Chapter 12

Chromatographic and Electrophoretic Methods

Chapter Overview

Drawing from an arsenal of analytical techniques—many of which were the subject of the preceding four chapters—analytical chemists design methods for the analysis of analytes at increasingly lower concentrations and in increasingly more complex matrices. Despite the power of these analytical techniques, they often suffer from a lack of selectivity. For this reason, many analytical procedures include a step to separate the analyte from potential interferents. Although effective, each additional step in an analytical procedure increases the analysis time and introduces uncertainty. In this chapter we consider two analytical techniques that avoid these limitations by combining the separation and analysis: chromatography and electrophoresis.

12A Overview of Analytical Separations

In Chapter 7 we examined several methods for separating an analyte from potential interferents. For example, in a liquid–liquid extraction the analyte and interferent are initially present in a single liquid phase. We add a second, immiscible liquid phase and mix them thoroughly by shaking. During this process the analyte and interferents partition themselves between the two phases to different extents, effecting their separation. After allowing the phases to separate, we draw off the phase enriched in analyte. Despite the power of liquid–liquid extractions, there are significant limitations.

12A.1 Two Limitations of Liquid–Liquid Extractions

From Chapter 7 we know that the distribution ratio, D, for a solute, S, is

$$D = \frac{[S]_{ext}}{[S]_{samp}}$$

where $[S]_{ext}$ is its equilibrium concentration in the extracting phase and $[S]_{samp}$ is its equilibrium concentration in the sample.

We can use the distribution ratio to calculate the fraction of S that remains in the sample, q_{samp}, after an extraction

$$q_{samp} = \frac{V_{samp}}{DV_{ext} + V_{samp}}$$

where V_{samp} is the volume of sample and V_{ext} is the volume of the extracting phase. For example, if $D = 10$, $V_{samp} = 20$, and $V_{ext} = 5$, the fraction of S remaining in the sample after the extraction is

$$q_{samp} = \frac{20}{10 \times 5 + 20} = 0.29$$

or 29%. The remaining 71% of the analyte is in the extracting phase.

Suppose we have a sample containing an analyte in a matrix that is incompatible with our analytical method. To determine the analyte's concentration we first separate it from the matrix using a simple liquid–liquid extraction. If we have several analytes, we may need to complete a separate extraction for each analyte. For a complex mixture of analytes this quickly becomes a tedious process. This is one limitation to a liquid–liquid extraction.

A more significant limitation is that the extent of a separation depends on the distribution ratio of each species in the sample. If the analyte's distribution ratio is similar to that of another species, then their separation becomes impossible. For example, let's assume that an analyte, A, and an interferent, I, have distribution ratios of, respectively, 5 and 0.5. If we use a liquid–liquid extraction with equal volumes of sample and extractant, then it is easy to show that a single extraction removes approximately 83% of the analyte and 33% of the interferent. Although we can remove 99% of the analyte with three extractions, we also remove 70% of the interferent. In fact, there is no practical combination of number of extractions or volumes of sample and extractant that produce an acceptable separation.

12A.2 A Better Way to Separate Mixtures

The problem with a liquid–liquid extraction is that the separation occurs in only one direction—from the sample to the extracting phase. Let's take a closer look at the liquid–liquid extraction of an analyte and an interferent with distribution ratios of, respectively, 5 and 0.5. Figure 12.1 shows that a single extraction using equal volumes of sample and extractant transfers 83% of the analyte and 33% of the interferent to the extracting phase. If the original concentrations of A and I are identical, then their concentration ratio in the extracting phase after one extraction is

$$\frac{[A]}{[I]} = \frac{0.83}{0.33} = 2.5$$

A single extraction, therefore, enriches the analyte by a factor of 2.5×. After completing a second extraction (see Figure 12.1) and combining the two

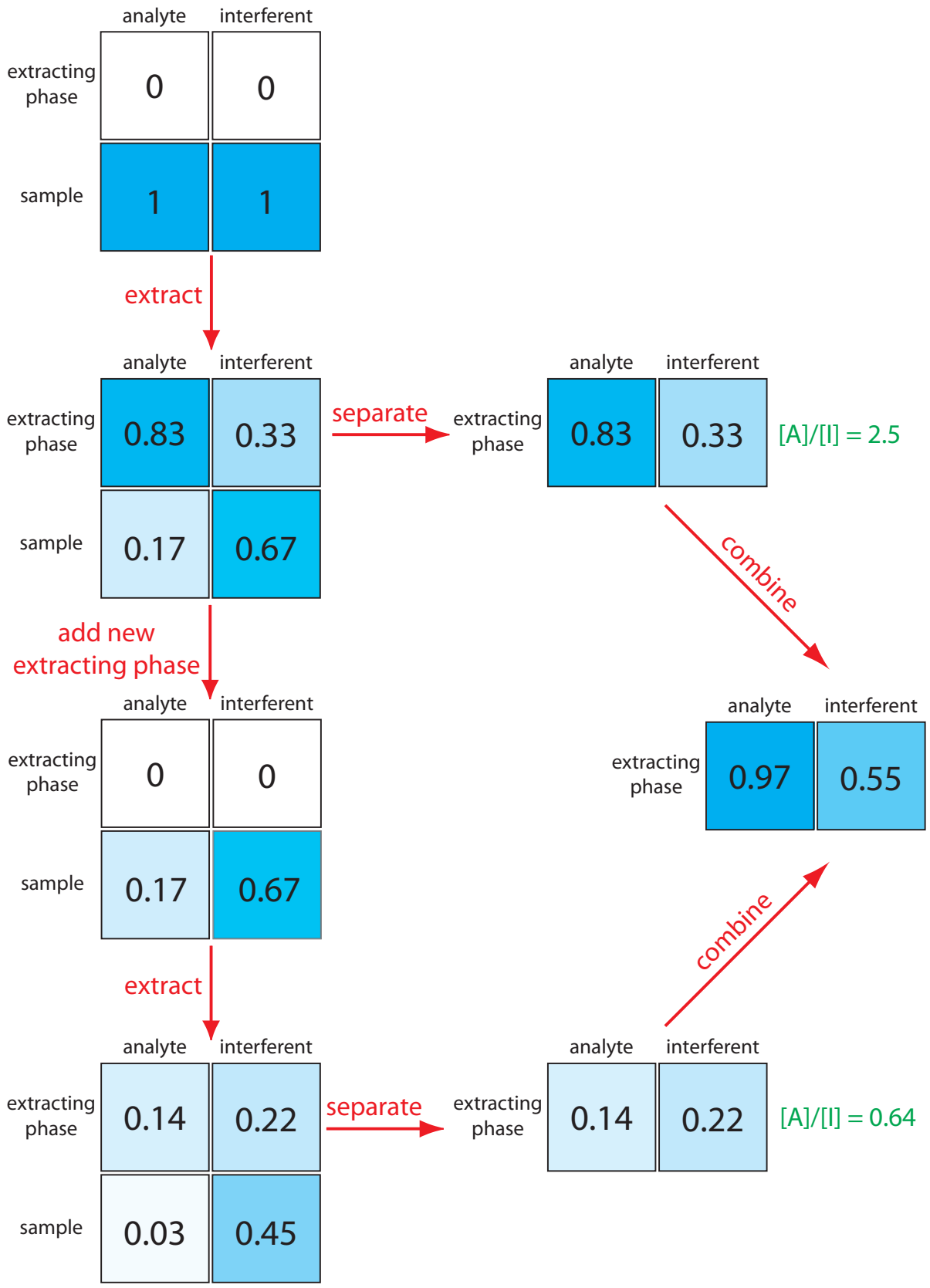


Figure 12.1 Progress of a traditional liquid–liquid extraction using two identical extractions of a single sample with fresh portions of the extractant. The numbers give the fraction of analyte and interferent in each phase assuming equal volumes of sample and extractant and distribution ratios of 5 and 0.5 for the analyte and the interferent, respectively.

extracting phases, the separation of the analyte and interferent is, surprisingly, less efficient.

$$\frac{[A]}{[I]} = \frac{0.97}{0.55} = 1.8$$

Figure 12.1 makes it clear why the second extraction results in a poorer overall separation—the second extraction actually favors the interferent!

We can improve the separation by first extracting the solutes from the sample into the extracting phase, and then extracting them back into a fresh portion of solvent that matches the sample's matrix (Figure 12.2). Because the analyte has the larger distribution ratio, more of it moves into the ex-

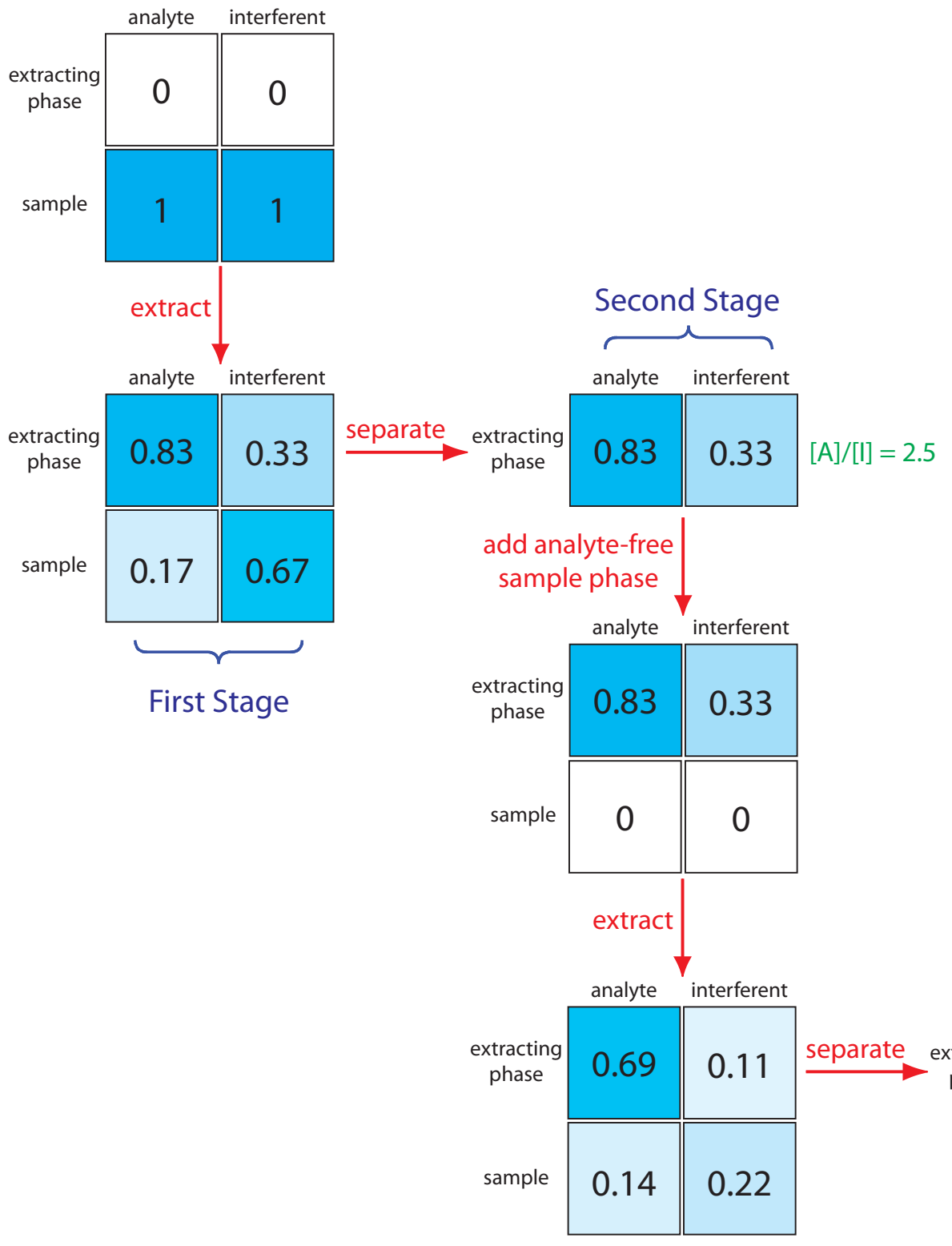


Figure 12.2 Progress of a liquid–liquid extraction in which we first extract the solutes into the extracting phase and then extract them back into an analyte-free portion of the sample's phase. The numbers give the fraction of analyte and interferent in each phase assuming equal volumes of sample and extractant and distribution ratios of 5 and 0.5 for the analyte and interferent, respectively.

tractant during the first extraction, and less of it moves back to the sample phase during the second extraction. In this case the concentration ratio in the extracting phase after two extractions is significantly greater.

$$\frac{[A]}{[I]} = \frac{0.69}{0.11} = 6.3$$

See Appendix 16 for a more detailed consideration of the mathematics behind a countercurrent extraction.

Not shown in Figure 12.2 is that we can add a fresh portion of the extracting phase to the sample that remains after the first extraction, beginning the process anew. As the number of extractions increases, the analyte and the interferent each spread out in space over a series of stages. Because the interferent's distribution ratio is smaller than that of the analyte, the interferent lags behind the analyte. With a sufficient number of extractions, a complete separation is possible. This process of extracting the solutes back

and forth between fresh portions of the two phases, which we call a COUNTERCURRENT EXTRACTION, was developed by Craig in the 1940s.[1] The same phenomenon forms the basis of modern chromatography.

12A.3 Chromatographic Separations

In CHROMATOGRAPHY we pass a sample-free phase, which we call the MOBILE PHASE, over a second sample-free STATIONARY PHASE that remains fixed in space (Figure 12.3). We inject or place the sample into the mobile phase. As the sample moves with the mobile phase, its components partition themselves between the mobile phase and the stationary phase. A component whose distribution ratio favors the stationary phase requires more time to pass through the system. Given sufficient time, and sufficient stationary and mobile phase, we can separate solutes even if they have similar distribution ratios.

There are many ways in which we can identify a chromatographic separation: by describing the physical state of the mobile phase and the stationary phase; by describing how we bring the stationary phase and the mobile phase into contact with each other; or by describing the chemical or physical interactions between the solute and the stationary phase. Let's briefly consider how we might use each of these classifications.

Types of Mobile Phases and Stationary Phases

The mobile phase is a liquid or a gas, and the stationary phase is a solid or a liquid film coated on a solid substrate. We often name chromatographic techniques by listing the type of mobile phase followed by the type of stationary phase. In gas–liquid chromatography, for example, the mobile phase is a gas and the stationary phase is a liquid film coated on a solid substrate. If a technique's name includes only one phase, as in gas chromatography, it is the mobile phase.

Contact Between the Mobile Phase and the Stationary Phase

There are two common methods for bringing the mobile phase and the stationary phase into contact. In COLUMN CHROMATOGRAPHY we pack the stationary phase into a narrow column and pass the mobile phase through the column using gravity or by applying pressure. The stationary phase is a solid particle or a thin, liquid film coated on either a solid particulate packing material or on the column's walls.

In PLANAR CHROMATOGRAPHY the stationary phase is coated on a flat surface—typically, a glass, metal, or plastic plate. One end of the plate is placed in a reservoir containing the mobile phase, which moves through the stationary phase by capillary action. In paper chromatography, for example, paper is the stationary phase.

We can trace the history of chromatography to the turn of the century when the Russian botanist Mikhail Tswett used a column packed with calcium carbonate and a mobile phase of petroleum ether to separate colored pigments from plant extracts. As the sample moved through the column the plant's pigments separated into individual colored bands. After the pigments were adequately separated, the calcium carbonate was removed from the column, sectioned, and the pigments recovered by extraction. Tswett named the technique chromatography, combining the Greek words for "color" and "to write."

There was little interest in Tswett's technique until Martin and Synge's pioneering development of a theory of chromatography (see Martin, A. J. P.; Synge, R. L. M. "A New Form of Chromatogram Employing Two Liquid Phases," *Biochem. J.* **1941**, *35*, 1358–1366). Martin and Synge were awarded the 1952 Nobel Prize in Chemistry for this work.

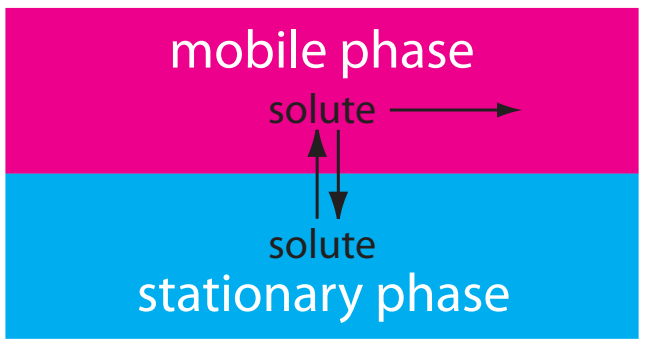


Figure 12.3 In chromatography we pass a mobile phase over a stationary phase. When we inject a sample into the mobile phase, the sample's components both move with the mobile phase and partition into the stationary phase. The solute spending the most time in the stationary phase takes the longest time to move through the system.

