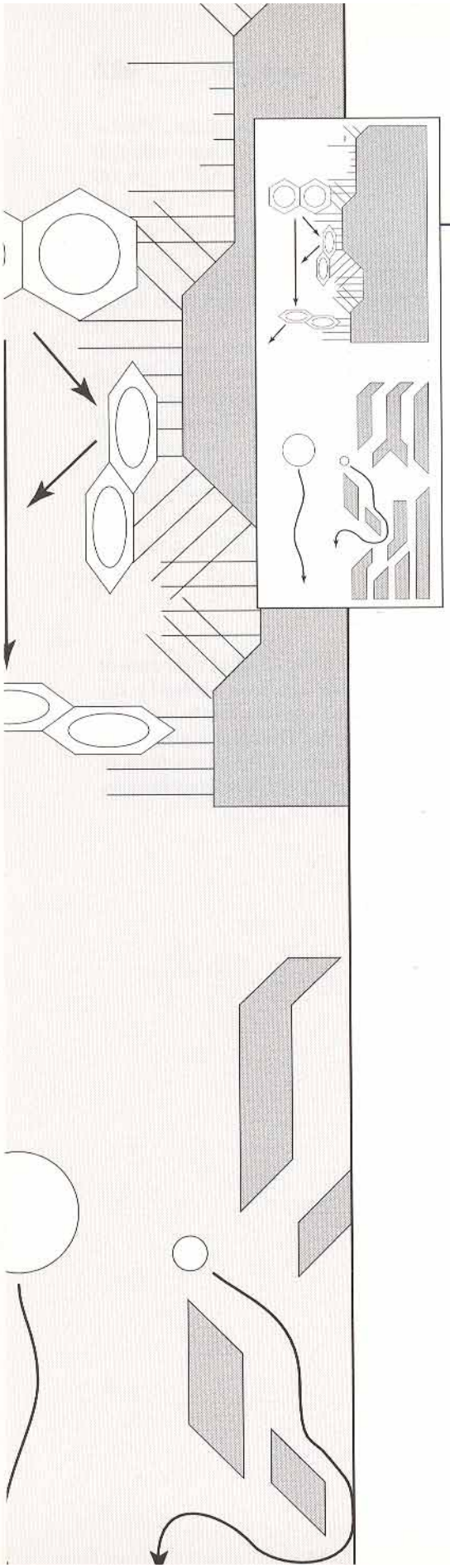


CHAPTER 14

Liquid Chromatography

Overview

14.1 Types of Liquid Chromatography

As its name implies, liquid chromatography (LC) has a liquid mobile phase. The great power of liquid chromatography resides in the combination of a wide range of possible mobile-phase properties together with the choice of numerous, significantly different kinds of stationary phases and a wide variety of detectors.

As a result, liquid chromatography really refers to a myriad of combinations, and many of them are classified with more than one name. The names focus on many different aspects of LC methods. For instance, one of the primary classification schemes of LC is by the overall physical shape of the stationary phase, such as *column* chromatography (like that done by Tswett and described at the beginning of Chapter 13), *thin-layer* chromatography (a layer on a plate), and *capillary* liquid chromatography (a layer on the inner surface of the capillary). Other names arise based on the direction of flow of the mobile phase: *ascending* chromatography, *descending* chromatography, and *flat-bed* chromatography. Classification is also based on the efficiency of the separations, such as *high-performance* liquid chromatography or *high-performance* thin-layer chromatography. Sometimes names of LC methods identify the solutes that are separated and detected, such as *ion* chromatography and *amino acid* analysis (both usually done on columns).

The types of liquid chromatography also are named after the general type of interaction that occurs between the stationary phase and the solutes in the eluent. The four basic types of interactions are shown in Figure 14.1. The classifications are denoted **normal-phase**, **reversed-phase**, **ion-exchange**, and **gel-filtration** (also called *exclusion* or *gel-permeation*) chromatographies. Some stationary phases are designed to interact with specific chemical groups. Chromatography with such site-specific groups on the stationary phase is called **affinity chromatography**. The stationary phase also may be made with **chiral** groups linked to it.

It is imperative to understand that no LC separation occurs by means of only one type of interaction between the analytes and the stationary phase. There are always interactions besides the named, predominant, type. So if you read "the separation was done by normal-phase chromatography," it means that polar adsorption was the *predominant* mechanism of interaction with the solid. However, there also was some contribution to the separation from at least one of the other mechanisms discussed here, such as ion exchange or gel filtration.

It is useful to be aware of all these special names. However, the underlying principles of all these methods are those you read about in Chapter 13. That is, doing effective liquid chromatography is an exercise in manipulating all the equilibria that affect the reaction

$$\text{analyte (mobile phase)} \rightleftharpoons \text{analyte (stationary phase)}$$

Any change in the solvent results in a shift of the equilibrium between the mobile and stationary phases. Chromatographic separations can be improved by switching from an **isocratic** elution (same eluent throughout) to a **gradient** elution. This is done by mixing two or more different eluents such that the mobile-phase composition changes over time. The gradient always changes the mobile phase toward better desorbing eluents.

Table 14.1 Solvent Strength Parameter e° for Alumina Supports: The Eluotropic Series[a]

In alphabetical order		In numerical order low to high	
Solvent	e°	**Solvent**	e°
Acetic acid	1.0	Pentane	0.00
Acetone	0.56	Petroleum ether	0.01
Acetonitrile	0.65	Hexane	0.01
Benzene	0.32	Cyclohexane	0.04
Carbon tetrachloride	0.18	Carbon tetrachloride	0.18
Chlorobenzene	0.30	Xylene	0.26
Chloroform	0.40	Toluene	0.29
Cyclohexane	0.04	Chlorobenzene	0.30
Dimethylsulfoxide	0.62	Benzene	0.32
Dioxane	0.56	Ethyl ether	0.38
Ethyl acetate	0.58	Chloroform	0.40
Ethylene dichloride	0.49	Methylene chloride	0.42
Ethyl ether	0.38	Tetrahydrofuran	0.45
Hexane	0.01	Ethylene dichloride	0.49
iso-Propanol	0.82	Methylethylketone	0.51
Methanol	0.95	Dioxane	0.56
Methylene chloride	0.42	Acetone	0.56
Methylethylketone	0.51	Ethyl acetate	0.58
Pentane	0.00	Dimethylsulfoxide	0.62
Petroleum ether	0.01	Acetonitrile	0.65
n-Propanol	0.82	Pyridine	0.71
Pyridine	0.71	*iso*-Propanol	0.82
Tetrahydrofuran	0.45	*n*-Propanol	0.82
Toluene	0.29	Methanol	0.95
Water	Large	Acetic acid	1.0
Xylene	0.26	Water	Large

[a] e° is the adsorption energy of the mobile phase per unit area of standard adsorbent surface.

Table 14.2 Representative Bonded-Phase Groups Used for Separations

Type	Application
Iminodiacetic acid–Ni^{2+}	Proteins, polypeptides
Nitrilotriacetate–Cu^{2+}	Proteins, polypeptides
Cyclodextrins (cyclic oligo D-glucopyranose)	Racemate separations
Bovine serum albumin (a protein)	Chiral separations
Amino acids (Cu^{2+} in mobile phase)	Chiral separations
Ferrocenylpropyl amine	Chiral separations

the surface can anchor carbon compounds onto the surface. Some of the groups that have usefully been bonded to silica surfaces for normal-phase separations are listed in Table 14.2. These packings with covalently linked surface groups are called **bonded phases**.

14.3 Reversed-Phase Liquid Chromatography

In reversed-phase chromatography, the stationary phase is less polar than the mobile phase. Two fundamental types of stationary phases are used, the most common being nonpolar groups bonded onto silica. Of these, the most often used are the organic groups $—CH_3$, $—C_8H_{17}$, and $—C_{18}H_{37}$. Of these the 18-carbon chain (the octadecyl group) is the most common. The abbreviations ODS and C18 are used for this type of stationary phase. These bonded organic groups have an effect similar to that which would be produced by an extremely thin organic solvent layer on the surface of the silica particles. Thus, the solutes partition between the surface coating and the mobile phase much like a liquid-liquid extraction. In addition, the longer the carbon chain length, the more "organic" these bonded layers become. As a result, the longer chains interact more strongly with solutes that prefer to dissolve in an organic phase. The effects of this difference are illustrated in Figure 14.3.

It turns out that binding of these long-chain phases over a surface is not complete; that is, some silica surface inevitably remains between the regions of hydrophobic surface. These remaining silica surfaces usually degrade the efficiency of separations by causing peak broadening or tailing. To make an improved reversed-phase surface, these areas are **end capped**. End capping means that, in a second reaction, a short organic group (usually $—CH_3$) is bound to the surface where the long-chain groups did not bind.

As described in Section 14.2, the silicon-oxygen bonds can hydrolyze. Of course, if the silica dissolves, any organic group bonded to it is lost from the surface. This leaves a normal-phase silica surface exposed. It follows that the retention indices such as k_i can change over time under the same experimental conditions as the surface changes. (The changes occur over hours to months depending on how rapidly the hydrolysis occurs.) In order to keep the separation as constant as possible, organic groups that bind strongly but not covalently to the silica surface can be added to the eluent. These organic reagents are retained on the open silica surface and, in effect, continuously regenerate an end cap. These materials are said to **deactivate** the silica. Examples of such **silanol suppressing additives** are amines such as triethylamine. A similar effect of suppressing the binding to the silica is achieved by raising the ionic strength. However, with high-quality end capping, these additives are not as necessary as they were in the earlier years of reversed-phase HPLC.

The second type of stationary phase used for reversed-phase chromatography is composed of organic polymer beads. A typical polymer is a resin composed of polystyrene and divinylbenzene. The divinylbenzene component forms bonds between the polystyrene polymer chains—**cross-links**. The cross-links cause a physical stiffening of the polymer; a greater density of cross-links is associated with a stiffer material. Stiffness is necessary to resist deforming under high pressure.

Reversed-phase chromatography is quite popular since the peaks in a reversed-phase separation tend to be narrow and symmetrical and the adsorption/desorption equilibrium reactions tend to be fast.

In contrast with the eluotropic series for stationary phases such as silica and alumina, less polar solvents are more powerful eluents in reversed-phase separations, as illustrated in Figure 14.4. In general, the reversed-phase eluting power is opposite the normal-phase order of Table 14.1. This reversed-phase eluting power is put on a numerical scale that is called the **polarity index**.

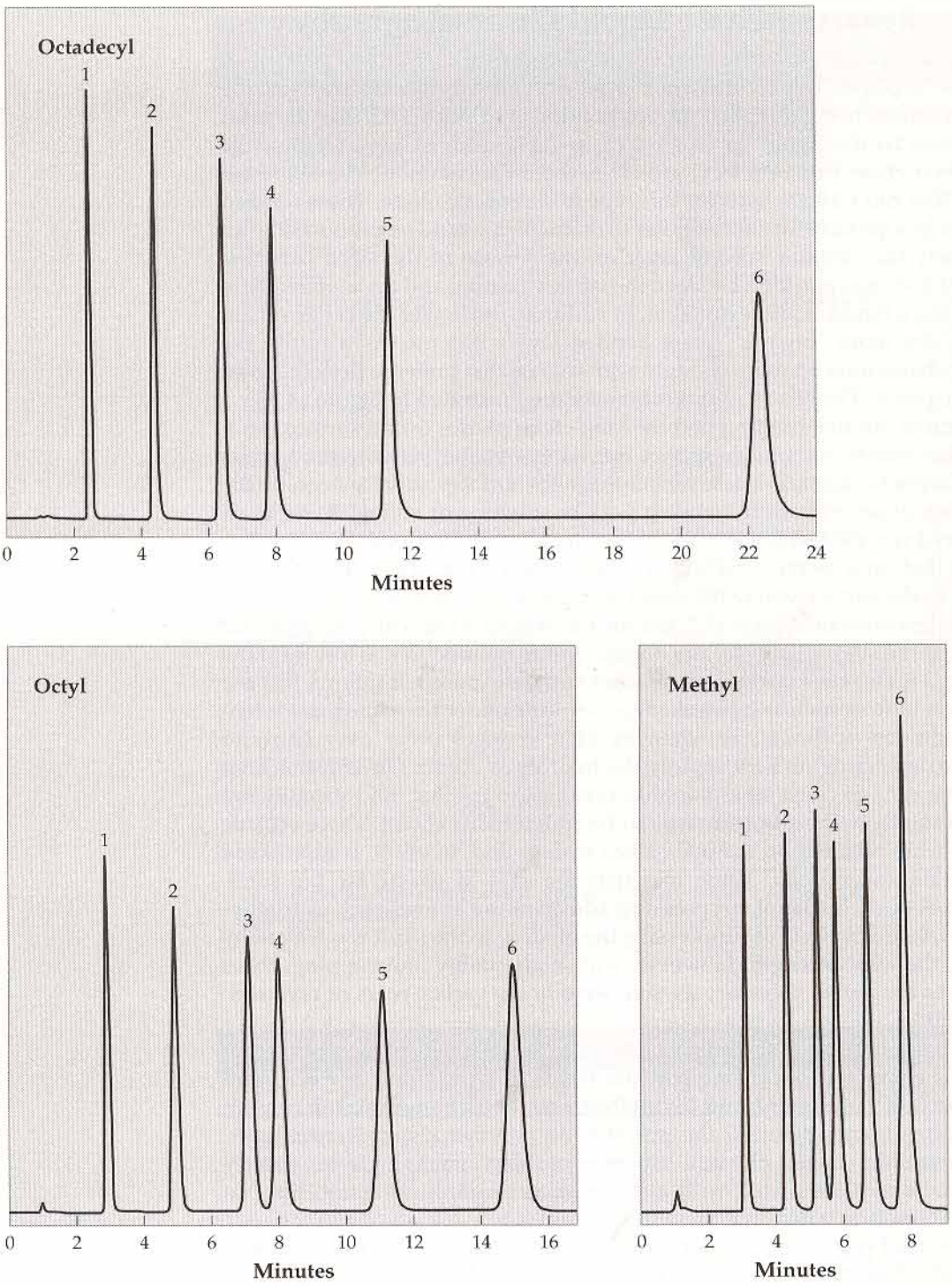


FIGURE 14.3 ▲

Comparison of reversed-phase chromatographic materials with different carbon chain lengths.

