by column chromatography. How much adsorbent should you use to pack the column? Estimate the appropriate column diameter and height.
7. In a particular sample, you wish to collect the component with the **highest** molecular weight as the **first** fraction. What chromatographic technique should you use?
8. A colored band shows an excessive amount of tailing as it passes through the column. What can you do to rectify this problem?
9. The two isomeric 4-*tert*-butylcyclohexanols, shown below, are present in a mixture. All efforts to separate these isomers by column chromatography have failed. Describe a possible method to separate the isomers.



10. How would you monitor the progress of a column chromatography when the sample is colorless? Describe at least two methods.

Technique 13

THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography (TLC) is a very important technique for the rapid separation and qualitative analysis of small amounts of material. It is ideally suited for the analysis of mixtures and reaction products in microscale experiments. The technique is closely related to column chromatography. In fact, TLC can be considered simply column chromatography **in reverse**, with the solvent ascending the adsorbent, rather than descending. Because of this close relationship to column chromatography, and because the principles governing the two techniques are similar, Technique 12, on column chromatography, should be read first.