1 Craig, L. C. *J. Biol. Chem.* **1944**, *155*, 519–534.

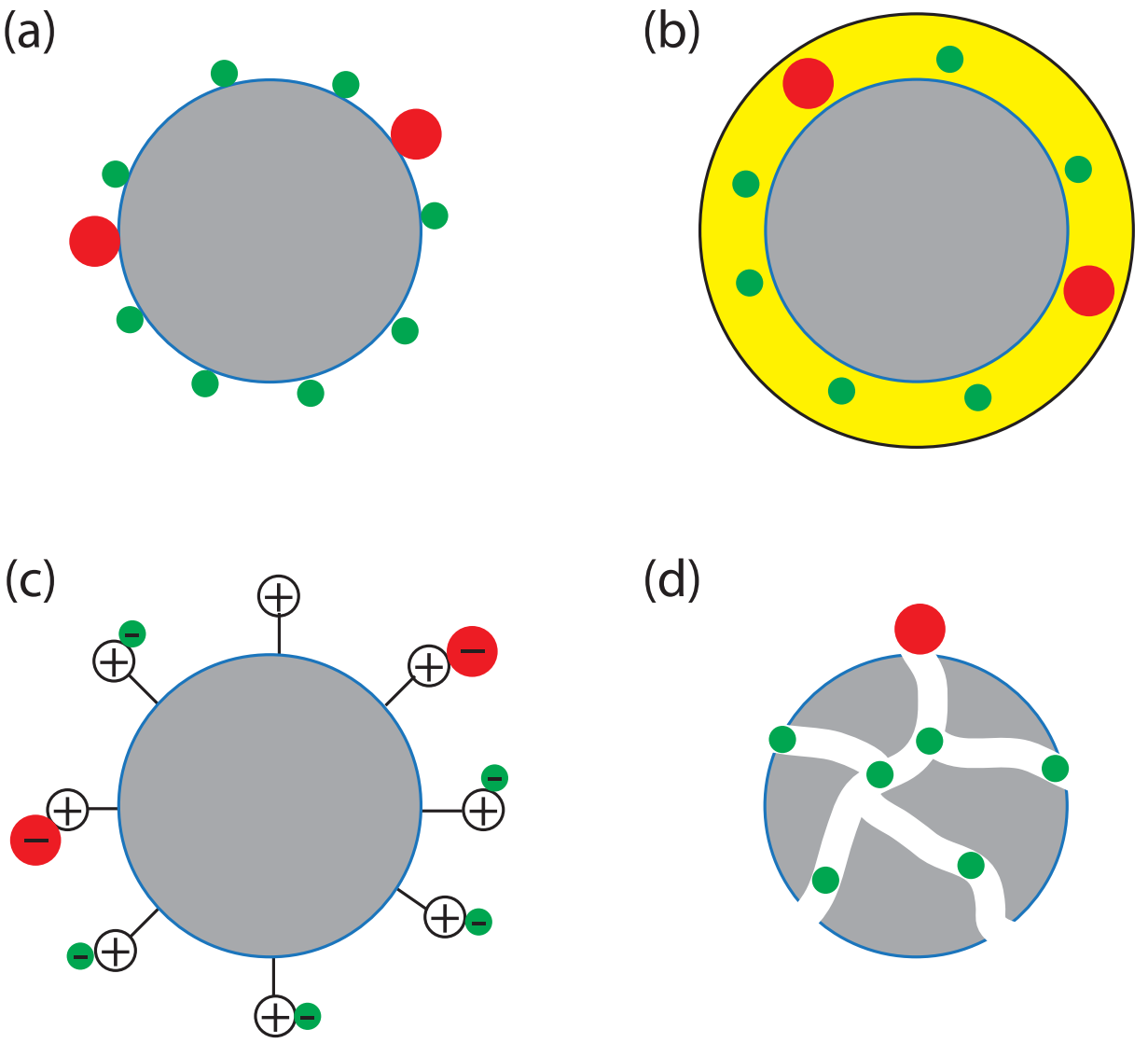


Figure 12.4 Four examples of interactions between a solute and the stationary phase: (a) adsorption on a solid surface, (b) partitioning into a liquid phase, (c) ion-exchange, and (d) size exclusion. For each example, the smaller, green solute is more strongly retained than the larger, red solute.

There are other interactions that can serve as the basis of a separation. In affinity chromatography the interaction between an antigen and an antibody, between an enzyme and a substrate, or between a receptor and a ligand can form the basis of a separation. See this chapter's additional resources for some suggested readings.

Interaction Between the Solute and the Stationary Phase

The interaction between the solute and the stationary phase provides a third method for describing a separation (Figure 12.4). In ADSORPTION CHROMATOGRAPHY, solutes separate based on their ability to adsorb to a solid stationary phase. In PARTITION CHROMATOGRAPHY, the stationary phase is thin, liquid film on a solid support. Separation occurs because of differences in the equilibrium partitioning of solutes between the stationary phase and the mobile phase. A stationary phase consisting of a solid support with covalently attached anionic (e.g., $-SO_3^-$) or cationic (e.g., $-N(CH_3)_3^+$) functional groups is the basis for ion-exchange chromatography. Ionic solutes are attracted to the stationary phase by electrostatic forces. In size-exclusion chromatography the stationary phase is a porous particle or gel, with separation based on the size of the solutes. Larger solutes, which are unable to penetrate as deeply into the porous stationary phase, move more quickly through the column.

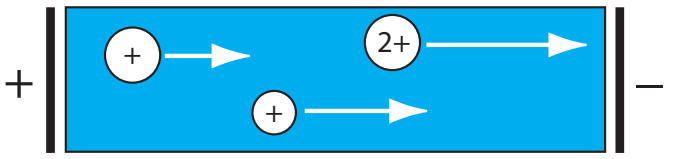


Figure 12.5 Movement of charged solutes under the influence of an applied potential. The lengths of the arrows indicate the relative speed of the solutes. In general, a larger solute moves more slowly than a smaller solute of equal charge, and a solute with a larger charge move more quickly than a solute with a smaller charge.

12A.4 Electrophoretic Separations

In chromatography, a separation occurs because there is a difference in the equilibrium partitioning of solutes between the mobile phase and the stationary phase. Equilibrium partitioning, however, is not the only basis for effecting a separation. In an electrophoretic separation, for example, charged solutes migrate under the influence of an applied potential. Separation occurs because of differences in the charges and the sizes of the solutes (Figure 12.5).

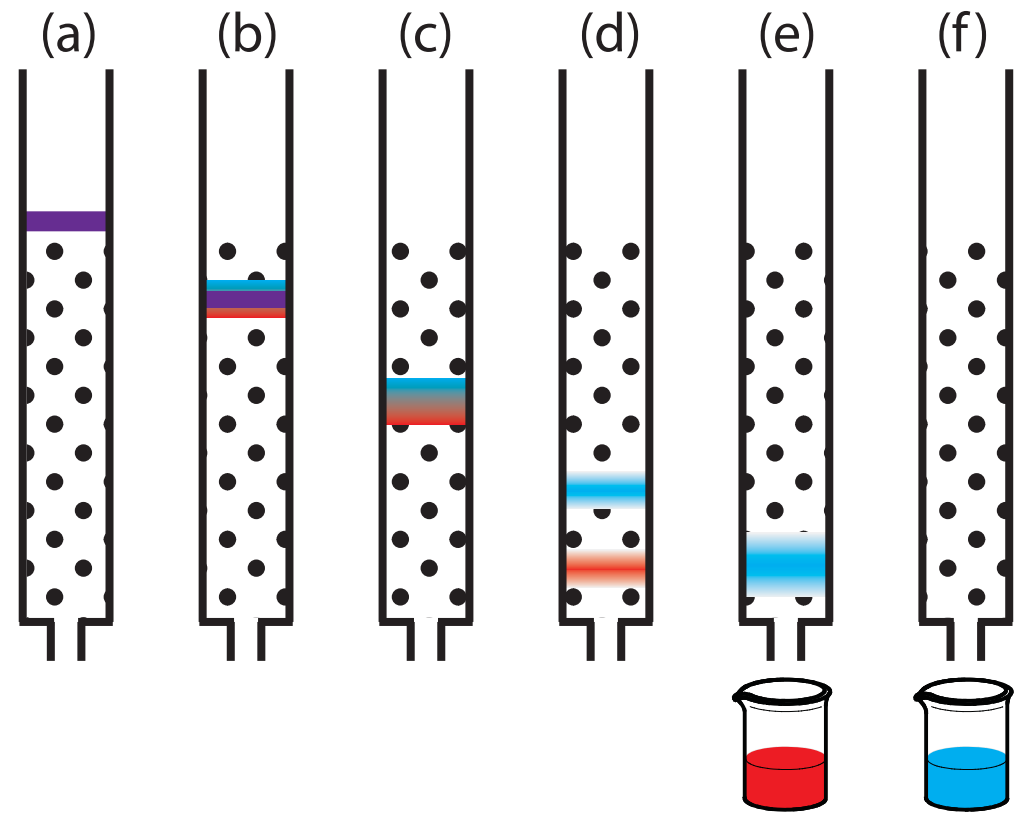


Figure 12.6 Progress of a column chromatographic separation of a two-component mixture. In (a) the sample is layered on top of the stationary phase. As mobile phase passes through the column, the sample separates into two solute bands (b–d). In (e) and (f), we collect each solute as it elutes from the column.

12B General Theory of Column Chromatography

Of the two methods for bringing the stationary phase and the mobile phases into contact, the most important is column chromatography. In this section we develop a general theory that we may apply to any form of column chromatography.

Figure 12.6 provides a simple view of a liquid–solid column chromatography experiment. The sample is introduced at the top of the column as a narrow band. Ideally, the solute's initial concentration profile is rectangular (Figure 12.7a). As the sample moves down the column the solutes begin to separate (Figures 12.6b,c), and the individual solute bands begin to broaden and develop a Gaussian profile (Figures 12.7b,c). If the strength of each solute's interaction with the stationary phase is sufficiently different, then the solutes separate into individual bands (Figure 12.6d and Figure 12.7d).

We can follow the progress of the separation either by collecting fractions as they elute from the column (Figure 12.6e,f), or by placing a suitable detector at the end of the column. A plot of the detector's response as a function of elution time, or as a function of the volume of mobile phase, is known as a CHROMATOGRAM (Figure 12.8), and consists of a peak for each solute.

There are many possible detectors that we can use to monitor the separation. Later sections of this chapter describe some of the most popular.

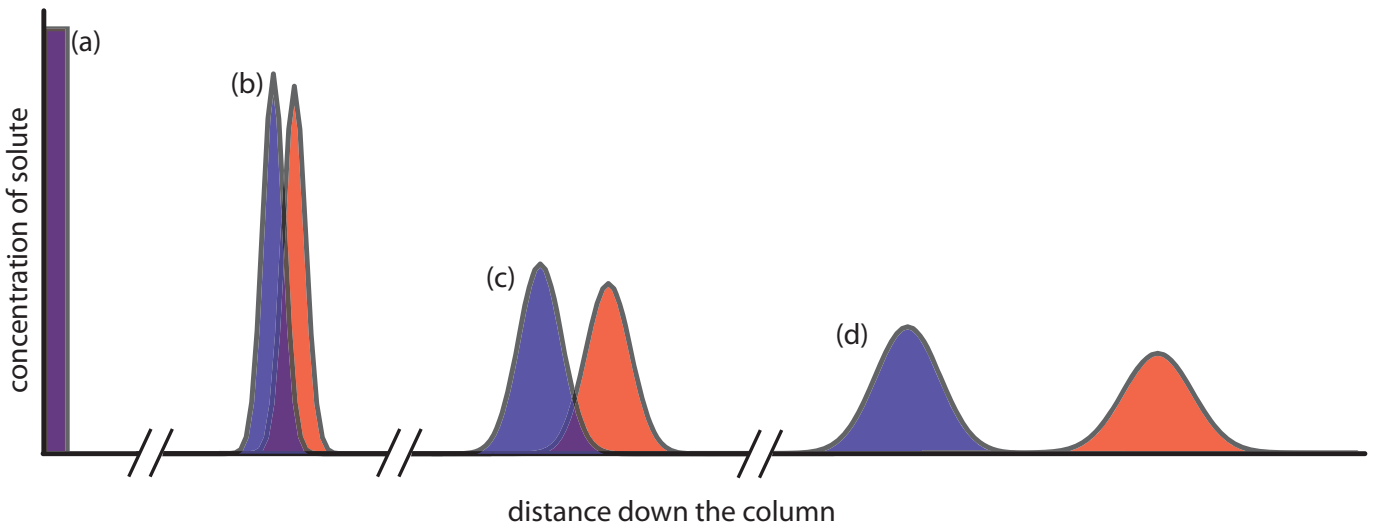


Figure 12.7 An alternative view of the separation in Figure 12.6 showing the concentration of each solute as a function of distance down the column.

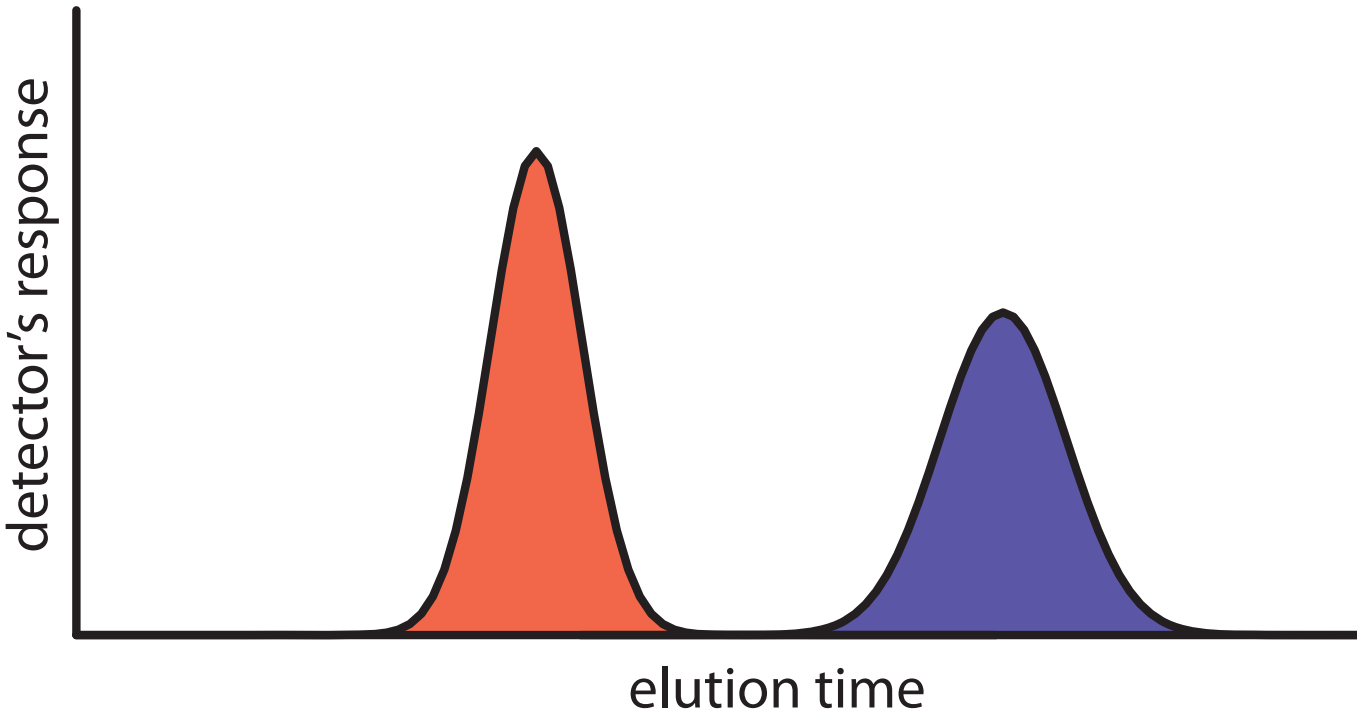


Figure 12.8 Chromatogram for the separation shown in Figure 12.6 and Figure 12.7, showing the detector's response as a function of the elution time.

A solute's chromatographic peak may be characterized in many ways, two of which are shown in Figure 12.9. RETENTION TIME, t_r, is the time between the sample's injection and the maximum response for the solute's peak. Another important parameter is the BASELINE WIDTH, w, which, as shown in Figure 12.9, is determined by extending tangent lines from the inflection points on either side of the chromatographic peak through the baseline. Although usually we report t_r and w using units of time, we can report them using units of volume by multiplying each by the mobile phase's velocity, or in linear units by measuring distances with a ruler.

In addition to the peak for the solute, Figure 12.9 also shows a small peak that elutes shortly after injecting the sample into the mobile phase. This peak is for NONRETAINED SOLUTES. Because these solutes do not interact with the stationary phase, they move through the column at the same rate as the mobile phase. The time required to elute nonretained solutes is called the column's VOID TIME, t_m.

For example, a solute's retention volume, V_r, is

$$V_r = t_r \times u$$

where u is the mobile phase's velocity through the column.