All three columns are the same except that the bonded phases have an 18 (octadecyl), 8 (octyl), or 1 (methyl) carbon chain bonded to the surface of silica. The elution times become progressively shorter with shorter carbon chains for the organic solutes listed below. In addition, their capacity factors change somewhat. Peak identification: (1) uracil; (2) phenol; (3) acetophenone; (4) nitrobenzene; (5) methyl benzoate; (6) toluene.

Experimental Conditions. Columns: IBM columns 4.5 × 250 mm. Mobile phase, 50/50 methanol/water isocratic elution; flow rate, 1.0 mL/min. Detection at 254 nm. [Figure courtesy of IBM Instruments.]

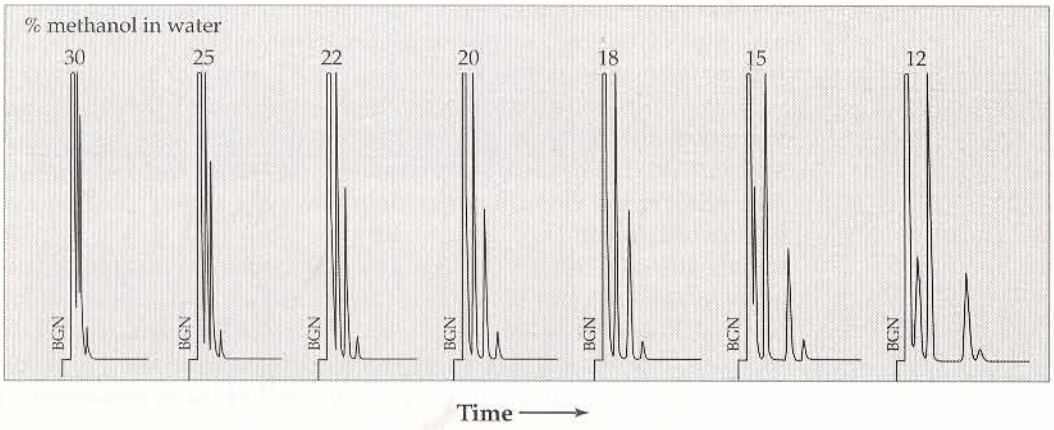


FIGURE 14.4 ▲
Illustration of the effects of changing solvent conditions on a reversed-phase column.

The sample is an analgesic preparation. Each run is isocratic, at the indicated percentage of methanol. The methanol is a stronger eluent than is the water.

Experimental Conditions. Columns: ODS column 0.26 × 25 cm. Eluent: methanol (% shown):water + 0.5% H_3PO_4; flow rate, 1.0 mL/min. Detection: ultraviolet light absorption at 250 nm. [Data courtesy of The Perkin-Elmer Corporation.]

In addition to the solvent polarity, the hydrophobicity of the solutes can be changed as illustrated by the differences in elution with pH shown in Figure 14.5. Not only do the retentions change with pH, but the order of retention does as well. As for any chromatographic separation, the retention times on an RP column depend on any factor that modifies the partitioning.

A comparatively new stationary phase is a form of graphite. The material is called **porous graphitic carbon** (PGC), which is essentially pure carbon, and the surfaces are molecularly flat. There are few non-carbon atoms, and those present are located at the edges of the large (on the molecular level) plates. PGC is less polar than the solvents, in general, and so falls into the class of reversed-phase supports. However, the most intriguing property of PGC is that the strength of the interaction between the PGC and solute molecules depends almost entirely on the molecular area that contacts the plate. As a result, large molecules tend to bind more tightly than small ones and flexible molecules more than rigid ones. Ionic charge does not affect the binding. And, finally, enantiomers are separated when a larger number of atoms of a structure can contact the surface in one enantiomeric form than another. This mixed mechanism of interaction offers an alternative to normal and bonded phases for certain classes of compounds.

Separation of enantiomers is discussed further in Section 14.6.

Reversed-phase chromatography evolved from an older type called **partition chromatography**. For partition chromatography, a solid support was coated with an organic solvent such as *n*-butanol, benzene, or chloroform, and the mobile phase was water and/or a polar organic solvent. Partition chromatography is, thus, truly a liquid-liquid extraction chromatography, but the unbonded, organic coating tends to wash off over time. You will see the name partition chromatography referring to the original form in the older literature, but now the term sometimes is used to refer to chromatography on C18 and similar phases.

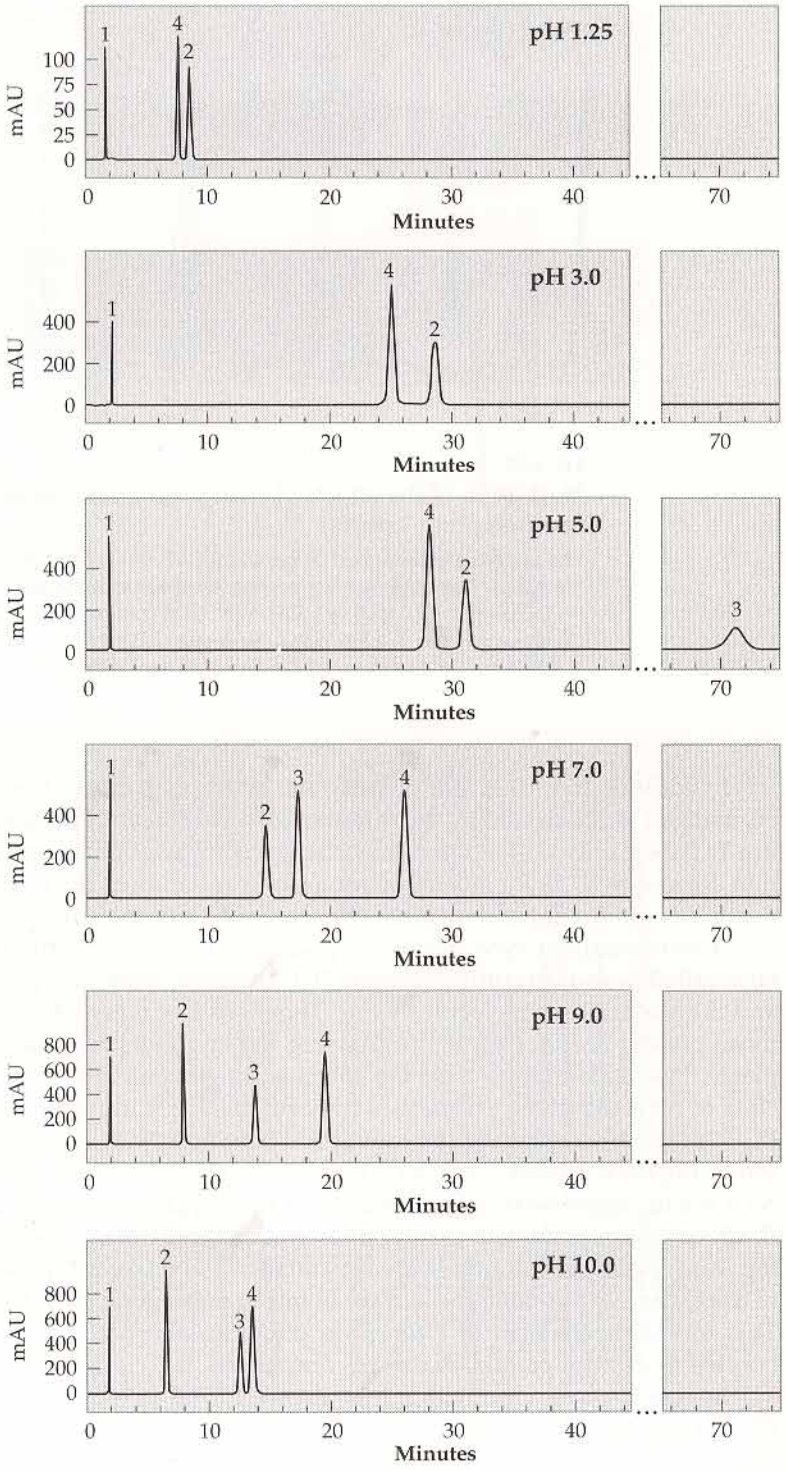


FIGURE 14.5 ▶
The effect of pH on a RP separation.

Here, the retentions of four sulfa drugs vary depending on the pH of the eluent. At pH 1.25 and 3.0, sulfisoxazole is unstable, and does not elute. Between pH 5.0 and 7.0 a change in order of elution occurs. The separation is excellent at pH 9.0, with a poorer separation at pH 10.0.

Experimental Conditions. Column: Phenomenex Luna™ 5 μm C18, 150 mm × 4.6 mm. Mobile Phase: 20 mM KH_2PO_4:acetonitrile 95:5 with pH as listed, flow rate 1.0 mL min^{-1}. Temperature: 40.0°C. Detector: UV at 270 nm. Sample: (1) Sulfanilic acid; (2) Sulfamerazine; (3) Sulfisoxazole; (4) Sulfapyridine. [Figure courtesy of Phenomenex, Torrance, CA, USA.]

14.4 Ion-Exchange Liquid Chromatography (Ion Chromatography)

In solution, one of the causes for two ions to bind is the attraction of unlike charges. As an example, we explain the strengths of acids by the magnitude of the negative charge at the site of proton binding. Highly charged sites tend

to hold the protons more strongly. In the same way, if, for example, positively charged groups are anchored to a stationary phase, then ions of the opposite charge will be attracted to them. These ionic interactions can be used to separate eluting species by differences in their average charge.

Stationary-phase materials with bound charges are called **ion-exchange resins**. A few are listed in Table 14.3. The reason they are called ion-*exchange* resins is because ionic media always contain equal numbers of positive and negative charges; that is, they are electrically neutral. As a result, the ions of the resin support are always associated with some ion of the opposite charge from the mobile phase. Thus, the ion initially associated with the site can leave only when it is exchanged with a new one, as illustrated in Figure 14.1c.

Positive ions (cations) exchange with stationary phases composed of cross-linked polymers with fixed negative charges. These are called **cation-exchange resins**. In analogy, polymers with covalently linked positive charges on them exchange negative ions (anions). These resins are called **anion-exchange resins**. Before an analysis, each resin is washed with a concentrated solution of an exchangeable ion. For example, an anion-exchange resin could be washed with a concentrated NaCl or KOH solution. Essentially all the sites would then be occupied by Cl^-, giving the *chloride form*, or OH^-, giving the *hydroxide form*. The process of washing in this way is called **regeneration**. Similarly, a cation-exchange resin could be regenerated with, for example, HCl or KNO_3 to produce the *hydrogen form* (*acid form*) or *potassium form* of the resin.

The ion exchange that occurs at a resin site is not quite as straightforward as it may sound since competitive chemical reactions occur simultaneously involving all the ionic species in the solution and on the resin. These ions include the bases and conjugate acids of the analytes as well as the bases and conjugate acids of the groups of the resin, and any buffers that may be present. Fortunately, we do not need to consider all the details of this complicated, simultaneous-equilibrium system, since the strengths of the various analytes' binding interactions with the stationary phase are proportional to the average charge of each acid-base pair involved. To the extent that we can control the fraction of each solute/analyte that is neutral, we can control the elution time for each. Because of the crucial dependence on pH, the mobile phase is almost inevitably buffered to a fixed and carefully chosen pH. Buffering also keeps the bands from spreading by eliminating variations in pH in different regions of the resin bed.

Table 14.3 Ion-Exchange Groups

Type	Active group	pH Range of operation	Application example
Strongly acidic cation exchanger	$-SO_3^-$	1–14	Amino acids, inorganic separations
Weakly acidic cation exchanger	$-COO^-$	5–14	Transition elements, organic bases
Strongly basic anion exchanger	For example, $-N^+(CH_3)_3$	1–12	Alkaloids, fatty acids
Weakly basic anion exchanger	For example, DEAE, $-C_2H_4N(C_2H_5)_2$	1–9	Organic acids, amino acids

As you can see from Table 14.3, the cation-exchange and anion-exchange resins are categorized into two types: strong and weak. The reasons for having four types is presented next.

What happens if the solute were, for example, NaCl, given that pH does not affect the charged fraction of Na^+ and Cl^- present? The way to control the retention of such ions is by changing the average charge on the stationary phase itself. In other words, the neutral fraction of the stationary-phase sites can be controlled. In the case of an anion exchanger, when the concentration of, for example, OH^- is made very high, it pairs with the positive charges of the resin and neutralizes its ionic charge. The ion-exchange properties of the resin are lost, and analyte anions are not retained. Intermediate levels of OH^- allow weak binding, and low OH^- allows the strongest binding. This property of the resins gives us significant control of the elution of salts since we can drive off strongly associated ions with enough H^+ or OH^- in the eluent. For such separations, the *weak* resins are used in order to allow convenient manipulation of the affinities.