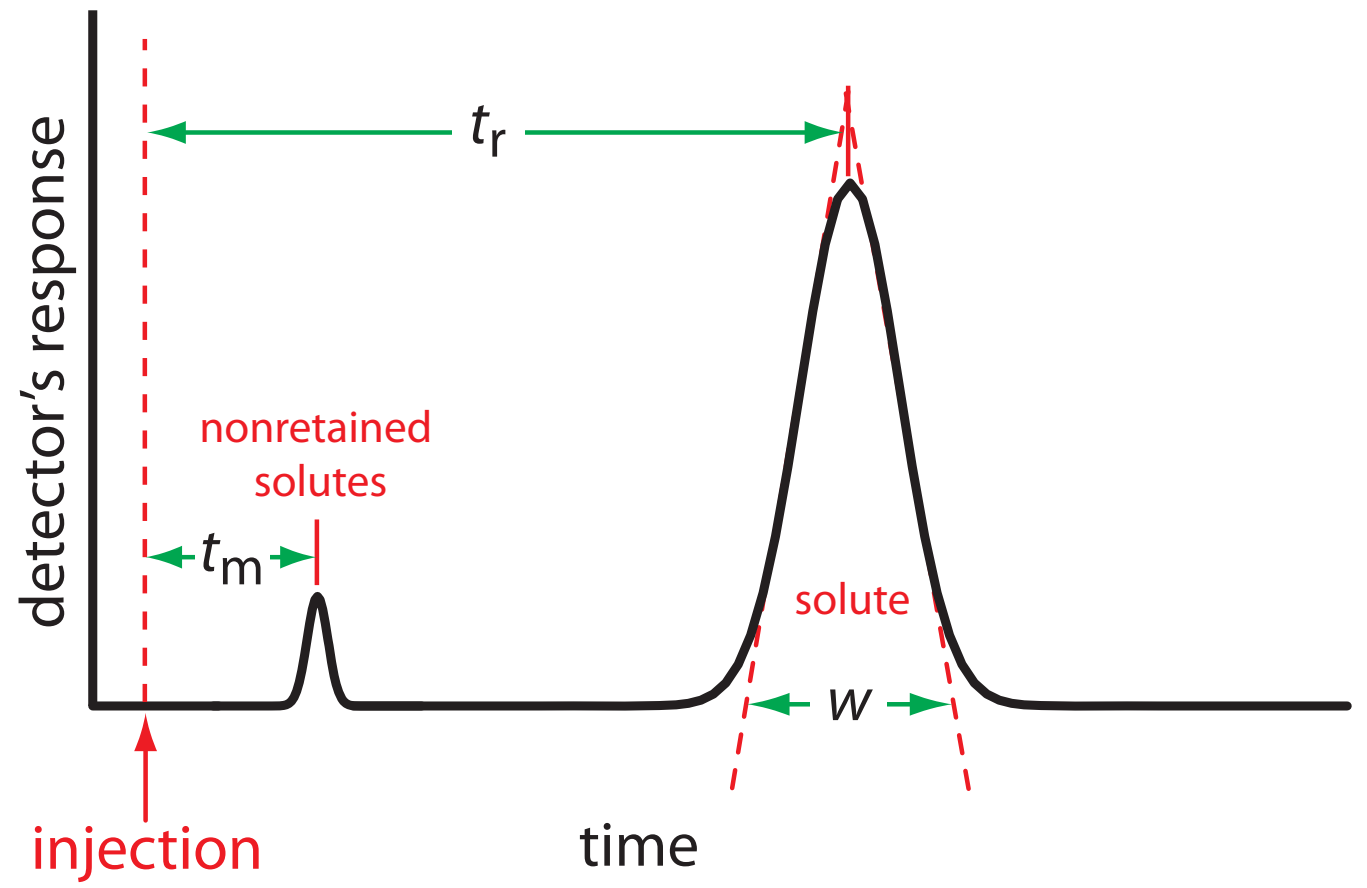


Figure 12.9 Chromatogram showing a solute's retention time, t_r, and baseline width, w, and the column's void time, t_m, for nonretained solutes.

12B.1 Chromatographic Resolution

The goal of chromatography is to separate a mixture into a series of chromatographic peaks, each representing a single component of the mixture. The RESOLUTION between two chromatographic peaks, R_{AB}, is a quantitative measure of their separation, and is defined as

$$R_{AB} = \frac{t_{r,B} - t_{r,A}}{0.5(w_B + w_A)} = \frac{2\Delta t_r}{w_B + w_A} \qquad 12.1$$

where B is the later eluting of the two solutes. As shown in Figure 12.10, the separation of two chromatographic peaks improves with an increase in R_{AB}. If the areas under the two peaks are identical—as is the case in Figure 12.10—then a resolution of 1.50 corresponds to an overlap of only 0.13% for the two elution profiles. Because resolution is a quantitative measure of a separation's success, it is a useful way to determine if a change in experimental conditions leads to a better separation.

Example 12.1

In a chromatographic analysis of lemon oil a peak for limonene has a retention time of 8.36 min with a baseline width of 0.96 min. γ-Terpinene elutes at 9.54 min with a baseline width of 0.64 min. What is the resolution between the two peaks?

SOLUTION

Using equation 12.1 we find that the resolution is

$$R_{AB} = \frac{2\Delta t_r}{w_B + w_A} = \frac{2(9.54\ \text{min} - 8.36\ \text{min})}{0.64\ \text{min} + 0.96\ \text{min}} = 1.48$$

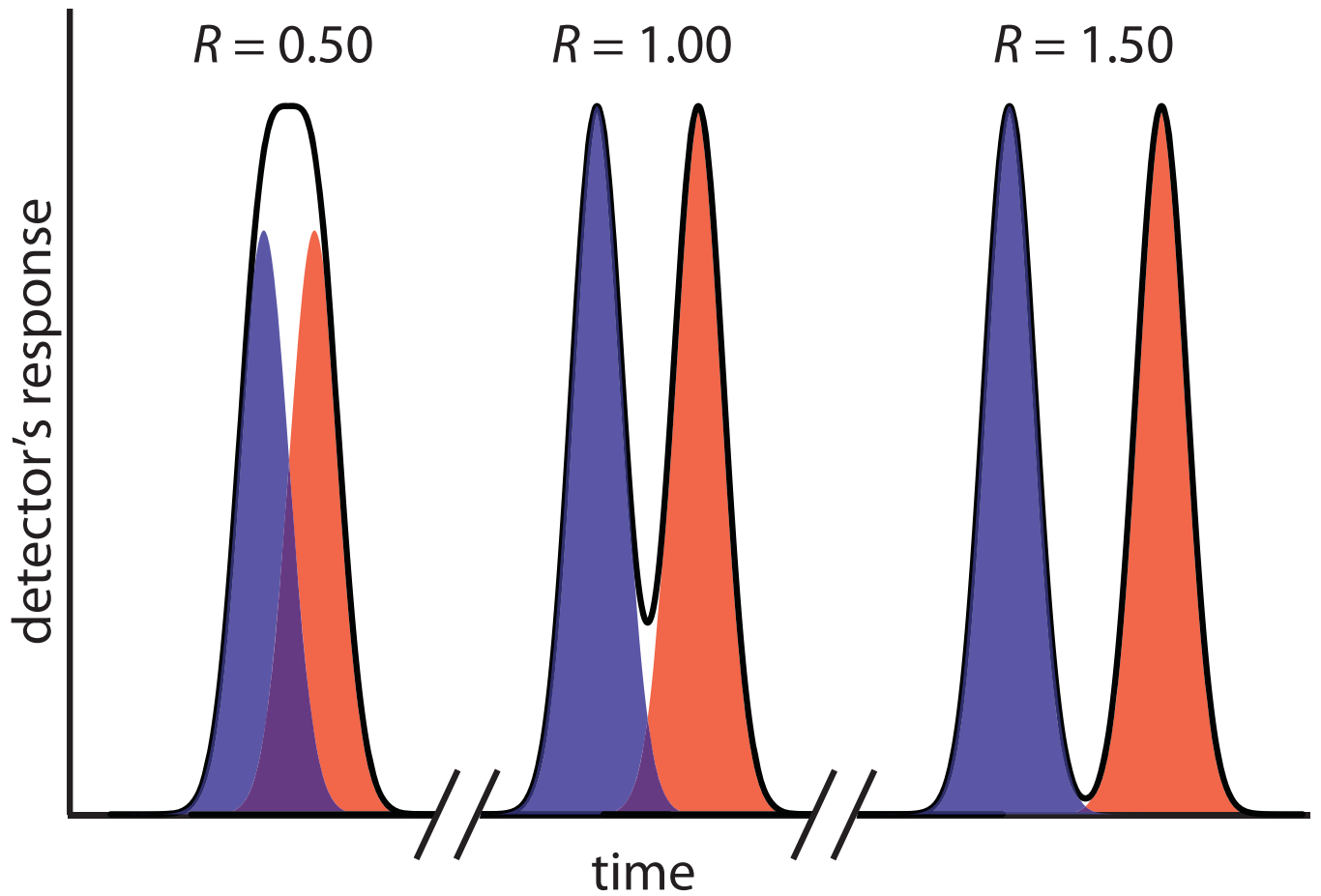


Figure 12.10 Three examples showing the relationship between resolution and the separation of a two component mixture. The blue and red peaks are the elution profiles for the two components. The chromatographic peak—which is the sum of the two elution profiles—is shown by the solid black line.

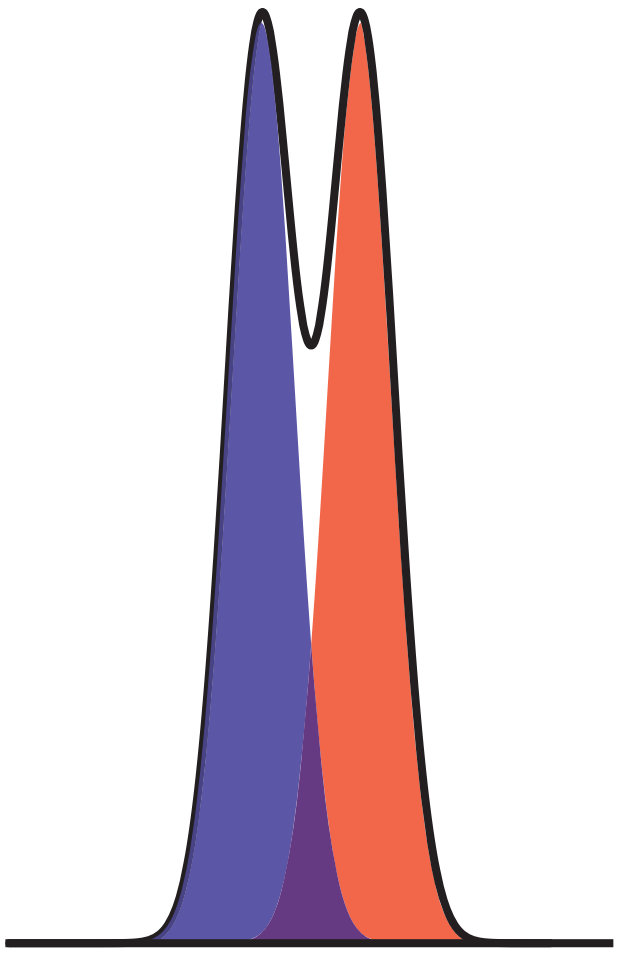


Figure 12.11 Chromatogram for Practice Exercise 12.1.

Practice Exercise 12.1

Figure 12.11 shows the separation of a two-component mixture. What is the resolution between the two components? Use a ruler to measure Δt_r, w_A, and w_B in millimeters.

Click here to review your answer to this exercise.

Equation 12.1 suggests that we can improve resolution by increasing Δt_r, or by decreasing w_A and w_B (Figure 12.12). To increase Δt_r we can use one of two strategies. One approach is to adjust the separation conditions so that both solutes spend less time in the mobile phase—that is, we increase each *solute's retention factor*—which provides more time to effect a separation. A second approach is to increase *selectivity* by adjusting conditions so that only one solute experiences a significant change in its retention time. The baseline width of a solute's peak depends on the solutes movement within and between the mobile phase and the stationary phase, and is governed by several factors that we collectively call *column efficiency*. We will consider each of these approaches for improving resolution in more detail, but first we must define some terms.

12B.2 Solute Retention Factor

Let's assume that we can describe a solute's distribution between the mobile phase and stationary phase using the following equilibrium reaction

$$S_m \rightleftharpoons S_s$$

where S_m is the solute in the mobile phase and S_s is the solute in the stationary phase. Following the same approach that we used in Section 7G.2 for liquid–liquid extractions, the equilibrium constant for this reaction is an equilibrium partition coefficient, K_D.

This is not a trivial assumption. In this section we are, in effect, treating the solute's equilibrium between the mobile phase and the stationary phase as if it is identical to the equilibrium in a liquid–liquid extraction. You might question whether this is a reasonable assumption.

There is an important difference between the two experiments we need to consider. In a liquid–liquid extraction, which takes place in a separatory funnel, the two phases remain in contact with each other at all times, allowing for a true equilibrium. In chromatography, however, the mobile phase is in constant motion. A solute moving into the stationary phase from the mobile phase equilibrates back into a different portion of the mobile phase; this does not describe a true equilibrium.

So, we ask again: Can we treat a solute's distribution between the mobile phase and the stationary phase as an equilibrium process? The answer is yes, if the mobile phase velocity is slow relative to the kinetics of the solute's moving back and forth between the two phase. In general, this is a reasonable assumption.

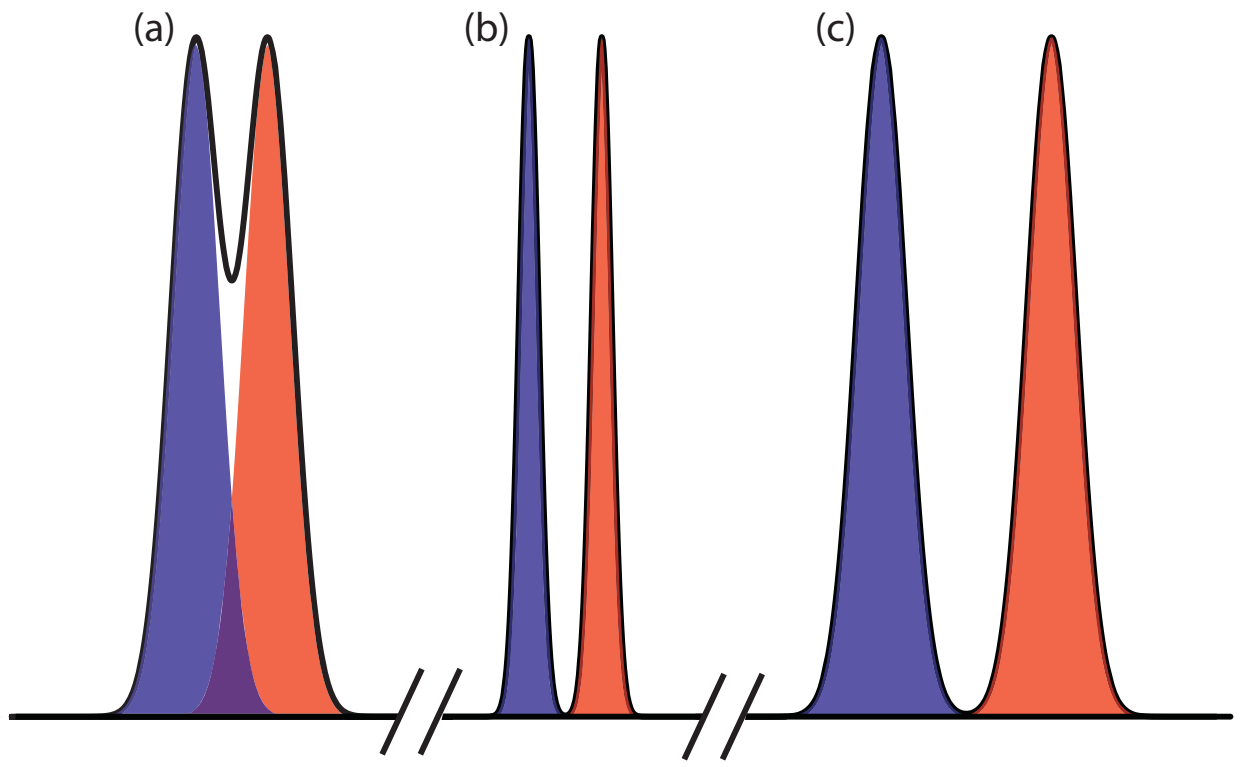


Figure 12.12 Two method for improving chromatographic resolution: (a) original chromatogram; (b) chromatogram after decreasing w_A and w_B by 4×; (c) chromatogram after increasing Δt_r by 2×.

$$K_D = \frac{[S_s]}{[S_m]}$$

In the absence of any additional equilibrium reactions in the mobile phase or stationary phase, K_D is equivalent to the distribution ratio, D,

$$D = \frac{[S_s]}{[S_m]} = \frac{(\text{mol S})_s / V_s}{(\text{mol S})_m / V_m} = K_D \qquad 12.2$$

where V_s and V_m are the volumes of the stationary phase and the mobile phase, respectively.

A conservation of mass requires that the total moles of solute remain constant throughout the separation; thus, we know that the following equation is true.

$$(\text{mol S})_{total} = (\text{mol S})_m + (\text{mol S})_s \qquad 12.3$$

Solving equation 12.3 for the moles of solute in the stationary phase and substituting into equation 12.2 leaves us with

$$D = \frac{\left\{(\text{mol S})_{total} - (\text{mol S})_s\right\} / V_s}{(\text{mol S})_m / V_m}$$

Rearranging this equation and solving for the fraction of solute in the mobile phase, f_m, gives a result

$$f_m = \frac{(\text{mol S})_m}{(\text{mol S})_{total}} = \frac{V_m}{DV_s + V_m} \qquad 12.4$$

that is identical to equation 7.26 for a liquid–liquid extraction. Because we may not know the exact volumes of the stationary phase and the mobile phase, we can simplify equation 12.4 by dividing both the numerator and the denominator by V_m; thus

$$f_m = \frac{V_m / V_m}{DV_s / V_m + V_m / V_m} = \frac{1}{DV_s / V_m + 1} = \frac{1}{1 + k} \qquad 12.5$$

where k

$$k = D \times \frac{V_s}{V_m} \qquad 12.6$$

is the solute's RETENTION FACTOR. Note that the larger the retention factor, the more the distribution ratio favors the stationary phase, leading to a more strongly retained solute and a longer retention time.