However, having to control this extra equilibrium of the stationary phase may complicate the choice of conditions for ion exchange of acids and bases. The strong anion and strong cation exchangers have a wider pH range over which they *do not change* their ion exchange properties, so they are used for ion exchange of acids and bases. Only the analyte equilibria, then, are important. As you would suspect, the strong ion exchangers require higher concentrations of the wash reagents to regenerate them.

Ion Chromatography

At present, the primary analytical use of ion exchangers is in **ion chromatography**. With contemporary methods, a single separation experiment allows us to quantify numerous anions or cations down to the ppb level, as shown in Figure 14.6a. The band positions are used for identification, and the areas under the bands are used to quantify the amounts of each ion in the mix. Ion detection is done by measuring the electrical conductivity of the effluent. When the bands containing the ions pass through the detector, the conductivity rises. The general setup of the equipment for the determination and the reactions that occur in each part of the chromatograph are shown in Figure 14.6b.

A conductivity detector is described in Figure 14.25.

Ion chromatography in the form described involves the use of two ion-exchange processes. The first is the ion exchange on the column to separate the ions. The second eliminates background ions so that the analyte peaks can be measured by conductimetry. The limit of detection is, then, determined by the background conductivity, which is due to the ions produced by the solvent's dissociation alone. (The conductivity of water is significantly less than a solution containing any ions present at 1 μm.) This second process is called **suppression**. Background suppression involves simply neutralizing the hydroxide ions of the effluent to produce water.

The suppressor chemistry is indicated in the circle. Two different reactions occur here. The sodium ions are substituted by protons, and hydroxide ions are neutralized to water. As mentioned above, by neutralizing the hydroxide, the background conductivity is significantly reduced so that trace analysis can be done. By substituting protons for sodium, the conductivity of the solution per ion is raised about tenfold—proportional to their ratio of molar conductivities. This increase in conductance lowers the limit of detection and improves the sensitivity by the same factor. The two processes are carried out simultaneously in the following way.

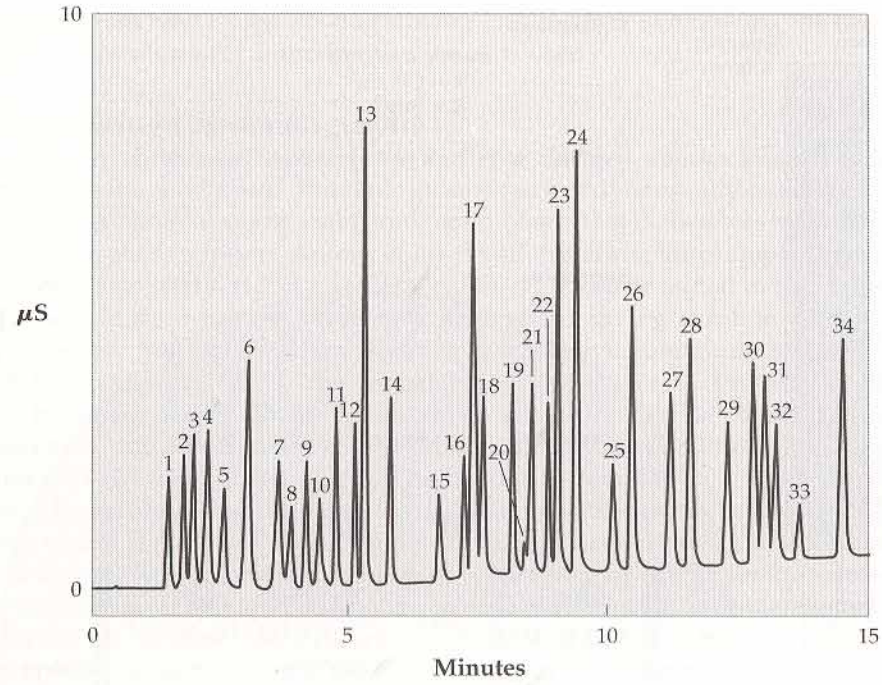


FIGURE 14.6(a) ▲
An ion chromatogram showing quantitation of 34 anions in fifteen minutes.

The ion chromatogram was run with a gradient program mixing three different eluents. The peak numbers, material identities, and their concentrations (in ppb w/v) in the injected sample are:

1. Isopropyl-methylphosphonate (5)
2. Quinate (5)
3. Fluoride (1)
4. Acetate (5)
5. Propionate (5)
6. Formate (5)
7. Methylsulfonate (5)
8. Pyruvate (5)
9. Chlorite (5)
10. Valerate (5)
11. Monochloroacetate (5)
12. Bromate (5)
13. Chloride (5)
14. Nitrite (5)
15. Trifloroacetate (5)
16. Bromide (3)
17. Nitrate (3)
18. Chlorate (3)
19. Selenite (5)
20. Carbonate (5)
21. Malonate (5)
22. Maleate (5)
23. Sulfate (5)
24. Oxalate (5)
25. Ketomalonate (10)
26. Tungstate (10)
27. Phthalate (10)
28. Phosphate (10)
29. Chromate (10)
30. Citrate (10)
31. Tricarballylate (10)
32. Isocitrate (10)
33. *cis*-Aconitate
34. *trans*-Aconitate

33 + 34 = (10)

Experimental Conditions. Column: Dionix IonPac™ AS11. Mobile phase: Eluent 1 = Deionized water; Eluent 2 = 5.0 mM NaOH; Eluent 3 = 100 mM NaOH. 2.0 mL min^{-1}. Gradient: 90% Eluent 1, 10% Eluent 2 for 2 minutes. Then, a linear gradient for three minutes to 0% Eluent 1, 100% Eluent 2. Finally, a linear gradient over 10 minutes to 65% Eluent 2, 35% Eluent 3. Detector: Conductivity on ion-suppressed output stream. [Chromatogram courtesy of Dionix Corp.]

On either side of the flow path are membranes that have covalently bound negative ion charges on them. The presence of these charges has the effect of making the membranes permeable only to positive ions and impermeable to negative ions. In the leftmost compartment an electrochemical anode oxidizes a flowing water solution to generate oxygen and protons.

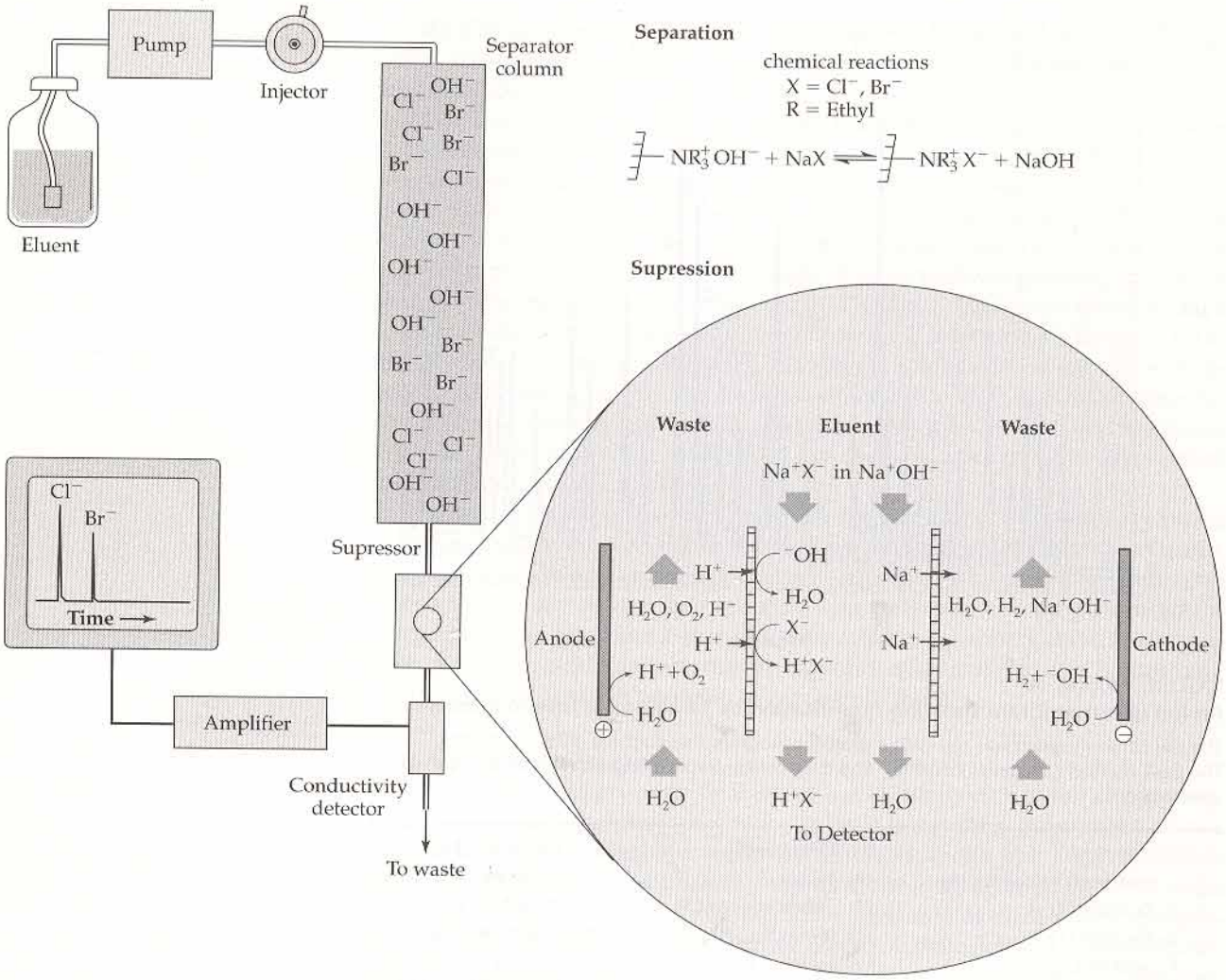


FIGURE 14.6(b) ▲
The mechanisms of ion chromatography.

Ion chromatography employs a standard HPLC apparatus and an ion-exchange column. For the anions used in this illustration, the separation is done using a quaternary ammonium resin (with $—NR_3^+$ groups). The eluent is aqueous sodium hydroxide solution. The reversible reaction shown in the figure determines the k_i for each ion. (If there were no OH^- in the eluent solution, the anions would not elute but merely bind with and remain on the resin sites with a simultaneous release of the ionically bound hydroxide that they displace.)

After many runs, the hydroxides of the chromatographic column will have been exchanged, leaving the resin predominantly in the multianion form. The resin must be **regenerated** periodically by passing a solution high in OH^- through the column, reversing the ion-exchange reaction and regenerating the hydroxy form.

The suppressor reduces the background by neutralizing the eluent OH^- as shown, and increases the signal by substituting higher mobility H^+ ions for Na^+. The suppression is followed by conductivity detection.

These protons pass through the membrane into the flowing eluent and neutralize the hydroxide to water (the upper reaction) and supply protons as counterions to the X^- ions (the lower reaction). At the same time, the sodium ions are passing through the membrane on the other side and into a basic solution that is generated by the reduction of water at a cathode. The sodium

ions are removed by the flowing stream. This method of removing the background ions is one of a number that are now used.

Ion-Pairing Chromatography

As noted above, ion-exchange resins have charged species permanently bound to the solid phase. But ionic groups can be "dynamically coated" onto *reversed-phase* columns by using ionic compounds that themselves bind to the reversed-phase support. Anions of alkyl sulfonic acids, for example, hexane sulfonic acid ($CH_3CH_2CH_2CH_2CH_2CH_2SO_3^-H^+$), can be added to the eluent. These coat the reversed-phase stationary phase, giving it cation-exchange properties. Similarly, cations such as tetrapropylammonium hydroxide, ($(CH_3CH_2CH_2)_4N^+OH^-$), can be added to the eluent to provide anion-exchange properties. These organic anions and cations are called **ion-pairing reagents**, and the separations carried out under these conditions are called **ion-pair chromatography** (IPC). The fraction of retardation of analytes can be adjusted by using different concentrations of the ion-pairing reagent in the eluent as well as changing the mobile phase's eluent power. These choices provide an added flexibility to separations involving ionic analytes. One problem is that long-chain ionic groups such as a C12-sulfonate may be difficult to remove from the reversed-phase columns. The ion-exchange properties remain as long as the ion-pairing reagents remain, and the column will not return to its original chemistry. On the other hand, a C6-sulfonate will readily wash off with, for instance, methanol. Ion-pairing chromatography is used to analyze ionic and highly polar, water-soluble compounds such as detergents and many drugs.

14.5 Size-Exclusion Liquid Chromatography

Size-exclusion chromatography (SEC) differs from the other three types since the molecules are not separated through differences in chemical interactions. The separation is made on the basis of the effective *sizes* of molecules. (Again, though, remember that no separation is accomplished due to a single, pure mechanism.) Other names you may find for size-exclusion are **gel-permeation chromatography** (GPC), **gel filtration**, and **exclusion chromatography**. Gel filtration is more commonly used when the mobile phase is water, and separations are of water-soluble molecules such as proteins and polypeptides. We shall simply refer to all of them as size-exclusion separations.

An example of a size-exclusion separation can be seen in Figure 14.7 for a wide molecular weight range of polyethylenes and alkane oligomers. Oligomers are molecules with sizes approximately in the range between tetramers and polymers.

The retention times of molecules follow an interesting behavior, which is illustrated in Figure 14.8. Plotted on the graph is the logarithm of the molecular weight *versus* the elution volume for a number of different neutral and ionic molecules. Notice that at low elution volumes the curve turns steeply upward. This means that all the polymers with masses *greater* than about 50 kDa elute together. At higher volumes, all the particles with masses *less* than about 2,000 Da elute together. In between, the elution volume varies nearly linearly with the logarithm of the molecular mass. The calibration is insensitive to molecular charge. How can we explain these characteristics?

The mechanism of size-exclusion separation is indicated in Figure 14.1d, which indicates the presence of pores in the beads making up the stationary

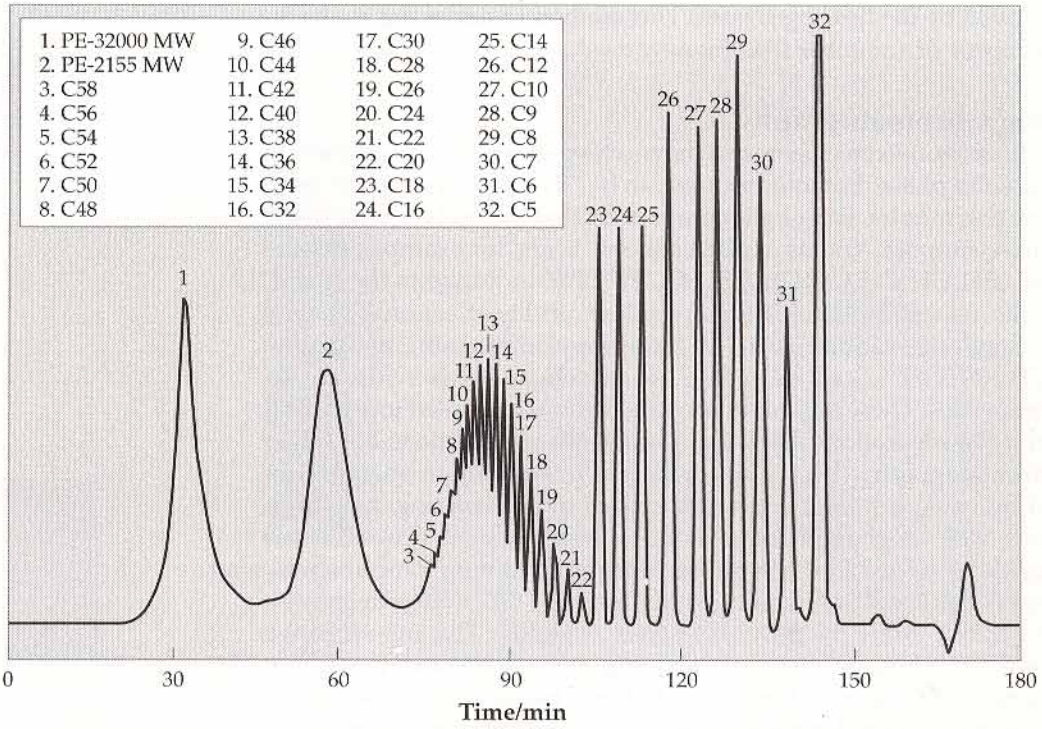


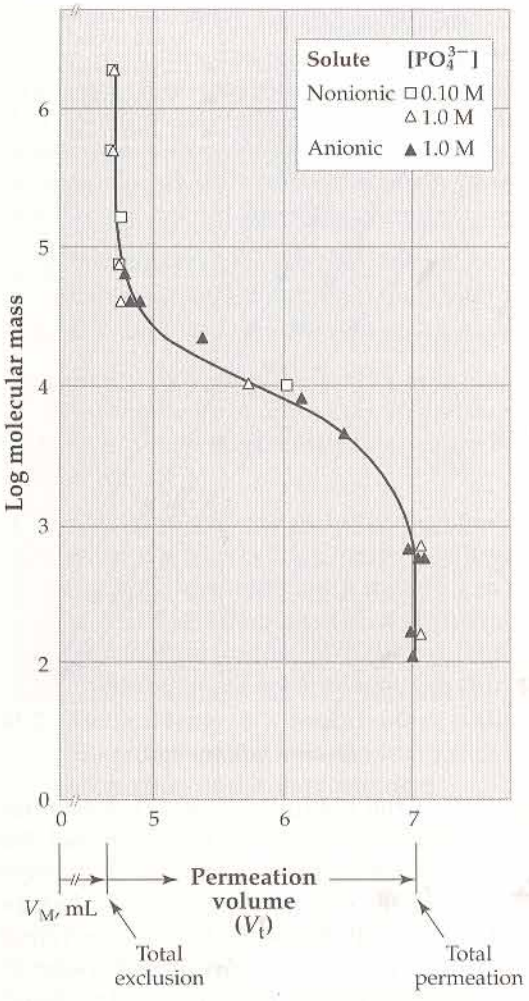
FIGURE 14.7 ▲
Example of gel permeation chromatogram.

Shown is the separation of a mixture of polymers and oligomers of ethylene and small hydrocarbons. The peak labels C_{xx} indicate the number of carbon atoms composing each chain. PE indicates polyethylene.

Experimental Conditions. Column: 500 mm × 10 mm packed with Jordi Gel DVB, 500 Å. Eluent: trichlorobenzene flow at 1.5 mL min^{-1}. Detection: refractive index. [Figure courtesy of Jordi Associates.]

phase. The pore cross sections are controlled as carefully and precisely as possible. Such pores permeate the beads packed in the columns. The separation occurs because only smaller molecules can enter the pores where they are sheltered from the flowing mobile phase. Thus, the larger molecules, which cannot enter the pores of the gel, flow along with the eluent. The larger molecules elute first, followed by the smaller molecules that are held up. This is the order you see in Figure 14.7. If a molecule is larger than the largest pore size, it will not enter into the pores during its passage through the column. It does not matter whether the molecules are 10% larger than the pores or twice the size of the pores. All molecules *over* a certain size limit flow continuously with the mobile phase and elute together in the hold-up volume V_M. This explains the lack of resolution at the hold-up volume of the column, as indicated at the left side of Figure 14.8. On the other hand, all molecules *below* a certain size spend an equal time tortuously making their way in and out of the pores of the static phase. Thus, there is also a lower limit to the molecular size resolution. These low molecular mass particles elute from the column in a volume called the **total permeation volume**, V_t. All molecules that interact exclusively through an exclusion mechanism elute from the column between these limits, V_M and V_t.

The resolution of size in the relatively linear part of the calibration is somewhat more subtle. This is indicated in Figure 14.9, which shows a representation of a cross section along a pore. The idea is that different sizes of



◀ FIGURE 14.8
Example of calibration curve for gel permeation chromatography (GPC).

The plot is semi-logarithmic: the molecular mass scale is logarithmic, the elution volume linear. The calibration was done with both ionic and nonionic solute standards. The packing is a controlled pore glass, which is a silica glass prepared under conditions so that it is permeated with molecular size pores that are 75 Å. Total exclusion occurs only for polymers over about 100,000 daltons. [Ref: Cooper, A. R., Matzinger, D. P. 1979. *J. Appl. Poly. Sci.* 23, 419. Copyright © 1979. Reprinted by permission of John Wiley & Sons, Inc.]

molecules have different ranges of motion within the pores. These are indicated by the shaded regions that extend to within a particle radius of the wall. The *volume* inside the pores is, in effect, larger for smaller molecules. Consequently, the *time* that smaller molecules spend inside the pores is greater. Of the molecules that can enter the pores, smaller molecules elute later. In the language of SEC, the smaller molecules have a larger **accessible volume** in the pores, and the elution order follows the accessible volume.

Most manufacturers produce packings with different **nominal pore sizes**. The pore size of the packing used in the experiment giving the results shown in Figure 14.7 was 500 Å; for the calibration curve of Figure 14.8, the pore size was 75 Å. Often the calibration is given as a molecular weight exclusion limit, which is the molecular mass slightly too large to enter the pores. These exclusion limits range from around m.w. 1000 to m.w. 20×10^6. The useful range in which the fractionation can be calibrated is from about 1/3 of the exclusion limit to the exclusion limit. For example, a packing with an exclusion limit of 100,000 daltons can be calibrated for masses between 30,000 and 100,000 daltons.

You will notice that the concept of an exclusion limit is particular to size-exclusion packings. The separation is unlike those based on differences in chemical bonding. When the chemical bonding determines the retention, we expect *some* interaction of all the molecules present. As a result, seldom do a

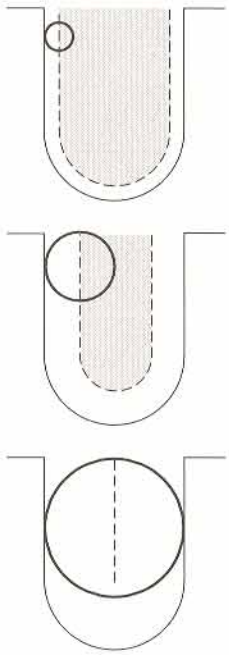


FIGURE 14.9 ▲
A simple model that explains gel filtration.

Consider a spherical molecule in a regularly shaped pore, as shown in longitudinal cross section. The accessible volume is the volume of the pore inside a surface that lies at a molecular radius inward from the true pore surface. Three molecules of different sizes are shown along with the cross-hatched accessible volume. When the pore radius equals the molecular radius, the accessible volume becomes zero. No entry to the pore is possible if the molecular radius is larger. Retardation of a molecular species is proportional to its accessible volume.

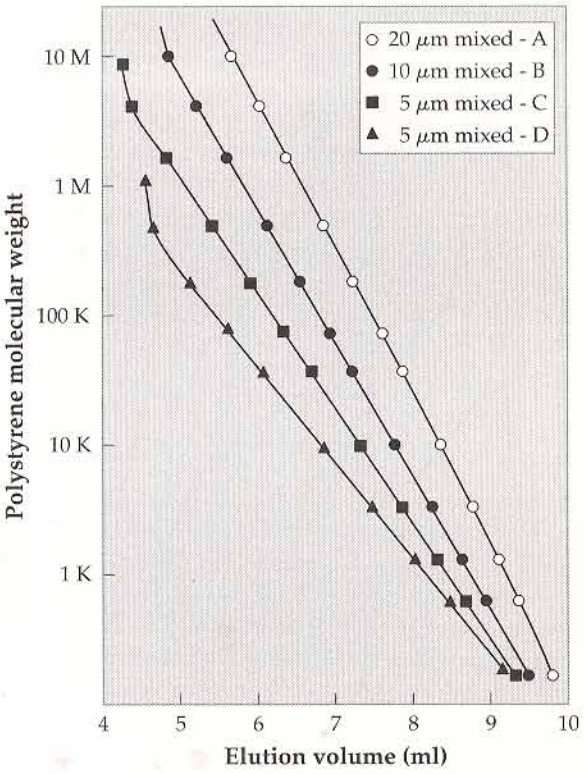


FIGURE 14.10 ▶
Calibration curves for four different mixed-bed exclusion chromatography columns.

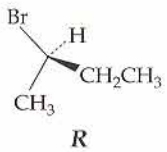
Notice the linear calibration ranges extend over 4–5 orders of magnitude compared to the calibration curve shown in Figure 14.8 that is linear over about an order of magnitude. [Figure courtesy of Polymer Laboratories.]

large number of species elute together in the holdup volume. However, for size exclusion, all the molecules above the exclusion limit elute together. We could put this group onto a second chromatographic column with a larger exclusion limit and separate them. The smaller set of molecules would be separated by the first column, and the larger ones by the second. However, rather than add columns, the same effect can be obtained by **mixed-bed** columns. Mixed-bed columns are packed with carefully chosen proportions of packings with different nominal pore sizes. These mixed-bed columns have log-linear calibration ranges up to factors of 10^5 in mass. Calibration curves for some are shown in Figure 14.10.

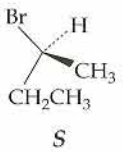
Size-exclusion chromatography can be used to determine the molecular size (and approximate molecular weight) of both biochemical and industrial polymers. Packings exist for both aqueous and nonaqueous systems.

14.6 Chiral Separations

As you know, isomeric molecules have the same atom content with different structures. **Chiral** isomers are **stereoisomers**: molecules that have the same atomic connectivities but differ in how the atoms are oriented in space. In addition, chiral isomers cannot be superimposed on their mirror images, just as your left and right hand cannot be superimposed on each other. The molecules in a set of chiral stereoisomers that are mirror images of each other are also called **enantiomers** or an **enantiomeric pair**. An example of enantiomers is the pair of 2-bromobutanes $CH_3—C^*HBr—CH_2—CH_3$, where the asterisk marks the chiral center: (*R*)-2-bromobutane and (*S*)-2-bromobutane shown in the margin. Details of the *R*,*S* nomenclature can be found in any organic chemistry textbook.