We can determine a solute's retention factor from a chromatogram by measuring the column's void time, t_m, and the solute's retention time, t_r (see Figure 12.9). Solving equation 12.5 for k, we find that

The retention factor is also called the capacity factor, the capacity ratio, and the partition ratio, and is sometimes given the symbol k'. Keep this in mind if you are using other resources. Retention factor is the approved name from the IUPAC Gold Book.

$$k = \frac{1 - f_m}{f_m} \qquad 12.7$$

Earlier we defined f_m as the fraction of solute in the mobile phase. Assuming a constant mobile phase velocity, we also can define f_m as

$$f_m = \frac{\text{time spent in mobile phase}}{\text{total time spent on column}} = \frac{t_m}{t_r}$$

Substituting back into equation 12.7 and rearranging leaves us with

$$k = \frac{1 - t_m / t_r}{t_m / t_r} = \frac{t_r - t_m}{t_m} = \frac{t_r'}{t_m} \qquad 12.8$$

where t_r' is the ADJUSTED RETENTION TIME.

Example 12.2

In a chromatographic analysis of low molecular weight acids, butyric acid elutes with a retention time of 7.63 min. The column's void time is 0.31 min. Calculate the retention factor for butyric acid.

SOLUTION

$$k_{but} = \frac{t_r - t_m}{t_m} = \frac{7.63 \text{ min} - 0.31 \text{ min}}{0.31 \text{ min}} = 23.6$$

Practice Exercise 12.2

Figure 12.13 is the chromatogram for a two-component mixture. Determine the retention factor for each solute assuming the sample was injected at time $t = 0$.

Click here to review your answer to this exercise.

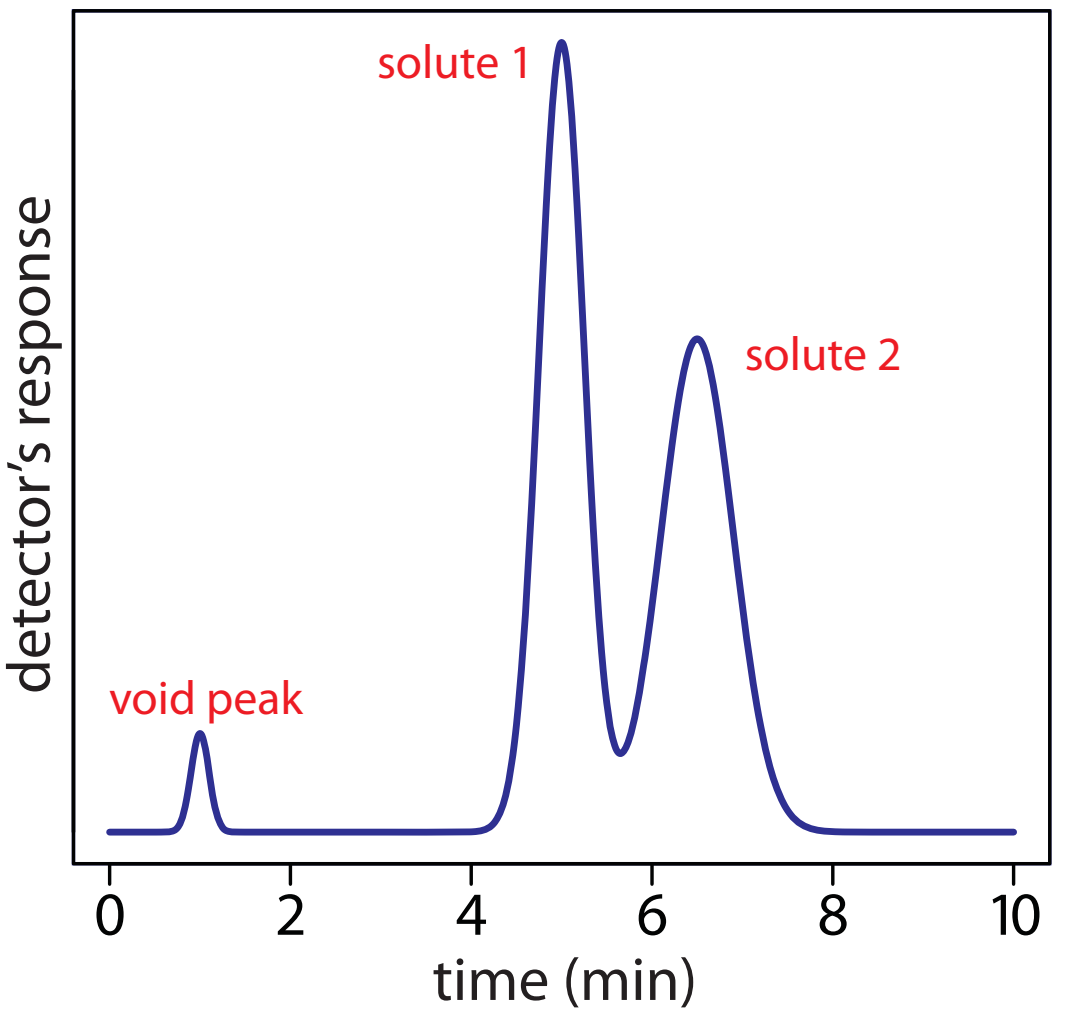


Figure 12.13 Chromatogram for Practice Exercise 12.2.

12B.3 Selectivity

Selectivity is a relative measure of the retention of two solutes, which we define using a SELECTIVITY FACTOR, α

$$\alpha = \frac{k_B}{k_A} = \frac{t_{r,B} - t_m}{t_{r,A} - t_m} \qquad 12.9$$

where solute A always has the smaller retention time. When two solutes elute with identical retention time, $\alpha = 1.00$; for all other conditions $\alpha > 1.00$.

Example 12.3

In the chromatographic analysis for low molecular weight acids described in Example 12.2, the retention time for isobutyric acid is 5.98 min. What is the selectivity factor for isobutyric acid and butyric acid?

SOLUTION

First we must calculate the retention factor for isobutyric acid. Using the void time from Example 12.2.

$$k_{iso} = \frac{t_r - t_m}{t_m} = \frac{5.98 \text{ min} - 0.31 \text{ min}}{0.31 \text{ min}} = 18.3$$

The selectivity factor, therefore, is

$$\alpha = \frac{k_{but}}{k_{iso}} = \frac{23.6}{18.3} = 1.29$$

Practice Exercise 12.3

Determine the selectivity factor for the chromatogram in Practice Exercise 12.2.

Click here to review your answer to this exercise.

12B.4 Column Efficiency

Suppose we inject a sample consisting of a single component. At the moment of injection the sample occupies a narrow band of finite width. As the sample passes through the column, the width of this band continually increases in a process we call BAND BROADENING. Column efficiency provides a quantitative measure of the extent of band broadening.

In their original theoretical model of chromatography, Martin and Synge divided the chromatographic column into discrete sections—what they called THEORETICAL PLATES—in which there is an equilibrium partitioning of the solute between the stationary phase and the mobile phase.[2] They described column efficiency in terms of the number of theoretical plates, N,

See Figure 12.6 and Figure 12.7. When we inject the sample it has a uniform, or rectangular concentration profile with respect to distance down the column. As it passes through the column, the band broadens and takes on a Gaussian concentration profile.

2 Martin, A. J. P.; Synge, R. L. M. *Biochem. J.* **1941**, *35*, 1358–1366.

$$N = \frac{L}{H} \qquad 12.10$$

where L is the column's length and H is the height of a theoretical plate. Column efficiency improves—and chromatographic peaks become narrower—when there are more theoretical plates.

If we assume that a chromatographic peak has a Gaussian profile, then the extent of band broadening is given by the peak's variance or standard deviation. The height of a theoretical plate is the variance per unit length of the column

$$H = \frac{\sigma^2}{L} \qquad 12.11$$

where the standard deviation, σ, has units of distance. Because retention times and peak widths are usually measured in seconds or minutes, it is more convenient to express the standard deviation in units of time, τ, by dividing σ by the solute's average linear velocity, $\bar{v}$.

The solute's average linear velocity is the distance it travels, L, divided by its retention time, t_r.

$$\tau = \frac{\sigma}{\bar{v}} = \frac{\sigma t_r}{L} \qquad 12.12$$

For a Gaussian peak shape, the width at the baseline, w, is four times its standard deviation, τ.

$$w = 4\tau \qquad 12.13$$

See Figure 12.9 for a review of how to determine values for t_r and w.

Combining equation 12.11, equation 12.12, and equation 12.13 defines the height of a theoretical plate in terms of the easily measured chromatographic parameters t_r and w.

$$H = \frac{Lw^2}{16t_r^2} \qquad 12.14$$

Combing equation 12.14 and equation 12.10 gives the number of theoretical plates.

$$N = 16\frac{t_r^2}{w^2} \qquad 12.15$$

Example 12.4

A chromatographic analysis for the chlorinated pesticide Dieldrin gives a peak with a retention time of 8.68 min and a baseline width of 0.29 min. What is the number of theoretical plates? Given that the column is 2.0 m long, what is the height of a theoretical plate in mm?

Solution

Using equation 12.15, the number of theoretical plates is

$$N = 16\frac{t_r^2}{w^2} = N = 16\frac{(8.68\text{ min})^2}{(0.29\text{ min})^2} = 14300\text{ plates}$$

Solving equation 12.10 for H gives the average height of a theoretical plate as

$$H = \frac{L}{N} = \frac{2.0\text{ m}}{14300\text{ plates}} \times \frac{1000\text{ mm}}{\text{m}} = 0.14\text{ mm/plate}$$

Practice Exercise 12.4

For each solute in the chromatogram for Practice Exercise 12.2, calculate the number of theoretical plates and the average height of a theoretical plate. The column is 0.5 m long.

Click here to review your answer to this exercise.

It is important to remember that a theoretical plate is an artificial construct and that a chromatographic column does not contain physical plates. In fact, the number of theoretical plates depends on both the properties of the column and the solute. As a result, the number of theoretical plates for a column may vary from solute to solute.

12B.5 Peak Capacity

One advantage of improving column efficiency is that we can separate more solutes with baseline resolution. One estimate of the number of solutes that we can separate is

$$n_c = 1 + \frac{\sqrt{N}}{4}\ln\frac{V_{max}}{V_{min}} \qquad 12.16$$

where n_c is the column's PEAK CAPACITY, and V_{min} and V_{max} are the smallest and the largest volumes of mobile phase in which we can elute and detect a solute.[3] A column with 10 000 theoretical plates, for example, can resolve no more than

The smallest volume we can use is the column's void volume. The largest volume is determined either by our patience—what is the maximum analysis time we can tolerate—or by our inability to detect solutes because there is too much band broadening.

$$n_c = 1 + \frac{\sqrt{10000}}{4}\ln\frac{30\text{ mL}}{1\text{ mL}} = 86\text{ solutes}$$

if V_{min} and V_{max} are 1 mL and 30 mL, respectively. This estimate provides an upper bound on the number of solutes and may help us exclude from consideration a column that does not have enough theoretical plates to separate a complex mixture. Just because a column's theoretical peak capacity is larger than the number of solutes, however, does not mean that a sepa-

3 Giddings, J. C. *Unified Separation Science*, Wiley-Interscience: New York, 1991.

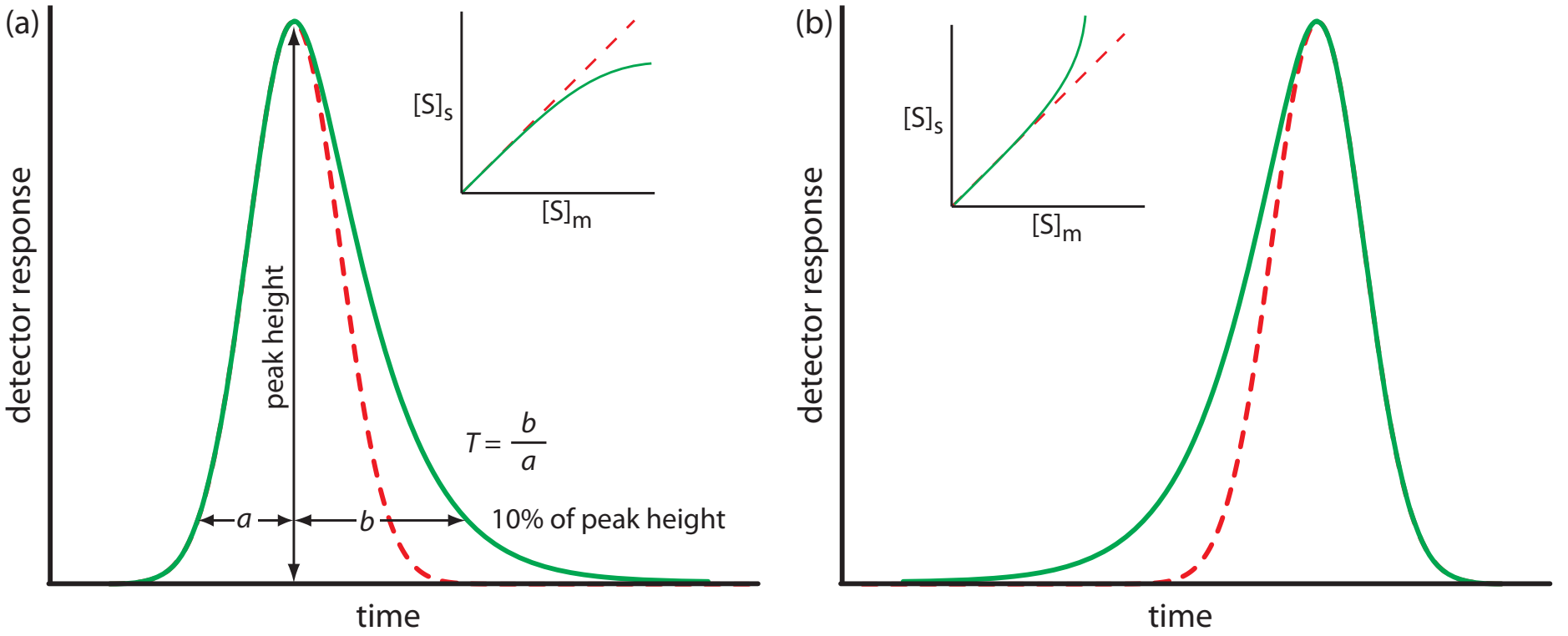


Figure 12.14 Examples of asymmetric chromatographic peaks showing (a) peak tailing and (b) peak fronting. For both (a) and (b) the green chromatogram is the asymmetric peak and the red dashed chromatogram shows the ideal, Gaussian peak shape. The insets show the relationship between the concentration of solute in the stationary phase, $[S]_s$, and its concentration in the mobile phase, $[S]_m$. The dashed red lines are for ideal behavior (K_D is constant for all conditions) and the green lines show nonideal behavior (K_D decreases or increases for higher total concentrations of solute). A quantitative measure of peak tailing, T, is shown in (a).

ration is feasible. In most situations the practical peak capacity is less than the estimated value because the retention characteristics of some solutes are so similar that a separation is impossible. Nevertheless, columns with more theoretical plates, or with a greater range of possible elution volumes, are more likely to separate a complex mixture.

12B.6 Asymmetric Peaks

Our treatment of chromatography in this section assumes that a solute elutes as a symmetrical Gaussian peak, such as that shown in Figure 12.9. This ideal behavior occurs when the solute's partition coefficient, K_D

$$K_D = \frac{[S_s]}{[S_m]}$$

is the same for all concentrations of solute. If this is not the case, then the chromatographic peak has an asymmetric peak shape similar to those shown in Figure 12.14. The chromatographic peak in Figure 12.14a is an example of peak TAILING, which occurs when some sites on the stationary phase retain the solute more strongly than other sites. Figure 12.14b, which is an example of peak FRONTING is most often the result of overloading the column with sample.

As shown in Figure 12.14a, we can report a peak's asymmetry by drawing a horizontal line at 10% of the peak's maximum height and measuring the distance from each side of the peak to a line drawn vertically through the peak's maximum. The asymmetry factor, T, is defined as

$$T = \frac{b}{a}$$

The number of theoretical plates for an asymmetric peak shape is approximately

$$N \approx \frac{41.7 \times \dfrac{t_r^2}{\left(w_{0.1}\right)^2}}{T + 1.25} = \frac{41.7 \times \dfrac{t_r^2}{\left(a+b\right)^2}}{T + 1.25}$$

where $w_{0.1}$ is the width at 10% of the peak's height.[4]

Asymmetric peaks have fewer theoretical plates, and the more asymmetric the peak the smaller the number of theoretical plates. For example, the following table gives values for N for a solute eluting with a retention time of 10.0 min and a peak width of 1.00 min.

b	a	T	N
0.5	0.5	1.00	1850
0.6	0.4	1.50	1520
0.7	0.3	2.33	1160
0.8	0.2	4.00	790
0.9	0.1	9.00	410

12C Optimizing Chromatographic Separations

Now that we have defined the solute retention factor, selectivity, and column efficiency we are able to consider how they affect the resolution of two closely eluting peaks. Because the two peaks have similar retention times, it is reasonable to assume that their peak widths are nearly identical. Equation 12.1, therefore, becomes

$$R_{AB} = \frac{t_{r,B} - t_{r,A}}{0.5(w_B + w_A)} \approx \frac{t_{r,B} - t_{r,A}}{0.5(2w_B)} = \frac{t_{r,B} - t_{r,A}}{w_B} \qquad 12.17$$

where B is the later eluting of the two solutes. Solving equation 12.15 for w_B and substituting into equation 12.17 leaves us with the following result.