R



S

When two chiral centers exist, four stereoisomers exist. For example, 2-bromo-3-chlorobutane has two chiral centers: $H_3C—C^*HBr—C^*HCl—CH_3$. Since each chiral center can have either the *R* or *S* configuration, there are four stereoisomers: 2*R*,3*S*; 2*R*,3*R*; 2*S*,3*S*; and 2*S*,3*R*. These four constitute two enantiomeric pairs. However, the two sets are not enantiomers of each other. Such stereoisomers that are not enantiomers are called **diastereomers**. An important point to understand about the chemical properties of stereoisomers is that

a. *Enantiomers have identical physical properties* (for instance melting and boiling points) other than their interaction with polarized light.
b. *Diastereomers have different physical properties.*

As a result, diastereomers may be separated by chromatography.

Separations of enantiomers (chiral separations) are usually carried out by utilizing their differential interaction with chiral reagents in one of two general ways.

1. A chiral reagent is added to the eluent—a **chiral mobile phase**. The reagent is also called a **chiral additive** or a **chiral selector**. The reagent reacts differentially with the stereoisomers, which allows a separation to be achieved on an achiral support, such as an ODS column. The explanation for the separation is that transient diastereomers are formed that are separable by the chiral reagent.
2. A chiral reagent is bound onto a solid support, and the differential interaction of the stereoisomers with this chiral phase causes a separation of the stereoisomers.

Figure 14.11 shows a chromatogram of a chiral separation along with the structures of the stationary phases and the chemical structure of cypermethrin. Chiral separations have become quite important in the area of pharmaceuticals since different stereoisomers tend to have different levels of biological effectiveness. For instance, one isomer may have beneficial effects, while another may cause mostly unwanted side effects.

The choice of conditions and reagents for chiral separations is not uncomplicated, so details are not presented here. You are referred to the Suggestions for Further Reading of this chapter for references.

14.7 Gradients

Finding the best conditions for a separation by liquid chromatography involves manipulating all the equilibria that affect the reactions

$$\text{analyte (mobile phase)} \rightleftharpoons \text{analyte (stationary phase)}$$

Changes in the solvent usually shift these equilibria, and, as a result, separations can be improved by switching from an **isocratic** elution (same eluent throughout) to a **gradient** elution. A gradient is formed by mixing two or more different eluents such that the mobile-phase composition changes over time. Gradients always are changed from the poorest eluting solvent mixture toward better eluting compositions. Why take the trouble? Because resolution can be improved and elution times of strongly retained species can be shortened. Different properties of mobile phases can be changed to affect a gradient elution, and some of these are listed in Table 14.4

Chromatograms run under isocratic conditions include those shown in Figures 14.3 through 14.5.

Figures 14.12 through 14.14 illustrate some of the improvements in separations obtained by the use of solvent gradient elutions. The change in

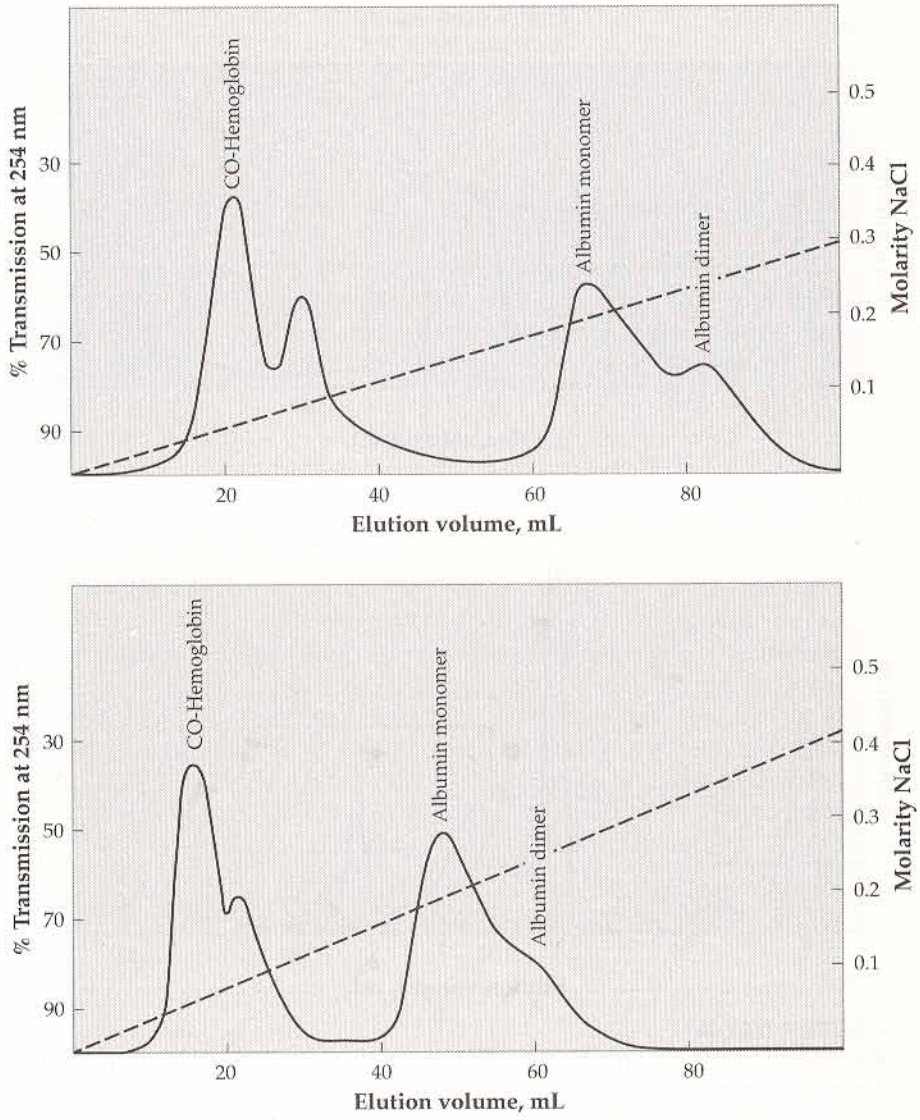


FIGURE 14.13 ▶
Illustration of the differences in a separation that can be made by changing the steepness of the gradient.

The gradient is made by increasing the ionic strength of NaCl. As can be seen, a significant change in the resolution occurs with a slight change in the gradient steepness. The separation is done on an ion exchange gel.

Experimental Conditions. Column: Diethylaminoethyl-Sephadex A-50. Eluent: 0.1-M Tris-HCl at pH 8.3 + NaCl (molarity noted on chromatogram); flow rate 8 mL cm^{-2} h^{-1} (that is, per cm^2 cross section). Sample: O_2-hemoglobin, CO-hemoglobin, albumin monomer, and albumin dimer. [Chromatograms courtesy of Pharmacia Fine Chemicals.]

Computer software available from a number of sources can be a useful adjunct in optimizing the gradient after a number of trial runs are made.

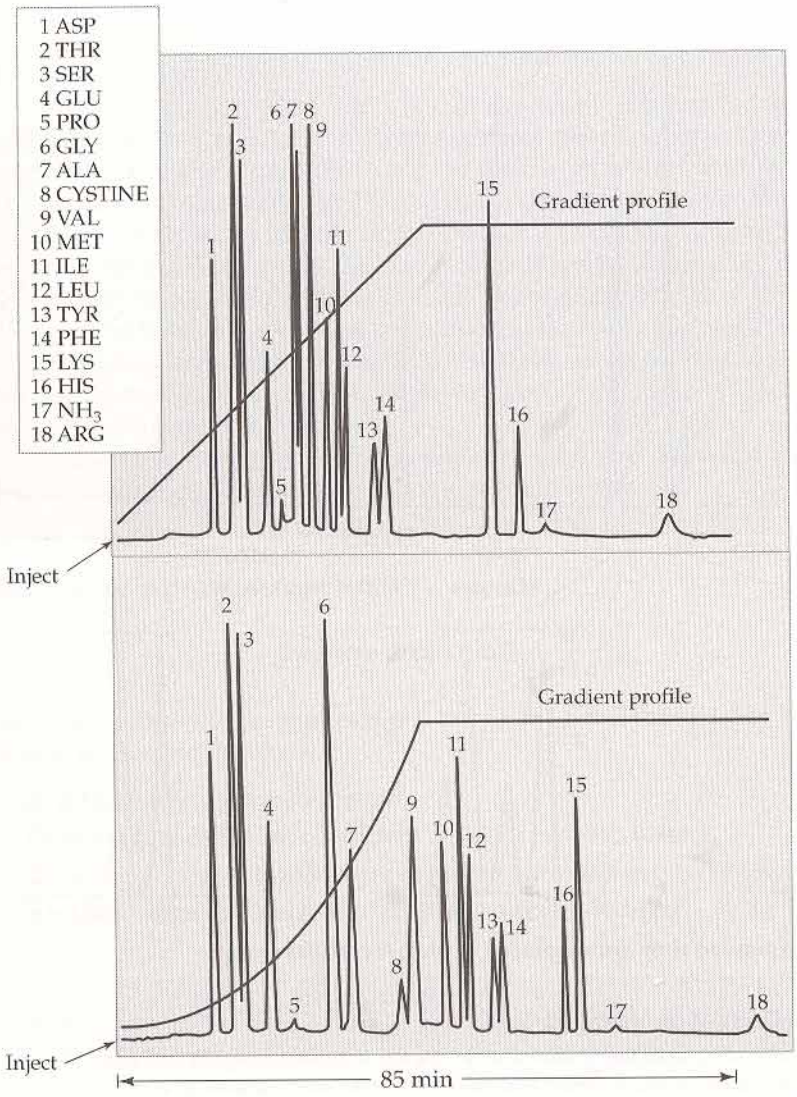
When solvents are combined into a gradient, they must be miscible, which means that they are soluble in each other at any concentration. A useful diagram to show which sets of solvents are miscible is a **miscibility table**, which is part of Appendix V.

14.8 Effects of Temperature

Temperature has a major effect on liquid chromatographic separations because at higher temperatures:

The solvent viscosity decreases, causing the eluent to flow more easily;

The rates of solute diffusion increase, causing faster diffusion in all directions;



◀ FIGURE 14.14
Illustration of the differences in a separation that can be made by changing the shape of a gradient from linear to nonlinear.

Separation of the amino acids is done by ion-exchange chromatography with an ionic strength and pH gradient elution. Buffer 1: 0.2 N Na^+, pH 3.08. Buffer 2: 1.0 N Na^+, pH 7.4. The primary improvement is in separating peaks 6-8. Note the reversal of the positions of peaks 15 and 16. [Figure courtesy of Waters Associates.]

The equilibria between the various solutes and the stationary phase are shifted by different amounts; and

The rates of exchange in those equilibria are generally faster.

In other words, all the equilibria shift and all the transport processes change with temperature. An example of the effects of temperature are shown in Figure 14.15. The differences between the separation at 20° and 30° are illustrated with three sets of analytes that are also shown in detail with the time scale expanded. As you can see, the separations are significantly affected by the 10° difference in temperature.

Two consequences of temperature control should be understood from this discussion. First, the temperature can be changed to improve liquid chromatographic separations. Second, if reproducibility is critical, the column should be held at a constant temperature. That is the reason the chromatograph illustrated in Figure 13.2 has a column thermostat.

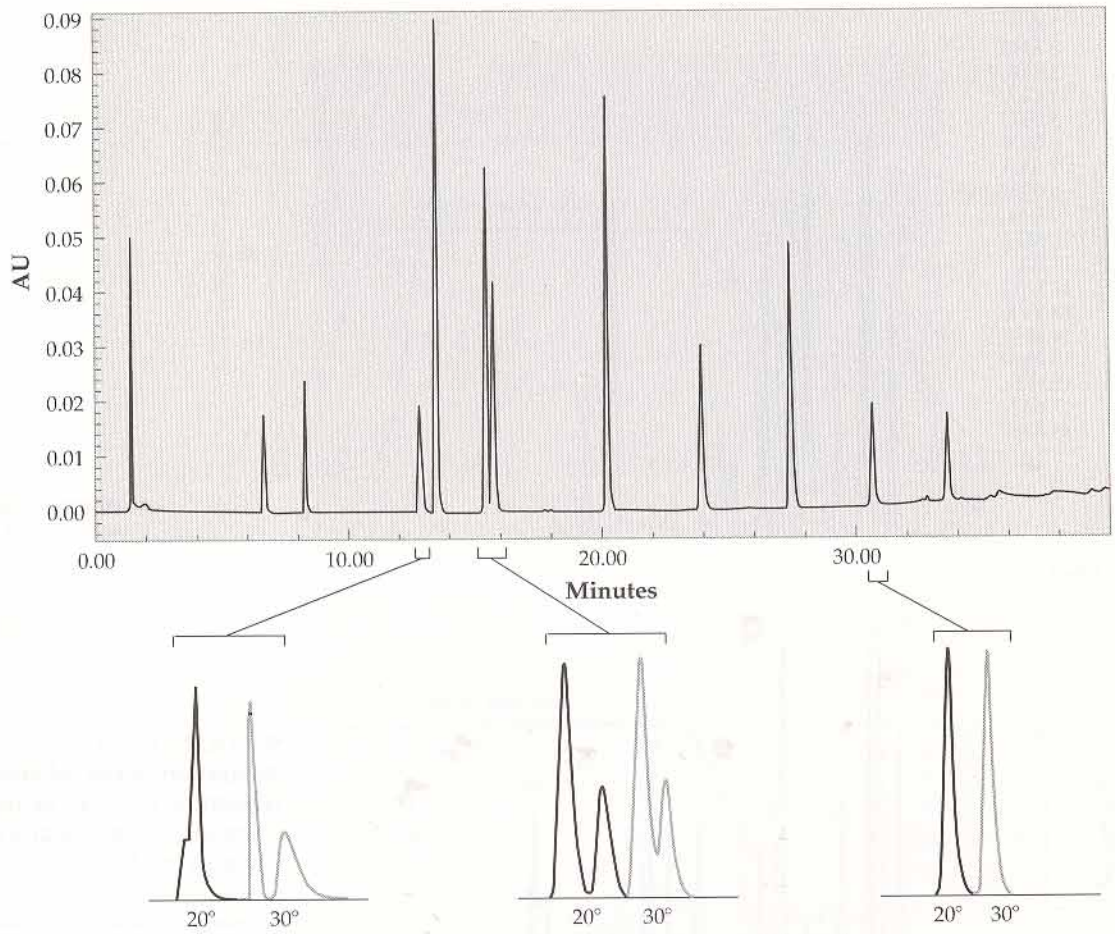


FIGURE 14.15 ▲
Illustration of the effects of temperature in LC.