If the number of theoretical plates is the same for all solutes—not strictly true, but not a bad assumption—then from equation 12.15, the ratio t_r/w is a constant. If two solutes have similar retention times, then their peak widths must be similar.

$$R_{AB} = \frac{\sqrt{N}}{4} \times \frac{t_{r,B} - t_{r,A}}{t_{r,B}} \qquad 12.18$$

Rearranging equation 12.8 provides us with the following equations for the retention times of solutes A and B.

$$t_{r,A} = k_A t_m + t_m \qquad t_{r,B} = k_B t_m + t_m$$

After substituting these equations into equation 12.18 and simplifying, we have

$$R_{AB} = \frac{\sqrt{N}}{4} \times \frac{k_B - k_A}{1 + k_B}$$

Finally, we can eliminate solute A's retention factor by substituting in equation 12.9. After rearranging, we end up with the following equation for the resolution between the chromatographic peaks for solutes A and B.

$$R_{AB} = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_B}{1 + k_B} \qquad 12.19$$

4 Foley, J. P.; Dorsey, J. G. *Anal. Chem.* **1983**, *55*, 730–737.

In addition to resolution, another important factor in chromatography is the amount of time needed to elute a pair of solutes, which we can approximate using the retention time for solute B.

$$t_{r,B} = \frac{16R_{AB}^2 H}{u} \times \left(\frac{\alpha}{\alpha - 1}\right)^2 \times \frac{(1 + k_B)^3}{k_B^2} \qquad 12.20$$

where u is the mobile phase's velocity.

Equation 12.19 and equation 12.20 contain terms corresponding to column efficiency, selectivity, and the solute retention factor. We can vary these terms, more or less independently, to improve resolution and analysis time. The first term, which is a function of the number of theoretical plates (for equation 12.19) or the height of a theoretical plate (for equation 12.20), accounts for the effect of column efficiency. The second term is a function of α and accounts for the influence of column selectivity. Finally, the third term in both equations is a function of k_B and accounts for the effect of solute B's retention factor. A discussion of how we can use these parameters to improve resolution is the subject of the remainder of this section.

12C.1 Using the Retention factor to Optimize Resolution

One of the simplest ways to improve resolution is to adjust solute B's retention factor. If all other terms in equation 12.19 remain constant, increasing k_B improves resolution. As shown by the green curve in Figure 12.15, however, the improvement is greatest if the initial value of k_B is small. Once k_B exceeds a value of approximately 10, a further increase produces only a marginal improvement in resolution. For example, if the original value of k_B is 1, increasing its value to 10 gives an 82% improvement in resolution; a further increase to 15 provides a net improvement in resolution of only 87.5%.

Any improvement in resolution by increasing the value of k_B generally comes at the cost of a longer analysis time. The red curve in Figure 12.15

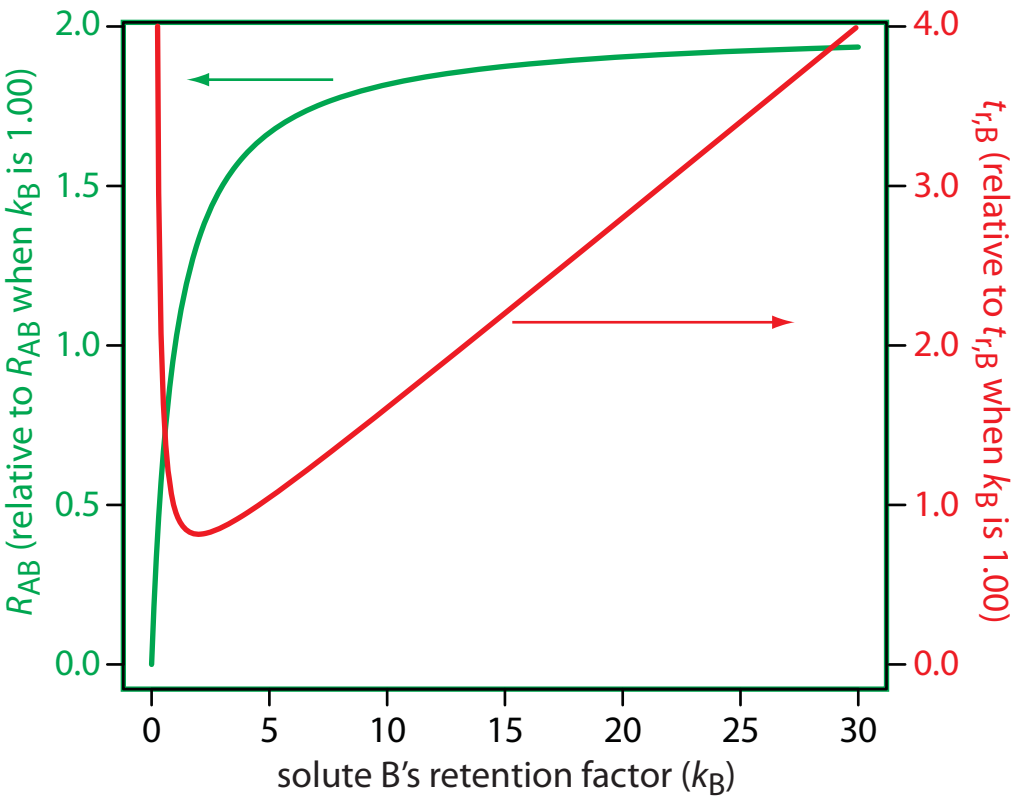


Figure 12.15 Effect of k_B on the resolution for a pair of solutes, R_{AB}, and the retention time for the later eluting solute, $t_{r,B}$. The y-axes display the resolution and retention time relative to their respective values when k_B is 1.00.

shows the relative change in solute B's retention time as a function of its retention factor. Note that the minimum retention time is for $k_B = 2$. Increasing k_B from 2 to 10, for example, approximately doubles solute B's retention time.

To increase k_B without significantly changing the selectivity, α, any change to the chromatographic conditions must result in a general, non-selective increase in the retention factor for both solutes. In gas chromatography, we can accomplish this by decreasing the column's temperature. Because a solute's vapor pressure is smaller at lower temperatures, it spends more time in the stationary phase and takes longer to elute. In liquid chromatography, the easiest way to increase a solute's retention factor is to use a mobile phase that is a weaker solvent. When the mobile phase has a lower solvent strength, solutes spend proportionally more time in the stationary phase and take longer to elute.

Adjusting the retention factor to improve the resolution between one pair of solutes may lead to unacceptably long retention times for other solutes. For example, suppose we need to analyze a four-component mixture with baseline resolution and with a run-time of less than 20 min. Our initial choice of conditions gives the chromatogram in Figure 12.16a. Although we successfully separate components 3 and 4 within 15 min, we fail to separate components 1 and 2. Adjusting the conditions to improve the resolution for the first two components by increasing $k_{B,2}$ provides a good separation of all four components, but the run-time is now too long (Figure 12.16b). This problem of finding a single set of acceptable operating conditions is known as the GENERAL ELUTION PROBLEM.

One solution to the general elution problem is to make incremental adjustments to the retention factor over time. Thus, we choose our initial chromatographic conditions to optimize the resolution for early eluting solutes. As the separation progresses, we adjust the chromatographic conditions to decrease k_B—and, therefore, decrease the retention time—for each of the later eluting solutes (Figure 12.16c). In gas chromatography this is accomplished by temperature programming. The column's initial temperature is selected such that the first solutes to elute are fully resolved. The temperature is then increased, either continuously or in steps, to bring off later eluting components with both an acceptable resolution and a reasonable analysis time. In liquid chromatography the same effect is obtained by increasing the solvent's eluting strength. This is known as a gradient elution. We will have more to say about each of these in later sections of this chapter.

The relationship between retention factor and analysis time in Figure 12.15 works to our advantage if a separation produces an acceptable resolution with a large k_B. In this case we may be able to decrease k_B with little loss in resolution and with a significantly shorter analysis time.

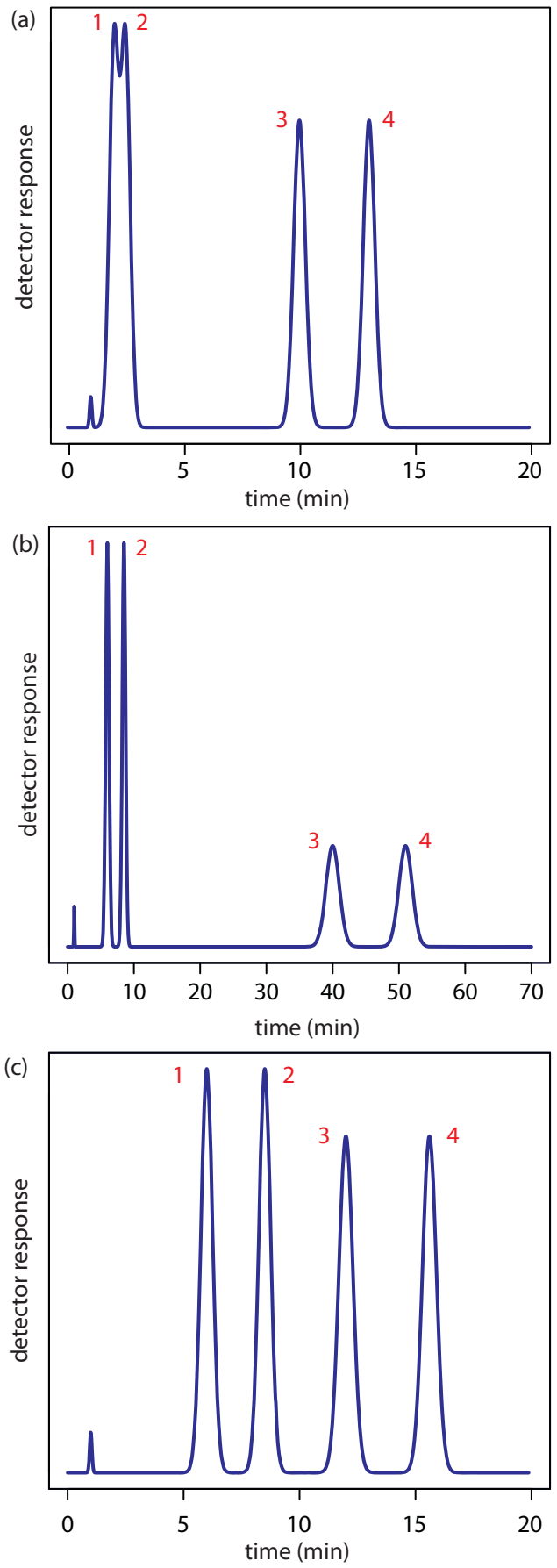


Figure 12.16 Example showing the general elution problem in chromatography. See text for details.

12C.2 Using Selectivity to Optimize Resolution

A second approach to improving resolution is to adjust the selectivity, α. In fact, for $\alpha \approx 1$ it usually is not possible to improve resolution by adjusting the solute retention factor, k_B, or column efficiency, N. A change in α often has a more dramatic effect on resolution than a change in k_B. For example,

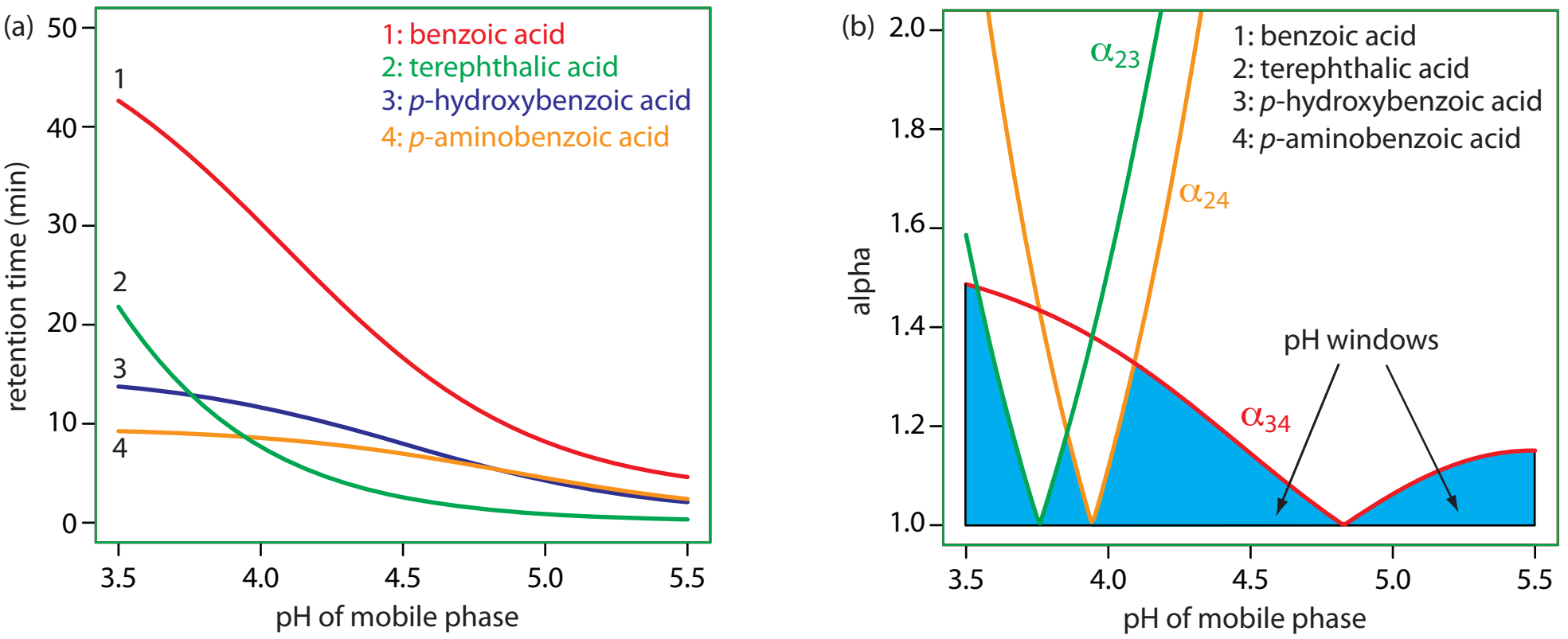


Figure 12.17 Example showing how the mobile phase pH in liquid chromatography affects selectivity: (a) retention times for four substituted benzoic acids as a function of the mobile phase's pH; (b) alpha values for three pairs of solutes that are difficult to separate. See text for details. The mobile phase is an acetic acid/sodium acetate buffer and the stationary phase is a nonpolar hydrocarbon. Data from Harvey, D. T.; Byerly, S.; Bowman, A.; Tomlin, J. "Optimization of HPLC and GC Separations Using Response Surfaces," *J. Chem. Educ.* **1991**, *68*, 162–168.

changing α from 1.1 to 1.5, while holding all other terms constant, improves resolution by 267%. In gas chromatography, we usually adjust α by changing the stationary phase, and we usually change the composition of the mobile phase in liquid chromatography.

To change α we need to selectively adjust individual solute retention factors. Figure 12.17 shows one possible approach for the liquid chromatographic separation of a mixture of substituted benzoic acids. Because the components are weak acids, their retention times vary with the pH of the mobile phase, as shown in Figure 12.17a. The intersections of these curves show pH values where two solutes co-elute. For example, at a pH of 3.8 terephthalic acid and *p*-hydroxybenzoic acid elute as a single chromatographic peak.

Figure 12.17a shows that there are many pH values where some separation is possible. To find the optimum separation, we plot α for each pair of solutes. The red, green, and orange curves in Figure 12.17b show the variation in α with pH for the three pairs of solutes that are hardest to separate (for all other pairs of solutes, $\alpha > 2$ at all pH levels). The blue shading shows windows of pH values in which at least a partial separation is possible—this figure is sometimes called a window diagram—and the highest point in each window gives the optimum pH within that range. The best overall separation is the highest point in any window, which, for this example, is a pH of 3.5. Because the analysis time at this pH is more than 40 min (see Figure 12.17a), choosing a pH between 4.1–4.4 might produce an acceptable separation with a much shorter analysis time.

Let's use benzoic acid, C_6H_5COOH, to explain why pH can affect a solute's retention time. The separation uses an aqueous mobile phase and a nonpolar stationary phase. At lower pHs, benzoic acid is predominately in its weak acid form, C_6H_5COOH, and easily partitions into the nonpolar stationary phase. At more basic pHs, however, benzoic acid is in its weak base form, $C_6H_5COO^-$. Because it now carries a charge, its solubility in the mobile phase increases and its solubility in the nonpolar stationary phase decreases. As a result, it spends more time in the mobile phase and has a shorter retention time.