Temperature strongly affects LC separations. Illustrated at the **top** is a chromatogram that was run with a joint pH and organic gradient. Below that appear three detailed illustrations with time expanded. Data from ~20°C and 30°C are shown, with the data from 30° shown in gray. **(Lower left)** At 20° the benzoic acid and tryptophan coelute; but at 30° the benzoic acid elutes ahead to provide baseline resolution. **(Lower center)** At 20° C2-phenone leads ethyl *p*-aminobenzoic acid (Ethyl Paba) to nearly baseline resolution. At 30° they both elute later and the resolution is decreased. **(Lower right)** A single component band from a neutral compound changes its retention significantly with the change in temperature.

Experimental Conditions. Column: Waters Nova Pak™ C18, 3.9 × 150 mm. No guard column. Mobile phase: linear gradient 0–60% neat acetonitrile in water (0.015% H_3PO_4) over thirty minutes, 1.0 mL min^{-1} flow. Detector: ultraviolet at 254 nm. Sample: in order of elution time, uracil, theophylline, caffeine, benzoic acid + tryptophan (coelution), methylparaben, butyrophenone, ethyl PABA, propylparaben, propiophenone, hexanophenone, heptanophenone, octanophenone. [Data courtesy of John Morawski, Waters Associates.]

14.9 Particle Size, Column Size, Pressure, and HETP

The particle size of the stationary phase is another factor of choice in chromatography—as noted in Table 13.2 on page 607. Reducing the particle size reduces the HETP as well. This means you should be able to obtain the same resolutions on shorter lengths of stationary phase. The benefit of reducing

the particle size is indicated in Figure 14.16, which shows van Deemter plots for three different particle sizes for column liquid chromatography. All other conditions remained the same.

However, changing the particle size cannot be effected independently without adjusting at least one other experimental variable: either the flow rate or the pressure required to force the mobile phase through the support. The three chromatograms in Figure 14.17 illustrate this interdependence. (Note that the column lengths differ for each chromatogram.) You can also see from the conditions that the pressure required to obtain a 1.0 mL min^{-1} flow rate with a shorter column containing smaller particles is greater than the pressure needed for a longer column packed with larger particles. In general, the pressure must increase approximately as the inverse square of the particle diameter. Typical HPLC pressures are in the range of a few thousand psi (~100 atmospheres).

These ideas can be written algebraically with two empirical equations. The dependence of the efficiency on the particle size is

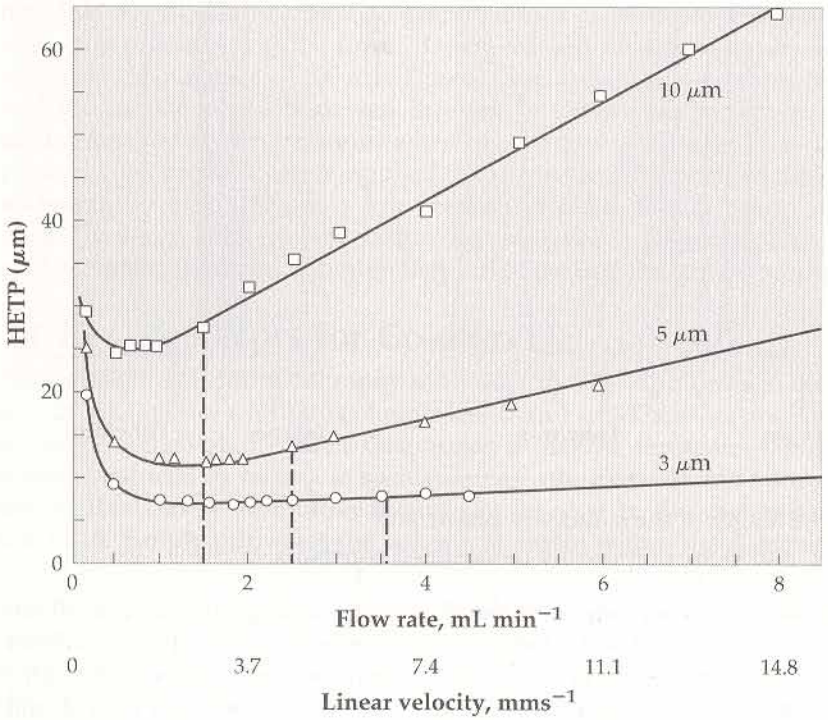
$$\text{HETP} = D_0 \bar{u}^n d_p^z \qquad \textbf{(14-1)}$$

The change in pressure required is

$$\text{pressure drop} = \Delta P = \frac{\bar{u} L \eta}{\Theta d_p^y} \qquad \textbf{(14-2)}$$

with the variables in the ranges $n = 0$ to 1, $y = 1.8$ to 2.0, $z = 1.4$ to 2.0, and $\Theta \approx 600$. In these equations

d_p is the particle diameter in cm,
D_0 is a solute diffusion coefficient ($\approx 10^{-5}$ cm s^{-1} in water),
ΔP is the pressure drop in atmospheres (1 atmosphere = 14.696 psi),
η is the viscosity in centipoise (cP) of the mobile phase (η_{H_2O} = 1 cP at 20°C),



◀ FIGURE 14.16
Graph of experimentally determined HETP *versus* flow rate for different particle diameters in HPLC.
The linear velocity in mm/s is related to the flow rate in mL/min. This column measures 4.6-mm inside diameter. The dashed lines indicate the optimum flow rates for each column. The reason that the optimum is not at the minimum of the curves is that the time required for an analysis has been taken into account. For instance, because there is such a small variation with flow rate in the HETP of the column with the 3-μm packing, the flow can be increased (and analysis time decreased) by a factor of about two with little loss of efficiency. [Figure courtesy of The Perkin-Elmer Corporation.]

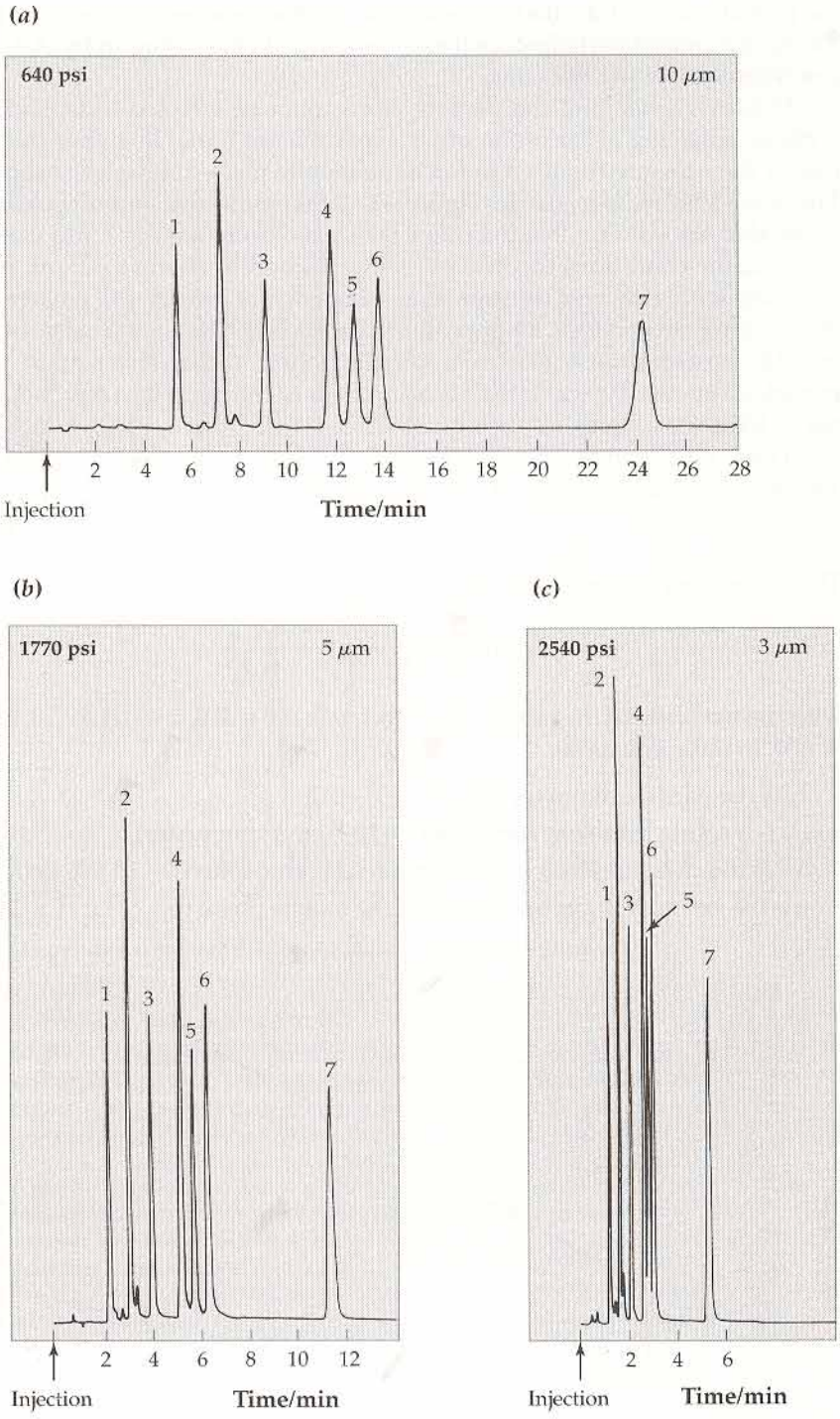


FIGURE 14.17 ▶
Illustration of the effect of particle size on analysis time.

As the particle size is reduced, the analysis time can be shortened. In this example the efficiency is kept approximately constant by reducing the column length as the particle size is reduced. The trade-off is the higher pressure required to keep the same flow rate for the three columns.

Experimental Conditions. Columns: Octadecylsilane packing of diameters noted with chromatograms; column A, 300 × 4.6 mm; column B, 150 × 4.6 mm; column C, 75 × 4.6 mm. Mobile phase: methanol/water 60/40; flow rate 1.0 mL/min; temperature 21°C. Pressure: on A = 640 psi, on B = 1770 psi, and on C = 2540 psi. Peak identification: 1, phenol; 2, acetophenone; 3, nitrobenzene; 4, methyl benzoate; 5, anisole; 6, benzene; 7, toluene. [Ref: Cook, N. H. C., Olsen, K. 1980. J. Chromatog. Sci. 18, 512–524. Reproduced from the *Journal of Chromatographic Science* by permission of Preston Publications, Inc.]

L is the length of the stationary phase, and
n, y, z, and Θ are empirically determined constants.

In addition, in keeping with the theme of reducing the amount of solvents used, less solvent is needed if columns are narrower. The same solvent velocity can be obtained with less solvent in a narrower column. Specifically, the amount of solvent needed changes as the inverse square of the column

Table 14.5 Characteristics of LC Microcolumns Compared to a Conventional Column

Column type	Typical dimensions		Volumetric flow rate	Sample capacity
	i.d.	Length		
Conventional column	4.6 mm	10–25 cm	1 mL/min	10–100 μg
Small-bore packed column	0.2–1 mm	1–10 cm	1–20 μL/min	1–10 μg
Packed capillary column	40–80 μm	1–100 cm	0.5–2 μL/min	100 ng–1 μg
Open-tubular capillary	15–50 μm	1–100 cm	<1 μL/min	<100 ng

Primarily from Novotny, M. V. 1986. "Toward Better Detection Techniques in Modern Liquid Chromatography." *Chromatography Forum*. May–June.

diameter. For example, if a column has half the diameter, the same linear velocity can be obtained with a fourth the solvent volume. This relationship simply follows the dependence of cross sectional area on diameter.

Since the total surface area per unit length of the static phase is proportional to the diameter of the column, narrower columns cannot hold as much analyte, and the column capacity is reduced. This is not a problem when the solutes are dilute, and there is no chance for saturation. In fact, if analyte is limited, it is worthwhile to change to a smaller column since the *concentration* in the detector will be higher for the same number of moles of solute. As long as the detector is adequate, using microbore columns can be useful both in solvent use and increased solute concentrations. Table 14.5 lists column characteristics and their sample capacities.