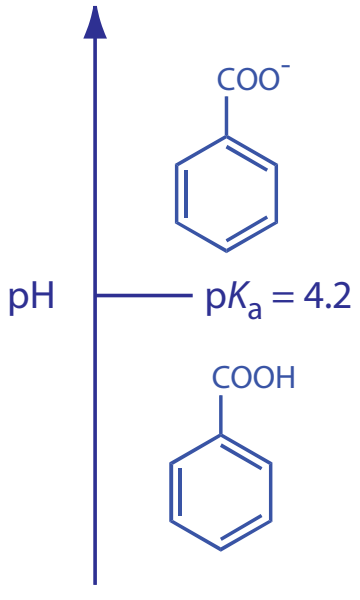


Table 12.1 Minimum Number of Theoretical Plates to Achieve Desired Resolution for Selected Values of k_B and α

	$R_{AB} = 1.00$		$R_{AB} = 1.25$		$R_{AB} = 1.50$	
k_B	$\alpha = 1.05$	$\alpha = 1.10$	$\alpha = 1.05$	$\alpha = 1.10$	$\alpha = 1.05$	$\alpha = 1.10$
0.5	63500	17400	99200	27200	143000	39200
1.0	28200	7740	44100	12100	63500	17400
1.5	19600	5380	30600	8400	44100	12100
2.0	15900	4360	24800	6810	35700	9800
3.0	12500	3440	19600	5380	28200	7740
5.0	10200	2790	15900	4360	22900	6270
10.0	8540	2340	13300	3660	19200	5270

12C.3 Using Column Efficiency to Optimize Resolution

A third approach to improving resolution is to adjust the column's efficiency by increasing the number of theoretical plates, N. If we have values for k_B and α, then we can use equation 12.19 to calculate the number of theoretical plates for any resolution. Table 12.1 provides some representative values. For example, if $\alpha = 1.05$ and $k_B = 2.0$, a resolution of 1.25 requires approximately 24800 theoretical plates. If our column provides only 12400 plates, half of what is needed, then a separation is not possible. How can we double the number of theoretical plates? The easiest way is to double the length of the column, although this also doubles the analysis time. A better approach is to cut the height of a theoretical plate, H, in half, providing the desired resolution without changing the analysis time. Even better, if we can decrease H by more than 50%, it may be possible to achieve the desired resolution with an even shorter analysis time by decreasing k_B or α.

To decrease the height of a theoretical plate we need to understand the experimental factors that affect band broadening. There are several theoretical treatments of band broadening. We will consider one approach that considers four contributions: variations in paths length, longitudinal diffusion, mass transfer in the stationary phase, and mass transfer in the mobile phase.

Multiple Paths: Variations in Path Length

As solute molecules pass through the column they travel paths that differ in length. Because of this difference in path length, solute molecules entering the column at the same time, exit the column at different times. The result, as shown in Figure 12.18, is band broadening. The contribution of MULTIPLE PATHS to the height of a theoretical plate, H_p, is

$$H_p = 2\lambda d_p \qquad 12.21$$

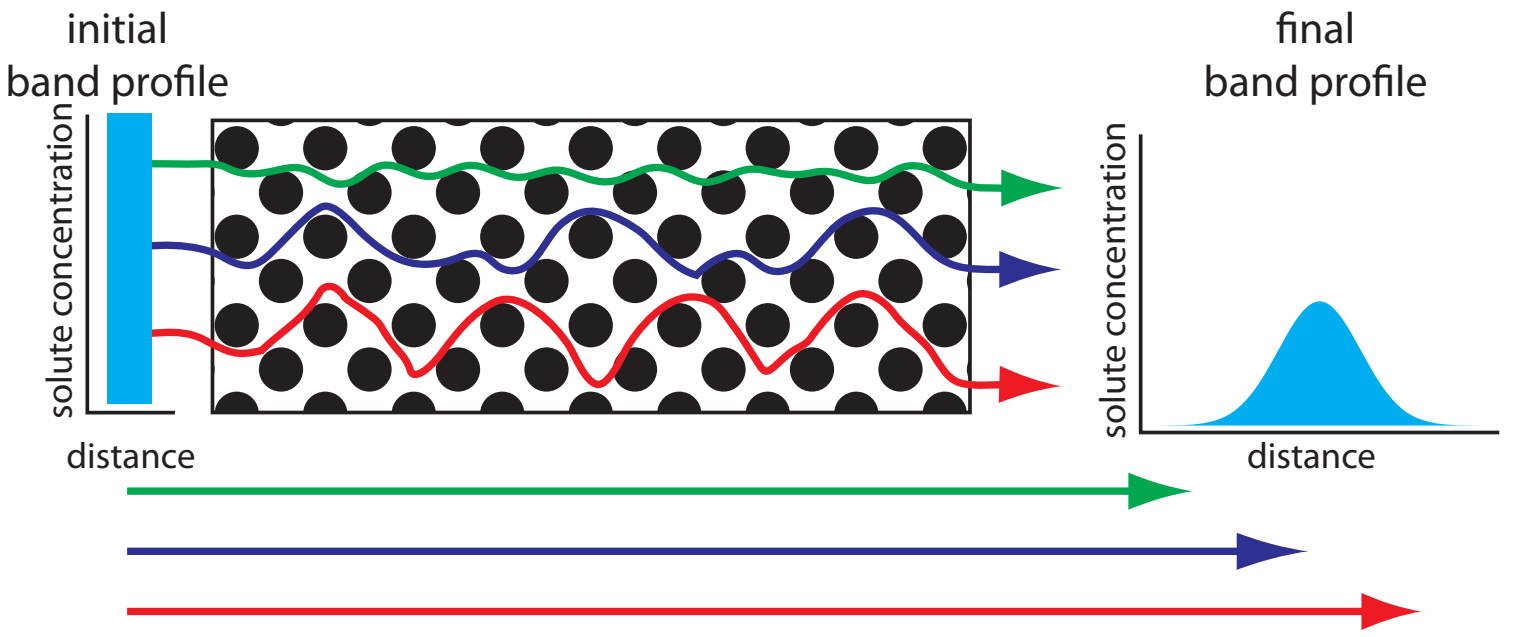


Figure 12.18 The effect of multiple paths on a solute's band broadening. The solute's initial band profile is rectangular. As this band travels through the column, individual solute molecules travel different paths, three of which are shown by the meandering colored paths (the actual lengths of these paths are shown by the straight arrows at the bottom of the figure). Most solute molecules travel paths with lengths similar to that shown in blue, with a few traveling much shorter paths (green) or much longer paths (red). As a result, the solute's band profile at the end of the column is broader and Gaussian in shape.

Inconsistent packing can produces channels through the column that allow some solute molecules to travel quickly through the column. It also can lead to pockets that can temporarily trap some solute molecules, slowing their progress through the column. A more uniform packing minimizes these two problems.

where d_p is the average diameter of the particulate packing material, and λ is a constant that accounts for the consistency of the packing. A smaller range of particle sizes and a more consistent packing produce a smaller value for λ. For a column without packing material, H_p is zero and there is no contribution to band broadening from multiple paths.

Longitudinal Diffusion

The second contribution to band broadening is the result of the solute's LONGITUDINAL DIFFUSION in the mobile phase. Solute molecules are constantly in motion, diffusing from regions of higher solute concentration to regions where the concentration of solute is smaller. The result is an increase in the solute's band width (Figure 12.19). The contribution of longitudinal diffusion to the height of a theoretical plate, H_d, is

$$H_d = \frac{2\gamma D_m}{u} \qquad 12.22$$

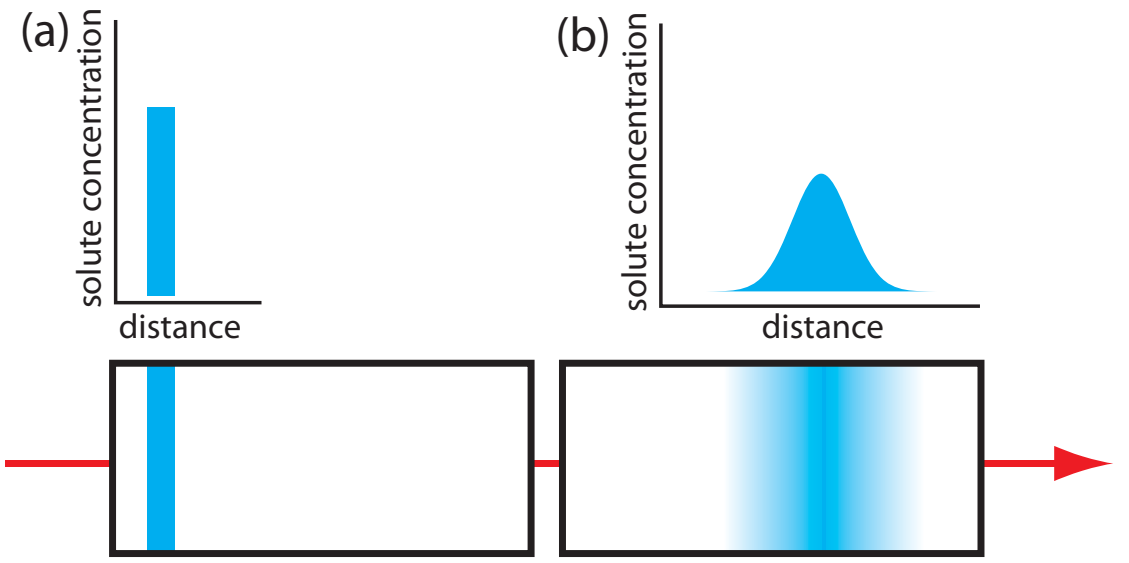


Figure 12.19 The effect of longitudinal diffusion on a solute's band broadening. Two horizontal cross-sections through the column and the corresponding concentration versus distance profiles are shown, with (a) being earlier in time. The red arrow shows the direction in which the mobile phase is moving.

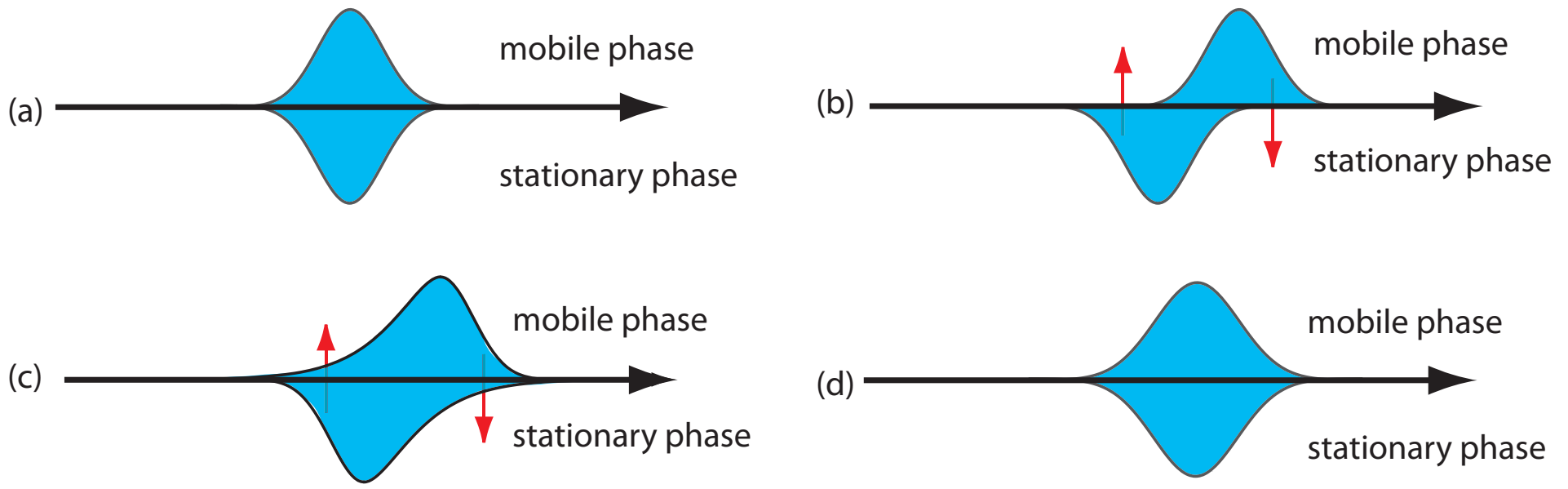


Figure 12.20 Effect of mass transfer on band broadening: (a) Ideal equilibrium Gaussian profiles for the solute in the mobile phase and in the stationary phase. (b, c) If we allow the solute's band to move a small distance down the column, an equilibrium between the two phases no longer exits. The red arrows show the movement of solute—what we call the mass transfer of solute—from the stationary phase to the mobile phase, and from the mobile phase to the stationary phase. (d) Once equilibrium is reestablished, the solute's band is now broader.

where D_m is the solute's diffusion coefficient in the mobile phase, u is the mobile phase velocity, and γ is a constant related to the efficiency of column packing. Note that the effect of H_d on band broadening is inversely proportional to the mobile phase velocity—a higher velocity provides less time for longitudinal diffusion. Because a solute's diffusion coefficient is larger in the gas phase than in a liquid phase, longitudinal diffusion is a more serious problem in gas chromatography.

Mass Transfer

As the solute passes through the column it moves between the mobile phase and the stationary phase. We call this movement between phases MASS TRANSFER. As shown in Figure 12.20, band broadening occurs if the solute's movement within the mobile phase or within the stationary phase is not fast enough to maintain an equilibrium partitioning of solute between the two phases. On average, solute molecules in the mobile phase move further down the column before passing into the stationary phase. Solute molecules in the stationary phase, on the other hand, take longer than expected to move back into the mobile phase. The contributions of mass transfer in the stationary phase, H_s, and mass transfer in the mobile phase, H_m, are given by the following equations

$$H_s = \frac{qkd_f^2}{(1+k)^2 D_s} u \qquad 12.23$$

$$H_m = \frac{fn(d_p^2, d_c^2)}{D_m} u \qquad 12.24$$

The abbreviation *fn* in equation 12.24 means "function of."

where d_f is the thickness of the stationary phase, d_c is the column's diameter, D_s and D_m are the solute's diffusion coefficient in the stationary phase and the mobile phase, k is the solute's retention factor, and q is a constant re-

lated to the column packing material. Although the exact form of H_m is not known, it is a function of particle size and column diameter. Note that the effect of H_s and H_m on band broadening is directly proportional to the mobile phase velocity—smaller velocities provide more time for mass transfer.

Putting it All Together

The height of a theoretical plate is a summation of the contributions from each of the terms affecting band broadening.

$$H = H_p + H_d + H_s + H_m \qquad 12.25$$

An alternative form of this equation is the VAN DEEMTER EQUATION

$$H = A + \frac{B}{u} + Cu \qquad 12.26$$

which emphasizes the importance of the mobile phase's velocity. In the van Deemter equation, A accounts for the contribution of multiple paths (H_p), B/u for the contribution of longitudinal diffusion (H_d), and Cu for the combined contribution of mass transfer in the stationary phase and mobile phase (H_s and H_m).

There is some disagreement on the best equation for describing the relationship between plate height and mobile phase velocity.[5] In addition to the van Deemter equation, other equations include

$$H = \frac{B}{u} + (C_s + C_m)u$$

where C_s and C_m are the mass transfer terms for the stationary phase and the mobile phase and

$$H = Au^{1/3} + \frac{B}{u} + Cu$$

All three equations, and others, have been used to characterize chromatographic systems, with no single equation providing the best explanation in every case.[6]

To increase the number of theoretical plates without increasing the length of the column we need to decrease one or more of the terms in equation 12.25. The easiest way to decrease H is by adjusting the velocity of the mobile phase. For smaller mobile phase velocities, column efficiency is limited by longitudinal diffusion, and at higher velocities efficiency is limited by the two mass transfer terms. As shown in Figure 12.21—which uses the van Deemter equation—the optimum mobile phase velocity is the minimum in a plot of H as a function of u.

The remaining parameters affecting the terms in equation 12.25 are functions of the column's properties and suggest other possible approaches

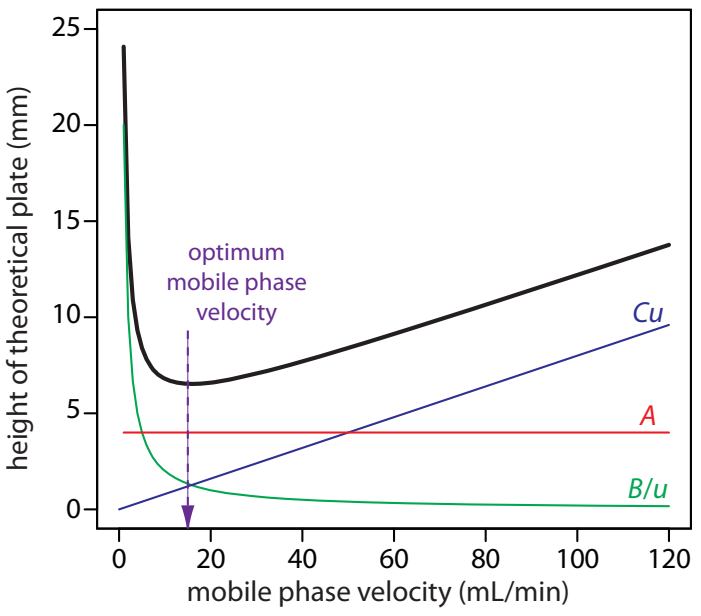


Figure 12.21 Plot showing the relationship between the height of a theoretical plate, H, and the mobile phase's velocity, u, based on the van Deemter equation.

5 Hawkes, S. J. *J. Chem. Educ.* **1983**, *60*, 393–398.
6 Kennedy, R. T.; Jorgenson, J. W. *Anal. Chem.* **1989**, *61*, 1128–1135.

to improving column efficiency. For example, both H_p and H_m are a function of the size of the particles used for packing the column. Decreasing particle size, therefore, is another useful method for improving efficiency.

The smaller the particles, the more pressure is needed to push the mobile phase through the column. As a result, for any form of chromatography there is a practical limit to particle size.

Perhaps the most important advancement in chromatography columns is the development of open-tubular, or CAPILLARY COLUMNS. These columns have very small diameters ($d_c \approx 50$–500 μm) and contain no packing material ($d_p = 0$). Instead, the interior wall of a capillary column is coated with a thin film of the stationary phase. Plate height is reduced because the contribution to H from H_p (equation 12.21) disappears and the contribution from H_m (equation 12.24) becomes smaller. Because the column does not contain any solid packing material, it takes less pressure to move the mobile phase through the column, allowing for much longer columns. The combination of a longer column and a smaller height for a theoretical plate increases the number of theoretical plates by approximately 100×. Capillary columns are not without disadvantages. Because they are much narrower than packed columns, they require a significantly smaller amount of sample, which may be difficult to inject reproducibly. Another approach to improving resolution is to use thin films of stationary phase, which decreases the contribution to H from H_s (equation 12.23).

12D Gas Chromatography

In GAS CHROMATOGRAPHY (GC) we inject the sample, which may be a gas or a liquid, into an gaseous mobile phase (often called the carrier gas). The mobile phase carries the sample through a packed or capillary column that separates the sample's components based on their ability to partition between the mobile phase and the stationary phase. Figure 12.22 shows an example of a typical gas chromatograph, which consists of several key

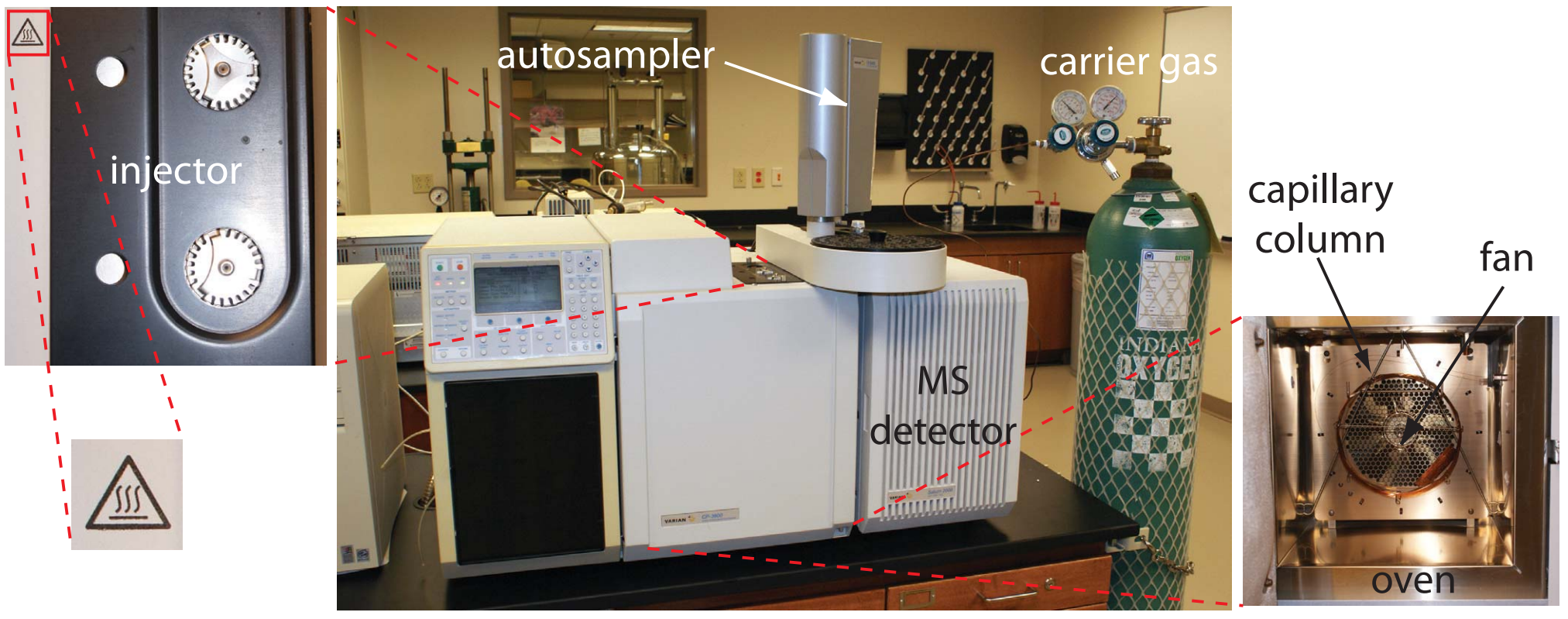


Figure 12.22 Example of a typical gas chromatograph with insets showing the heated injection ports—note the symbol indicating that it is hot—and the oven containing the column. This particular instrument is equipped with an autosampler for injecting samples, a capillary column, and a mass spectrometer (MS) as the detector. Note that the carrier gas is supplied by a tank of compressed gas.

components: a supply of compressed gas for the mobile phase; a heated injector, which rapidly volatilizes the components in a liquid sample; a column, which is placed within an oven whose temperature we can control during the separation; and a detector for monitoring the eluent as it comes off the column. Let's consider each of these components.

12D.1 Mobile Phase

The most common mobile phases for gas chromatography are He, Ar, and N_2, which have the advantage of being chemically inert toward both the sample and the stationary phase. The choice of carrier gas is often determined by the instrument's detector. For a packed column the mobile phase velocity is usually 25–150 mL/min. The typical flow rate for a capillary column is 1–25 mL/min.

12D.2 Chromatographic Columns

There are two broad classes of chromatographic columns: packed columns and capillary columns. In general, a packed column can handle larger samples and a capillary column can separate more complex mixtures.

Packed Columns

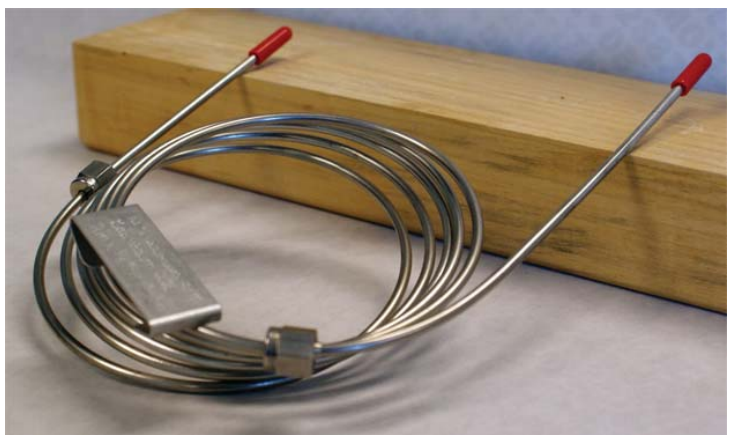
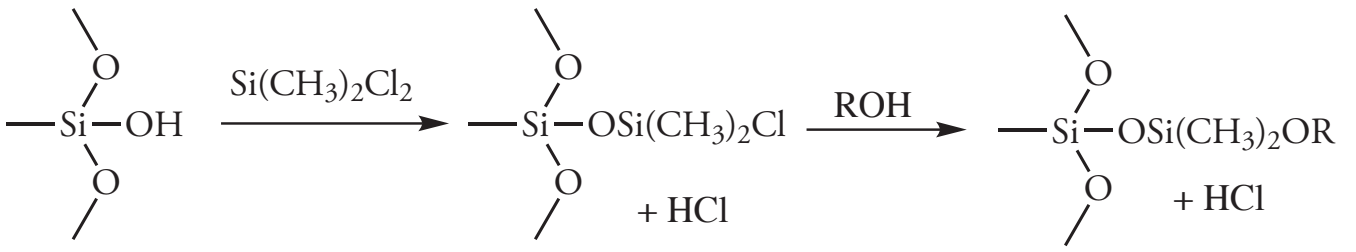


Figure 12.23 Typical example of a packed column for gas chromatography. This column is made from stainless steel and is 2 m long with an internal diameter of 3.2 mm. The packing material in this column has a particle diameter of 149–177 μm. To put this in perspective, beach sand has a typical diameter of 700 μm and the diameter of fine grained sand is 250 μm.

PACKED COLUMNS are constructed from glass, stainless steel, copper, or aluminum, and are typically 2–6 m in length with internal diameters of 2–4 mm. The column is filled with a particulate solid support, with particle diameters ranging from 37–44 μm to 250–354 μm. Figure 12.23 shows a typical example of a packed column.

The most widely used particulate support is diatomaceous earth, which is composed of the silica skeletons of diatoms. These particles are very porous, with surface areas ranging from 0.5–7.5 m^2/g, providing ample contact between the mobile phase and the stationary phase. When hydrolyzed, the surface of a diatomaceous earth contains silanol groups (–SiOH), which serve as active sites for absorbing solute molecules in GAS-SOLID CHROMATOGRAPHY (GSC).

In GAS-LIQUID CHROMATOGRAPHY (GLC), we coat the packing material with a liquid mobile phase. To prevent any uncoated packing material from adsorbing solutes, which degrades the quality of the separation, the surface silanols are deactivated by reacting them with dimethyldichlorosilane and rinsing with an alcohol—typically methanol—before coating the particles with stationary phase.



Other types of solid supports include glass beads and fluorocarbon polymers, which have the advantage of being more inert than diatomaceous earth.

To minimize the effect on plate height from multiple path and mass transfer, the diameter of the packing material should be as small as possible (see equation 12.21 and 12.25) and loaded with a thin film of stationary phase (see equation 12.23). Compared to capillary columns, which are discussed below, a packed column can handle larger sample volumes, typically 0.1–10 μL. Column efficiencies range from several hundred to 2000 plates/m, with a typical column having 3000–10 000 theoretical plates. The column in Figure 12.23, for example, has approximately 1800 plates/m, or a total of approximately 3600 theoretical plates. If we assume a $V_{max}/V_{min} \approx 50$, then it has a peak capacity of

$$n_c = 1 + \frac{\sqrt{3600}}{4}\ln(50) \approx 60$$

You can use equation 12.16 to estimate a column's peak capacity.

Capillary Columns

A capillary, or OPEN TUBULAR COLUMN is constructed from fused silica and is coated with a protective polymer coating. Columns range from 15–100 m in length with an internal diameter of approximately 150–300 μm. Figure 12.24 shows a typical example of a capillary column.

Figure 12.24 Typical example of a capillary column for gas chromatography. This column is 30 m long with an internal diameter of 247 μm. The interior surface of the capillary has a 0.25 μm coating of the liquid phase.

Capillary columns are of three principle types. In a WALL-COATED OPEN TUBULAR COLUMN (WCOT) a thin layer of stationary phase, typically 0.25 μm thick, is coated on the capillary's inner wall. In a POROUS-LAYER OPEN TUBULAR COLUMN (PLOT), a porous solid support—alumina, silica gel, and molecular sieves are typical examples—is attached to the capillary's inner wall. A SUPPORT-COATED OPEN TUBULAR COLUMN (SCOT) is a PLOT column that includes a liquid stationary phase. Figure 12.25 shows the differences between these types of capillary columns.

A capillary column provides a significant improvement in separation efficiency because it has more theoretical plates per meter and is longer than a packed column. For example, the capillary column in Figure 12.24 has almost 4300 plates/m, or a total of 129 000 theoretical plates. If we assume a $V_{max}/V_{min} \approx 50$, then it has a peak capacity of approximately 350. On the other hand, a packed column can handle a larger sample. Because of its smaller diameter, a capillary column requires a smaller sample, typically less than 10^{-2} μL.

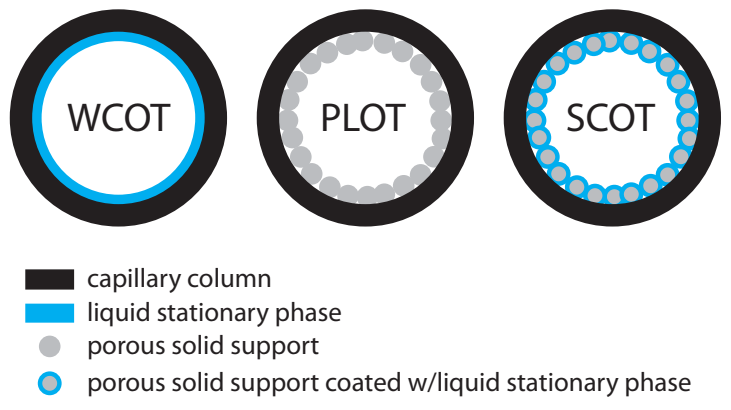


Figure 12.25 Cross-sections through the three types of capillary columns.

Stationary Phases for Gas–Liquid Chromatography

Elution order in gas–liquid chromatography depends on two factors: the boiling point of the solutes, and the interaction between the solutes and the stationary phase. If a mixture's components have significantly different boiling points, then the choice of stationary phase is less critical. If two

Table 12.2 Selected Examples of Stationary Phases for Gas–Liquid Chromatography

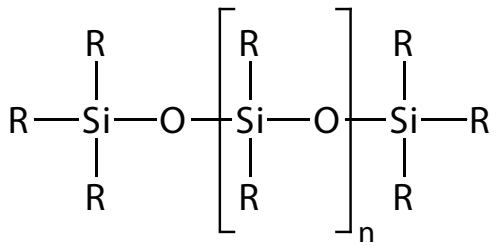
stationary phase	polarity	trade name	temperature limit (°C)	representative applications
squalane	nonpolar	Squalane	150	low-boiling aliphatics hydrocarbons
Apezion L	nonpolar	Apezion L	300	amides, fatty acid methyl esters, terpenoids
polydimethyl siloxane	slightly polar	SE-30	300–350	alkaloids, amino acid derivatives, drugs, pesticides, phenols, steroids
phenylmethyl polysiloxane (50% phenyl, 50% methyl)	moderately polar	OV-17	375	alkaloids, drugs, pesticides, polyaromatic hydrocarbons, polychlorinated biphenyls
trifluoropropylmethyl polysiloxane (50% trifluoropropyl, 50% methyl)	moderately polar	OV-210	275	alkaloids, amino acid derivatives, drugs, halogenated compounds, ketones
cyanopropylphenylmethyl polysiloxane (50%cyanopropyl, 50% phenylmethyl)	polar	OV-225	275	nitriles, pesticides, steroids
polyethylene glycol	polar	Carbowax 20M	225	aldehydes, esters, ethers, phenols

solutes have similar boiling points, however, then a separation is possible only if the stationary phase selectively interacts with one of the solutes. As a general rule, nonpolar solutes are more easily separated with a nonpolar stationary phase, and polar solutes are easier to separate when using a polar stationary phase.

There are several important criteria for choosing a stationary phase: it must not react chemically with the solutes, it must be thermally stable, it must have a low volatility, and it must have a polarity that is appropriate for the sample's components. Table 12.2 summarizes the properties of several popular stationary phases.

Many stationary phases have the general structure shown in Figure 12.26a. A stationary phase of polydimethyl siloxane, in which all the –R groups are methyl groups, $-CH_3$, is nonpolar and often makes a good first choice for a new separation. The order of elution when using polydimethyl siloxane usually follows the boiling points of the solutes, with lower boiling solutes eluting first. Replacing some of the methyl groups with other substituents increases the stationary phase's polarity and provides greater selectivity. For example, replacing 50% of the $-CH_3$ groups with phenyl groups, $-C_6H_5$, produces a slightly polar stationary phase. Increasing polar-

(a)



(b)

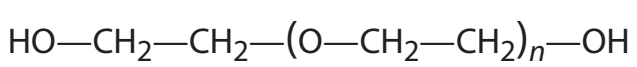


Figure 12.26 General structures of common stationary phases: (a) substituted polysiloxane; (b) polyethylene glycol.

ity is provided by substituting trifluoropropyl, $-C_3H_6CF_3$, and cyanopropyl, $-C_3H_6CN$, functional groups, or by using a stationary phase of polyethylene glycol (Figure 12.26b).

An important problem with all liquid stationary phases is their tendency to elute, or BLEED from the column when it is heated. The temperature limits in Table 12.2 minimize this loss of stationary phase. Capillary columns with bonded or cross-linked stationary phases provide superior stability. A bonded stationary phase is chemically attached to the capillary's silica surface. Cross-linking, which is done after the stationary phase is in the capillary column, links together separate polymer chains, providing greater stability.

Another important consideration is the thickness of the stationary phase. From equation 12.23 we know that separation efficiency improves with thinner films of stationary phase. The most common thickness is 0.25 μm, although a thicker films may be used for highly volatile solutes, such as gases, because it has a greater capacity for retaining such solutes. Thinner films are used when separating low volatility solutes, such as steroids.

A few stationary phases take advantage of chemical selectivity. The most notable are stationary phases containing chiral functional groups, which can be used for separating enantiomers.[7]

12D.3 Sample Introduction

Three considerations determine how we introduce a sample to the gas chromatograph. First, all of the sample's constituents must be volatile. Second, the analytes must be present at an appropriate concentration. Finally, the physical process of injecting the sample must not degrade the separation.

Preparing a Volatile Sample

Not every sample can be injected directly into a gas chromatograph. To move through the column, the sample's constituents must be volatile. A solute of low volatility may be retained by the column and continue to elute during the analysis of subsequent samples. A nonvolatile solute will condense at the top of the column, degrading the column's performance.

We can separate a sample's volatile analytes from its nonvolatile components using any of the extraction techniques described in Chapter 7. A liquid–liquid extraction of analytes from an aqueous matrix into methylene chloride or another organic solvent is a common choice. Solid-phase extractions also are used to remove a sample's nonvolatile components.