In concluding this section on packing particle and column sizes, let us note that not all column chromatography is HPLC. When larger particles are used, the pressures can be lower. **Open-column** chromatography is done with particle diameters $\geq$100 μm. The columns are usually mounted upright, and the mobile phase is driven through by gravity as in Tswett's early separation. To speed separations when the columns get longer, a few atmospheres of gas pressure can be applied to the solvent. This is called **flash chromatography**. Alternatively, a low-pressure, fixed-flow-rate pump may be used. However, neither open column nor flash chromatography are considered analytical methods, although they might be used for sample preparation.

14.10 Detectors for Column LC

For conventional chromatography most detectors have volumes in the range of 5–20 μL, and for capillary columns less than 1 μL. There are good reasons for keeping the volume small. One reason is that the chromatographic resolution is reduced by mixing in such volumes—the **extracolumn volume**. The reason this loss of resolution occurs can be seen in the cartoons in Figure 14.18. Simply put, since the volume of eluent within the detector cell is mixed by the flow of liquid, the earlier and later eluting volumes intermix. The final result is that each band is broadened due to this mixing, and the larger the cell the larger the eluent volume that is mixed. For a cell volume of a few microliters, the effect of mixing produces only a minor broadening of the narrowest bands. Larger cell volumes cause quite obvious decreases in

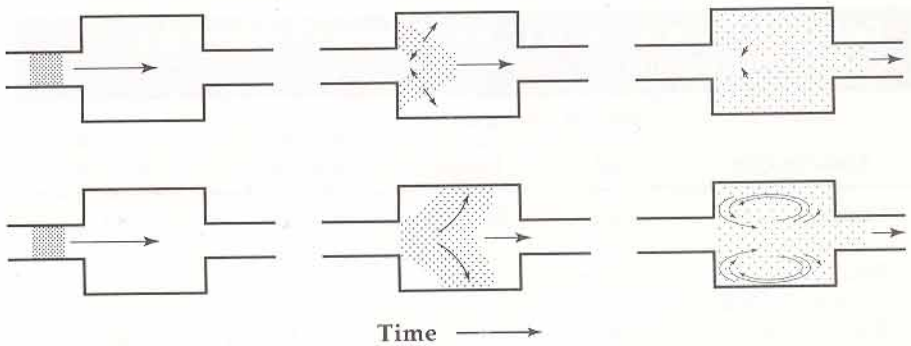


FIGURE 14.18 ▲
Mechanisms of mixing.
When a flow through an expanded volume occurs, the larger volume allows mixing through two different mechanisms. **(Top)** At low flow speed, the sample has a larger range to diffuse and mixes with whatever is already in the volume. **(Bottom)** At faster flow speeds, the flow is forced into the volume and can mix with vortices as shown on the right representing a later time. At flow rates in between, the mixing occurs by both mechanisms simultaneously. Not only does this process occur in detection cells, but also in any spaces at the column couplings or open volumes in the columns themselves.

the resolution that is achieved on the column. The greatest effect of the mixing, of course, is seen on narrow, closely spaced, adjacent bands.

In this section are illustrated the construction and general mechanisms of operation of seven of the most commonly used types of detectors. Table 14.6 lists their names, sensitivities, and linear ranges. Mass spectrometers are also important as detectors, and the interfaces connecting liquid chromatographs to mass spectrometers are described in Chapter 12.

UV-Visible Absorption Detectors

Figure 14.19 illustrates the construction and operation of a dual-beam UV detector that uses a filter to detect one wavelength only. (Variable wavelength operation would require a monochromator.) The reference cell is filled with eluent, but since the reference cannot be changed, gradients might cause a baseline drift. A single-beam system is likely to be found with a polychromator.

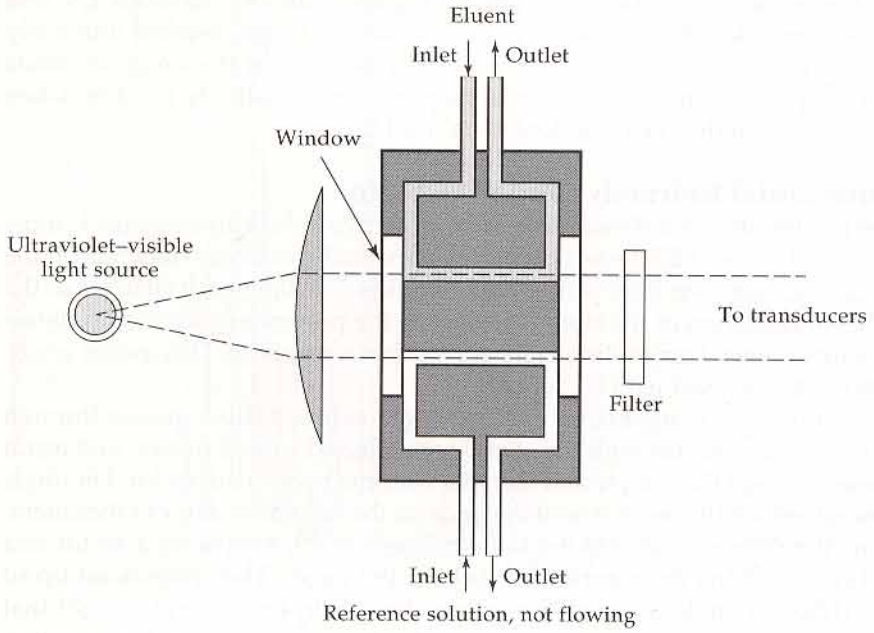
The volume of sample needed is kept low by using narrow-bore tubing where possible, and sensitivity is increased by constructing the longest possible path length within the constraints of the optics. Other parts of the detector are similar to a normal spectrophotometer in this wavelength region.

For capillary columns, the detection must be accomplished by passing the light through the capillary itself, since any cell would cause an unacceptable decrease in efficiency due to mixing of adjacent volumes. The optics shown in Figure 14.20 are used to focus the light through the sample and prevent it from leaking around the sample volume through the surrounding glass. (Any light that does not pass through the sample is clearly stray light.) A transparent sphere of the appropriate size placed adjacent to the capillary acts as a lens to focus the light through a short section of the middle of the capillary and onto a transducer. The best sensitivity is obtained when the light passes only through the center of the cross section, where the sample path is the longest. For all UV-vis cells, both solid-state detectors and photomultipliers are used, depending on the design.

Table 14.6 Characteristics of Selected Liquid Chromatography Detectors

Type	Approximate limit of detection*	Approximate linear range†	Comments
Ultraviolet and visible absorption	10^{-11} g	10^4	Specific for light-absorbing compounds (*Cf.* Chapter 11)
Differential refractive index	10^{-9}–10^{-10} g	10^3	Universal detector. Measures changes in refractive index. Cannot be used with gradients.
Electrochemical: amperometric	10^{-10}–10^{-11} g	10^5	Specific detector. Compound must be electroactive. (*Cf.* Chapter 15)
Electrochemical: conductometric	10^{-8} g/mL‡	10^5	Specific detector but for all ions (*Cf.* Chapter 15)
Fluorescence	10^{-14} g§	10^5	Specific detector. Compound must be fluorescent. (*Cf.* Chapter 11)
Mass spectrometry	10^{-7}–10^{-9} g	10^5¶	Universal detector. Also can be used to identify analytes with great certainty. (*Cf.* Chapter 12)
Solution light scattering	10^{-6} g/mL	10^5	See footnote *a*.
Evaporative light scattering	10^{-9} g	10^6φ	Universal except for volatile analytes. Not a linear response.

*Depends on the peak width. Value for a measurement without preconcentration of the sample. The lower limit of their usefulness in every case is in the trace to ultratrace range.
†Of a detector (transducer + electronics) without chemical interferences present.
‡Limited by the inherent conductance of the eluent.
§This value is for strongly fluorescing compounds without interferents.
¶For a sector instrument. Quadrupole, ion-trap, and Fourier-transform instruments are narrower.
a. Light scattering is used to determine the molecular weights of polymers (range 500–10^8 daltons) as they elute. The concentrations are usually far above the limit of detection.
φThe response is of the form (constant · [analyte]^m), so a log-log calibration plot is linear.



◀ FIGURE 14.19
Diagram of the light path and liquid flow path for a dual-beam LC sample cell for packed columns.

This is one of a number of designs. Light from the source on the left passes through a window and two tubes filled with liquid. One of the tubes contains a static sample of the mobile phase to avoid waste. The effluent from the column passes through the other tube. The difference in light absorption between the two is amplified and plotted as the chromatogram. The wavelength of the light is selected by the filter. The cell volume through which the light passes is generally in the range of 10–20 μL.

precise as possible, the instruments must be able to quantitate the measurement *over the whole area of the spot*. The material is generally more concentrated at the center of the spot, with decreasing concentrations toward the perimeter. As a result, the most precise measurements are made by scanning the analyte and reference spots in a series of narrow regions (analogous to the raster scanning of a television screen), recording the output as a function of position, and integrating the total intensity associated with each spot. Alternatively, quantitative imaging can be done with a CCD detector. Precisions in the range of $\pm 1\%$ are possible under the best conditions of sample application, development, and quantitation with parallel standards, but $\pm 5\%$ is more common.

Suggestions for Further Reading

BIDLINGMEYER, B. A. 1992. *Practical HPLC Methodology and Applications*. New York: John Wiley & Sons.

The best next place to go if you are planning to begin doing HPLC separations. A wealth of useful information and wisdom. The chapters on strategies are unique.

KATZ, E. D. 1996. *High Performance Liquid Chromatography: Principles and Methods in Biotechnology*. Chichester: John Wiley & Sons.

A surprisingly practical book of techniques that have been used for separations of proteins, glycoproteins, and nucleic acids. Not a "cookbook." Especially welcome are sections on sample preparation in a number of chapters. A chapter on electrophoresis is included.

NEUE, U. D. 1997. *HPLC Columns: Theory, Technology, and Practice*. New York: Wiley-VCH.

This book is written at approximately the same level as this chapter, but with much greater detail. A wealth of information from an author with a deep knowledge of the subject. Besides the columns' chemistry and selecting their conditions for use, methods development is included but not as a "cookbook." Classic papers in the field are cited and provide an excellent entry into the literature.

SADEK, P. C. 1996. *The HPLC Solvent Guide*. New York: John Wiley & Sons.

After reading Chapter 1, which presents the basic ideas, go to the index and find the initial conditions to develop the separation for your analytes. A great place to start.

SCOTT, R. P. W. 1994. *Liquid Chromatography for the Analyst*. New York: Dekker.

Clearly written with practical descriptions, this monograph covers most LC technologies.

MCMASTER, M. C. 1994. *HPLC, a practical user's guide*. New York: VCH Publishers.

A book full of practical information about setting up, running, and troubleshooting HPLC. The short chapter on column care is especially noteworthy.

PARRIOTT, D. 1993. *A Practical Guide to HPLC Detection*. San Diego: Academic Press.

Worthwhile reading for thorough but nonmathematical coverage of essentially every detection method used for HPLC. A unique reference.

WEISS, J. 1995. *Ion Chromatography*. Weinheim: VCH Verlag.

This single-author book covers the field of ion chromatography thoroughly and well: instrumentation, reagents, theory, applications. The next place to go if you want to do ion chromatography.

SMALL, H. 1989. *Ion Chromatography*. New York: Plenum.

An outstanding book covering the details of every essential aspect of ion chromatography (and fine descriptions of the essentials of column chromatography in general) at a level that you should be able to understand. By the inventor of ion chromatography.

JANCA, J., ed. 1984. *Steric Exclusion Liquid Chromatography of Polymers*. Dekker: New York.

A collection of chapters by different authors on size-exclusion chromatography. They cover the details and problems of GPC. Especially recommended is Chapter 7 on precision and accuracy.

SUBRAMANIAN, G. 1994. *A Practical Approach to Chiral Separations by Liquid Chromatography*. Weinheim: VCH.

The first few chapters give overviews of chiral selection. The rest are specific and, since general rules are not developed yet, tend to be descriptive. The index lists many of the separated compounds.

ZIEF, M., CRANE, L. J. 1988. *Chromatographic Chiral Separations*. New York: Marcel Dekker.

An edited book that has a mixture of chapters from highly specific to more general. A number of different approaches to chiral adsorbents are presented. However, there is not a truly broad chapter integrating the principles of the field and comparing various approaches.

LOUGH, W. J. 1989. *Chiral Liquid Chromatography*. Glasgow and New York: Blackie and Chapman and Hall.

Chapters provide in-depth description of each chiral stationary phase and chiral mobile phase method. However, it is difficult to find a method for a specific compound type. The short chapter (Chapter 14) on the choice of systems is helpful. This is an excellent specialist book.

POOLE, C. F., SCHUETTE, S. A. 1984. *Contemporary Practice of Chromatography*. Elsevier: Amsterdam.

An encyclopedic textbook/reference on gas, liquid, and thin-layer chromatography including equipment, sample preparation, and theory. It is exhaustive and contains large numbers of literature references. However, you will need to know what you are looking for

since the organization is complex. It should be easily readable after studying the topics in this chapter. Some parts are now dated.

BERRIDGE, J. C. 1985. *Techniques for the Automated Optimization of HPLC Separations*. Chichester: John Wiley & Sons.

A well written book that is exemplary as far as bringing a mostly mathematical subject to its applications in chromatography. Most of the approaches to optimization are presented.

GRINBERG, N. 1990. *Modern Thin-Layer Chromatography*. New York: Marcel Dekker.

A fine book on the supports, solvents, sample application, detection, theory, and instrumental techniques of TLC and HPTLC. (Much is applicable to all liquid chromatography.) The chapter on mobile phases is excellent for all of LC. The chapter on quantitation for TLC is also particularly well done.

TOUCHSTONE, J. C. 1992. *Practice of Thin Layer Chromatography*. New York: John Wiley & Sons.

An excellent book full of useful, descriptive, information about all the details of plates, sample spotting, development, and quantitation in TLC. Some of the information, however, should be considered more as history than contemporary practice.

FRIED, B., SHERMA, J. 1994. *Thin-Layer Chromatography, Techniques and Applications*. New York: Marcel Dekker.

A laboratory-oriented book with how-to-do-it pictures and practical details. The book includes an extensive collection of methods for biochemistry: lipids, carbohydrates, pigments, nucleic acids, steroids.

JORK, H., FUNK, W., FISCHER, W., WIMMER, H. 1990. *Thin-Layer Chromatography, Reagents and Detection Methods*. Weinheim: VCH.

This book contains thorough coverage of detection of analytes on thin-layer chromatography.

HUF, F. A. 1987. *In Situ Evaluation of Thin-Layer Chromatograms. Quantitative Thin-Layer Chromatography and Its Industrial Applications*. L. R. TREIBER, ed. New York: Marcel Dekker.

A thorough and mathematical treatment of the factors involved in quantitation in TLC by reflectance spectrophotometry.

Exercises

14.1 From the data supplied in Figure 14.17, use band 7 to determine the exponent for d_p in Equations 14-1 and 14-2. Assume that $\bar{u}$ does not depend on particle size. Are y and z approximately equal?

14.2 On the size-exclusion chromatogram in Figure 14.2.1, four of the five peaks are labeled with the molecular weights of the fractions (278,000, etc.). What is the average molecular weight of the middle fraction? [Ref: Fuller, E. N., et al. 1982. *J. Chromatog. Sci. 20*, 120. Reproduced from *Journal of Chromatographic Science* by permission of Preston Publications, Inc.]

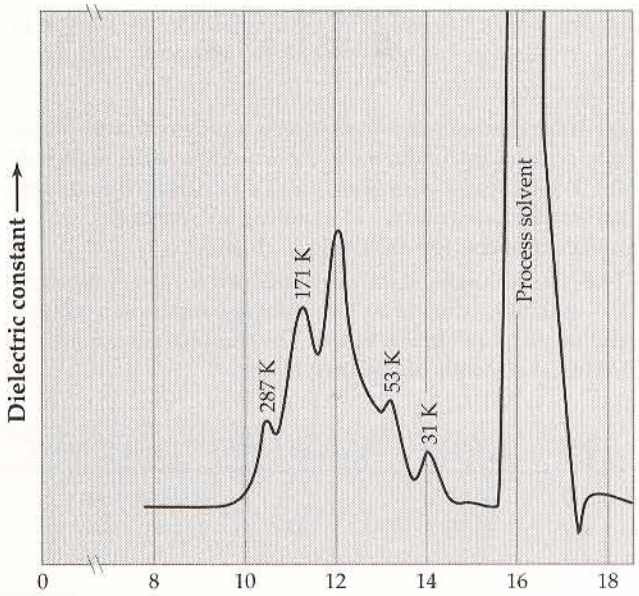


FIGURE 14.2.1 ▲

14.3 The graph in Figure 14.3.1 shows the changes in retention times for a number of amino acids as they

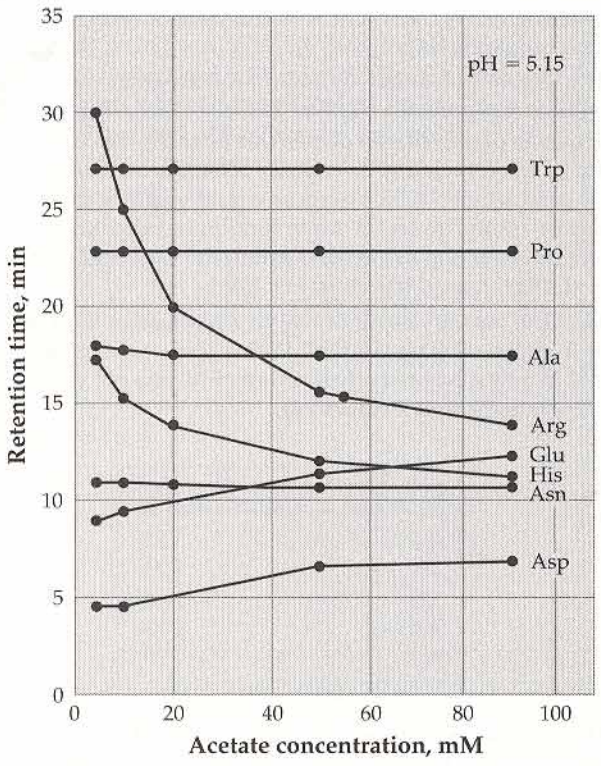


FIGURE 14.3.1 ▲

change with acetate concentration in the eluent. This is predominately an ionic-strength effect. Which of the amino acids are neutral at pH 5.15, the pH of the experiments? [Ref: Jackson, M. A., Fisher, J. E. 1983. *Ind Res. & Devel.* February, 130. © 1983, Industrial Research & Development.]

14.4 This problem refers to Figure 13.5.1 on pg. 624. Since the bands are somewhat asymmetric, the operator decided that the R_s value should be increased to 2.0. Since the conditions were already optimized, a longer column was needed.

(a) What column length is needed to achieve the desired separation?

(b) What pressure do you expect will be needed to keep the flow rate constant?

(c) What pressure do you expect will be needed to keep the same retention time?

14.5 From band 6 of the chromatograms in Figure 14.3,

(a) estimate the ratio of partition constants of methyl: octyl: octadecyl with toluene under the conditions of the separation. Assume V_S remains constant. (You may wish to make a photocopy enlargement.)

(b) Let us assume that the reversed-phase adsorbent surfaces are homogeneously covered with the chains sticking out like bristles on a brush. The V_S would vary with chain length (1:8:18). What are the ratios of K_D for toluene with V_S varying this way? Does this simple picture produce results consistent with the ideas of reversed-phase separations?

14.6 Figure 14.6.1 shows an ion chromatogram run without a suppressor column. There are three different detectors in the effluent stream one after the other. They are (1) photometric at 280 nm; (2) refractive index; and (3) conductivity. Which of these would yield the most precise results under the same conditions but with a tenfold reduction in ion concentrations? [Ref: Jenke, D. R., et al. 1983. *Anal. Chim. Acta. 155*, 279.]

14.7 The capacity factor of DL-tyrosine changes with pH on a reversed-phase support. With a phosphate buffer of 0.02 M and solvent of ethanol:water 5:95, the pH values and the associated k values are:

pH	k
2.3	3.33
4.8	1.75
6.5	1.56
8.9	1.94
11.4	7.28

The pK_a values of tyrosine are $pK_1 = 2.20$, $pK_2 = 9.11$, and $pK_3 = 10.07$. At low pH, the molecule is a monocation. At high pH values, it is a dianion. [Data from Kroeff, E. P., Pietrzyk, D. J. 1978. *Anal. Chem. 50*, 502.]

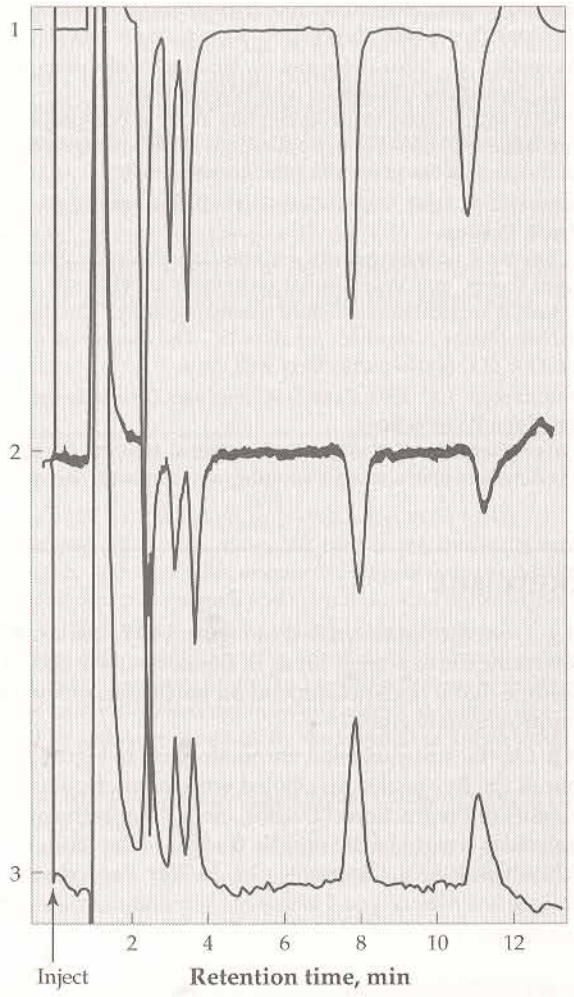


FIGURE 14.6.1 ▲

(a) If the mechanism of retention is a pure reversed-phase one, which form of the molecule would have the highest k?

(b) What type(s) of interaction(s) is/are occurring on this reversed-phase column: H–bonding (adsorption), reversed-phase, ion exchange, exclusion?

14.8 The following data were obtained for the elution of Cl^- and NO_3^- during an ion-exchange separation using phthalate as an elution buffer. The samples contained identical amounts of the analyte ions.

pH	t_M (min)	$t_{R,\,chloride}$ (min)	$t_{R,\,nitrate}$ (min)
2.7	0.50	2.75	3.50
3.7	0.50	1.06	1.93
4.5	0.50	0.75	1.50

[Data from Jupille, T. 1986. *American Laboratory. 18*, 114.]

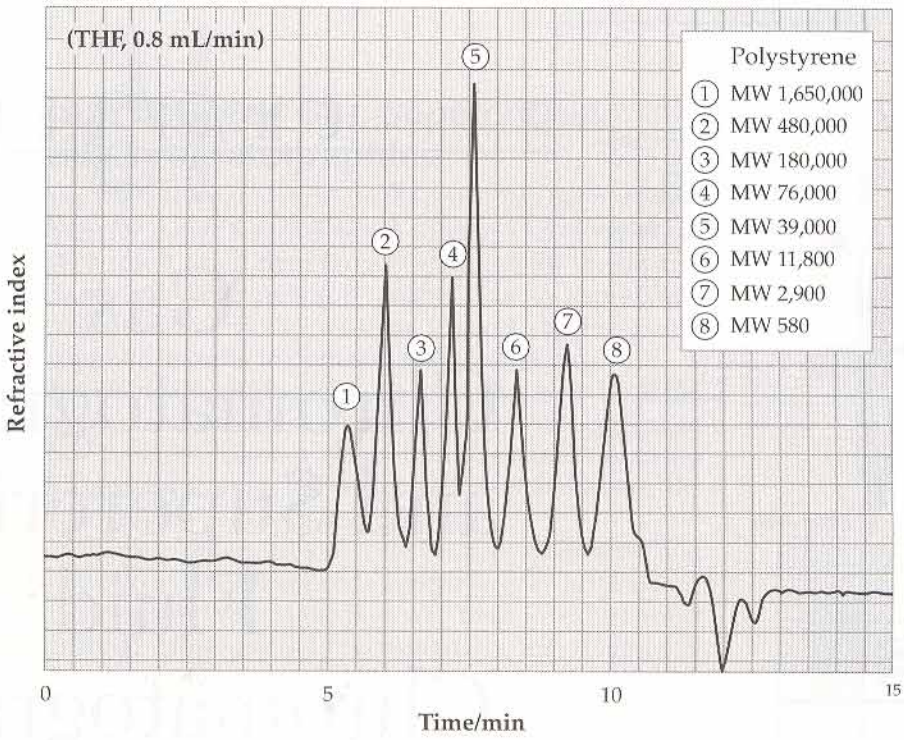


FIGURE 14.9.1 ▲

(a) What would be the most likely form of phthalate present at each of the pH values used?
(b) Plot the *k* values *versus* the $[H^+]$ for each ion on a log-log plot. Is the shape of each curve consistent with the elution time being a function of the concentration of H^+?

14.9 The GPC chromatogram in Figure 14.9.1 shows the separation of a series of narrow-range polystyrene standards. What would be the predicted elution time for a polystyrene standard with an average molecular weight of 55,000? [Ref: Reprinted from Reuter, W. M., Dong, M. W., and McConville, J. 1991. *American Laboratory*. 23, 45. Copyright 1991 by International Scientific Communications, Inc.]

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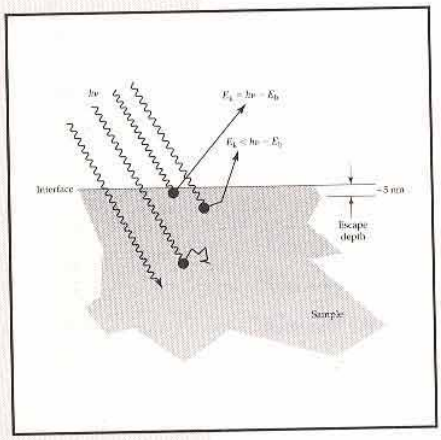

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