An attractive approach to isolating analytes is a SOLID-PHASE MICROEXTRACTION (SPME). In one approach, which is illustrated in Figure 12.27, a fused-silica fiber is placed inside a syringe needle. The fiber, which is coated with a thin film of an adsorbent, such as polydimethyl siloxane, is lowered into the sample by depressing a plunger and is exposed to the sample for

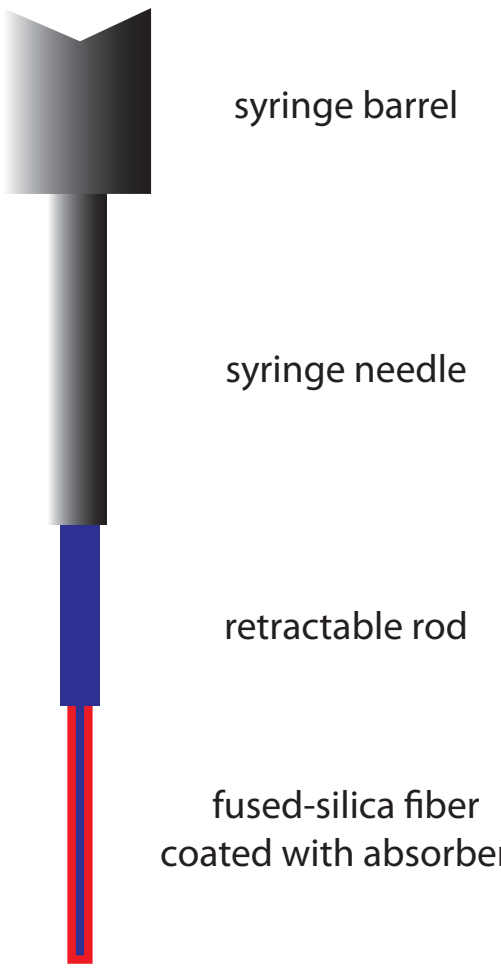


Figure 12.27 Schematic diagram of a solid-phase microextraction device.

7 Hinshaw, J. V. *LC•GC* **1993**, *11*, 644–648.

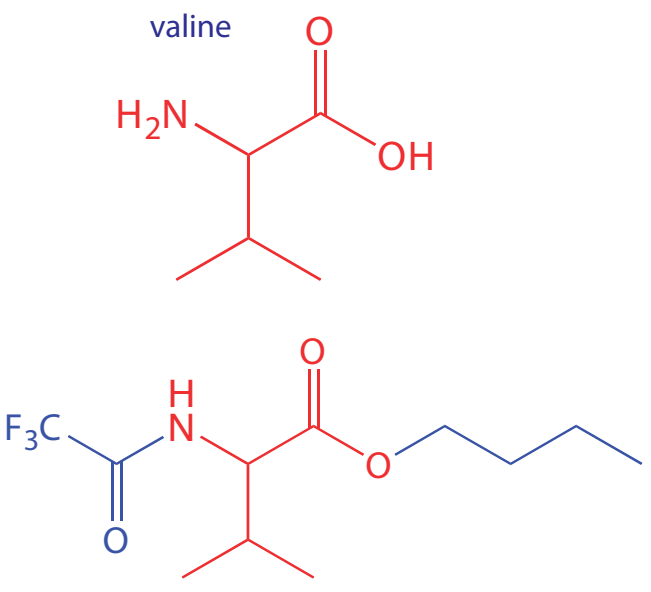
a predetermined time. After withdrawing the fiber into the needle, it is transferred to the gas chromatograph for analysis.

Two additional methods for isolating volatile analytes are a purge-and-trap and headspace sampling. In a PURGE-AND-TRAP (see Figure 7.25 in Chapter 7), we bubble an inert gas, such as He or N_2, through the sample, releasing—or purging—the volatile compounds. These compounds are carried by the purge gas through a trap containing an absorbent material, such as Tenax, where they are retained. Heating the trap and back-flushing with carrier gas transfers the volatile compounds to the gas chromatograph. In HEADSPACE SAMPLING we place the sample in a closed vial with an overlying air space. After allowing time for the volatile analytes to equilibrate between the sample and the overlying air, we use a syringe to extract a portion of the vapor phase and inject it into the gas chromatograph. Alternatively, we can sample the headspace with an SPME.

Thermal desorption is a useful method for releasing volatile analytes from solids. We place a portion of the solid in a glass-lined, stainless steel tube. After purging with carrier gas to remove any O_2 that might be present, we heat the sample. Volatile analytes are swept from the tube by an inert gas and carried to the GC. Because volatilization is not a rapid process, the volatile analytes are often concentrated at the top of the column by cooling the column inlet below room temperature, a process known as CRYOGENIC FOCUSING. Once the volatilization is complete, the column inlet is rapidly heated, releasing the analytes to travel through the column.

To analyze a nonvolatile analyte we must chemically convert it to a volatile form. For example, amino acids are not sufficiently volatile to analyze directly by gas chromatography. Reacting an amino acid with 1-butanol and acetyl chloride produces an esterfied amino acid. Subsequent treatment with trifluoroacetic acid gives the amino acid's volatile N-trifluoroacetyl-*n*-butyl ester derivative.

The reason for removing O_2 is to prevent the sample from undergoing an oxidation reaction when it is heated.



L-valine N-trifluoroacetyl-*n*-butyl ester

Adjusting the Analyte's Concentration

In an analyte's concentration is too small to give an adequate signal, then it needs to be concentrated before injecting the sample into the gas chromatograph. A side benefit of many extraction methods is that they often concentrate the analytes. Volatile organic materials isolated from an aqueous sample by a purge-and-trap, for example, may be concentrated by as much as 1000×.

If an analyte is too concentrated it is easy to overload the column, resulting in peak fronting (see Figure 12.14) and a poor separation. In addition, the analyte's concentration may exceed the detector's linear response. Injecting less sample or diluting the sample with a volatile solvent, such as methylene chloride, are two possible solutions to this problem.

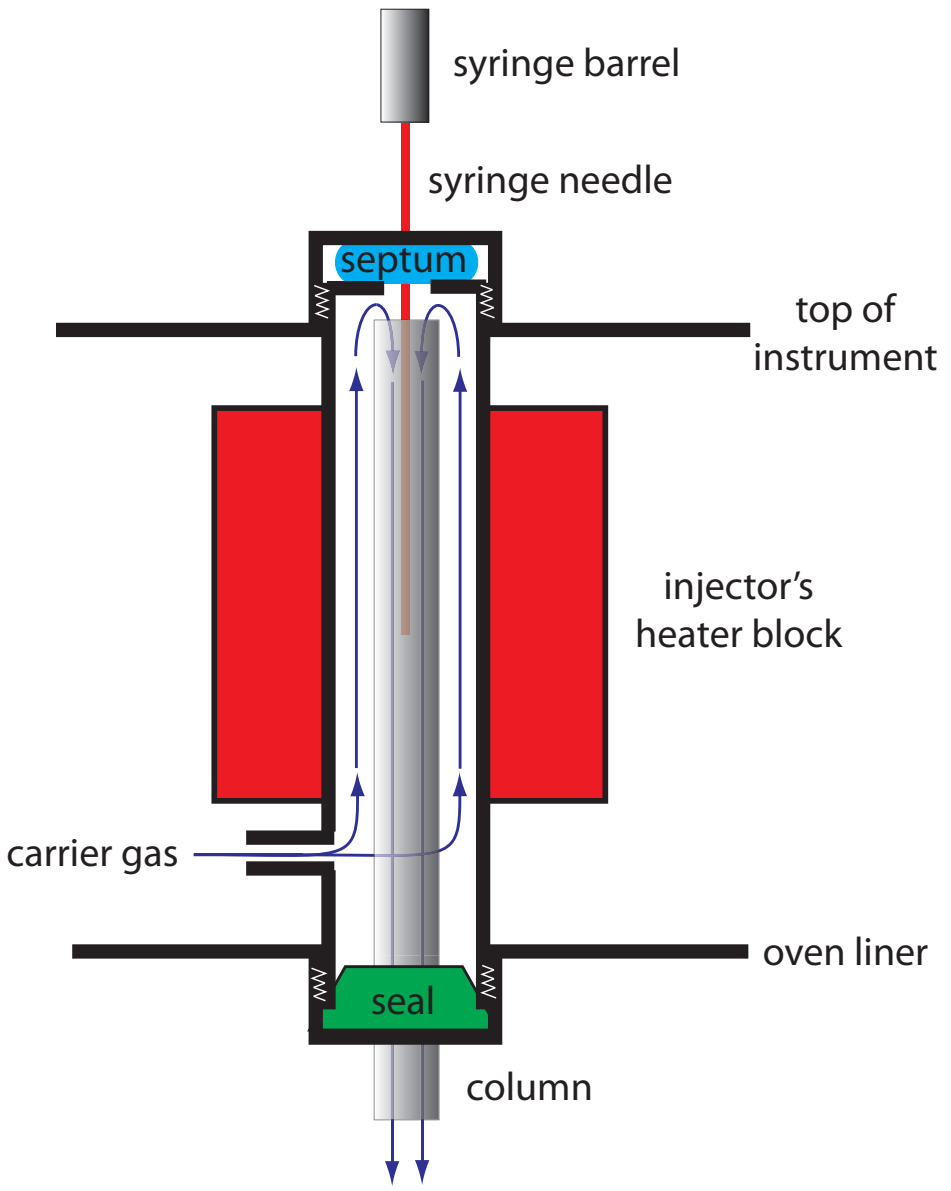


Figure 12.28 Schematic diagram showing a heated GC injector port for use with packed columns. The needle pierces a rubber septum and enters into the top of the column, which is located within a heater block.

Injecting the Sample

In Section 12C.3 we examined several explanations for why a solute's band increases in width as it passes through the column, a process we called band broadening. We also introduce an additional source of band broadening if we fail to inject the sample into the minimum possible volume of mobile phase. There are two principal sources of this precolumn band broadening: injecting the sample into a moving stream of mobile phase and injecting a liquid sample instead of a gaseous sample. The design of a gas chromatograph's injector helps minimize these problems.

An example of a simple injection port for a packed column is shown in Figure 12.28. The top of the column fits within a heated injector block, with carrier gas entering from the bottom. The sample is injected through a rubber septum using a microliter syringe such as the one shown in Figure 12.29. Injecting the sample directly into the column minimizes band broadening by mixing the sample with the smallest possible amount of carrier gas. The injector block is heated to a temperature that is at least 50 °C above the sample component with the highest boiling point, ensuring a rapid vaporization of the sample's components.

Because a capillary column's volume is significantly smaller than that for a packed column, it requires a different style of injector to avoid over-

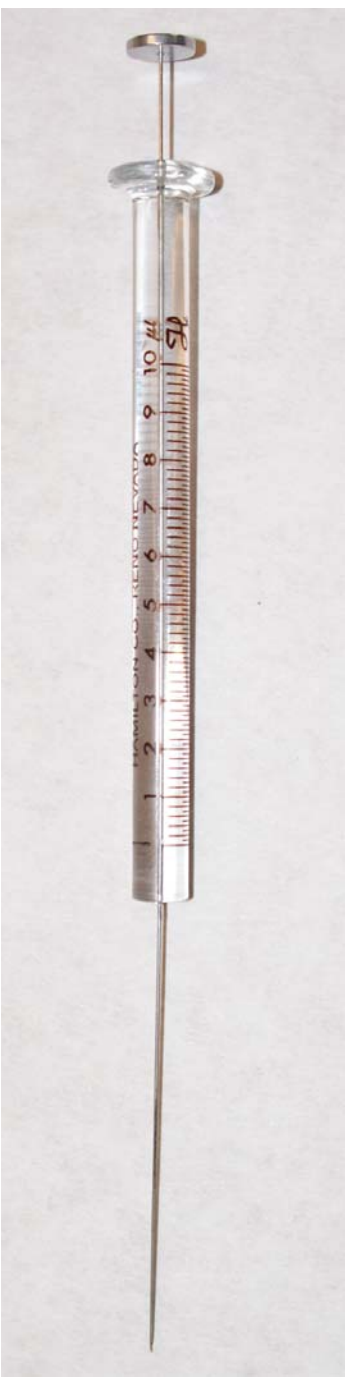


Figure 12.29 Example of a syringe for injecting samples into a gas chromatograph. This syringe has a maximum capacity of 10 μL with graduations every 0.1 μL.

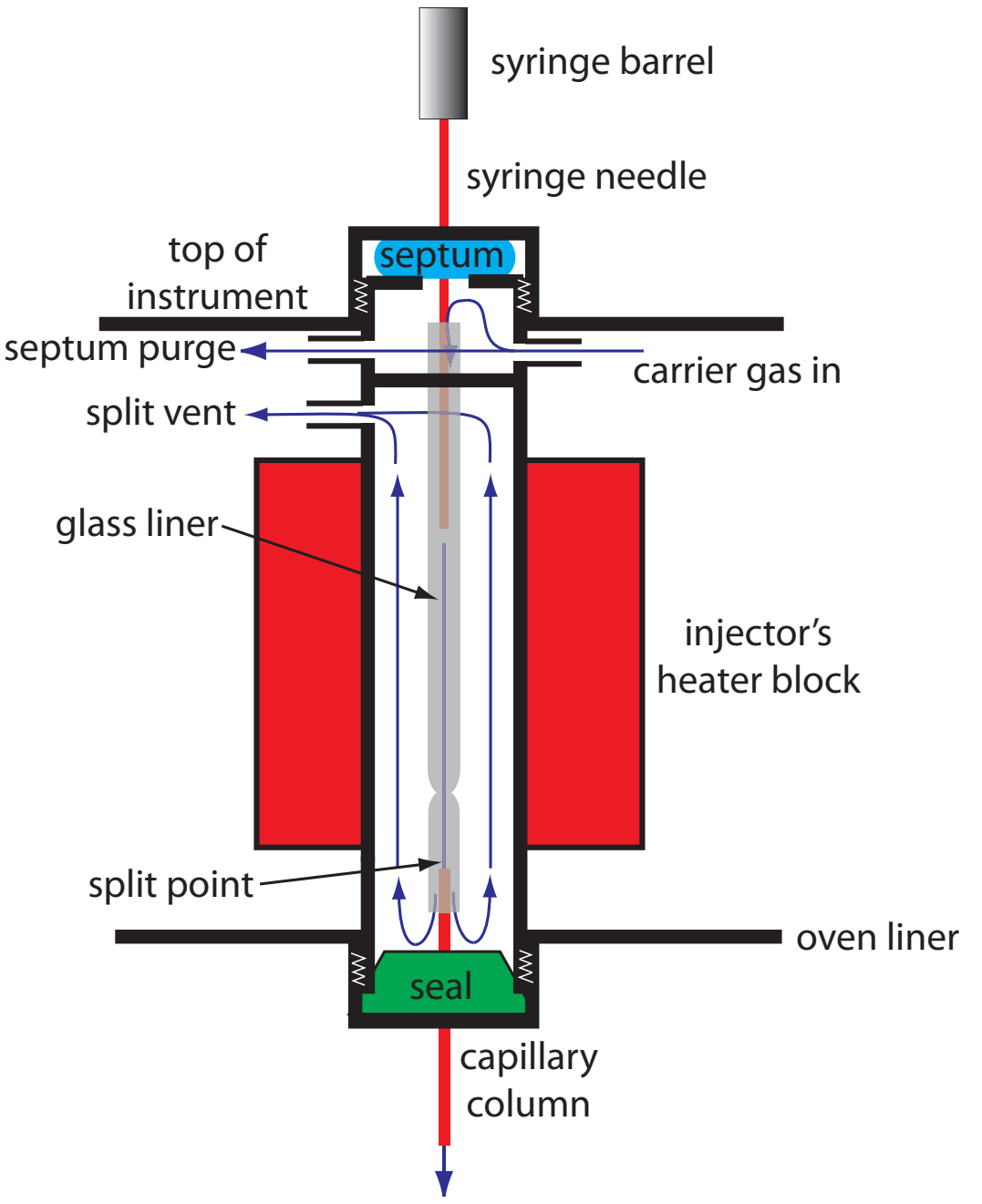


Figure 12.30 Schematic diagram showing a split/splitless injection port for use with capillary columns. The needle pierces a rubber septum and enters into a glass liner, which is located within a heater block. In a split injection the split vent is open; the split vent is closed for a splitless injection.

loading the column with sample. Figure 12.30 shows a schematic diagram of a typical split/splitless injector for use with capillary columns.

In a SPLIT INJECTION we inject the sample through a rubber septum using a microliter syringe. Instead of injecting the sample directly into the column, it is injected into a glass liner where it mixes with the carrier gas. At the split point, a small fraction of the carrier gas and sample enters the capillary column with the remainder exiting through the split vent. By controlling the flow rate of the carrier gas entering the injector, and the flow rates through the septum purge and the split vent, we can control what fraction of the sample enters the capillary column, typically 0.1–10%.

For example, if the carrier gas flow rate is 50 mL/min, and the flow rates for the septum purge and the split vent are 2 mL/min and 47 mL/min, respectively, then the flow rate through the column is 1 mL/min (=50–2–46). The ratio of sample entering the column is 1/50, or 2%.

In a SPLITLESS INJECTION, which is useful for trace analysis, we close the split vent and allow all the carrier gas passing through the glass liner to enter the column—this allows virtually all the sample to enters the column. Because the flow rate through the injector is low, significant precolumn band broadening is a problem. Holding the column's temperature approximately 20–25 °C below the solvent's boiling point allows the solvent to condense at the entry to the capillary column, forming a barrier that traps the solutes. After allowing the solutes to concentrate, the column's temperature is increased and the separation begins.

For samples that decompose easily, an ON-COLUMN INJECTION may be necessary. In this method the sample is injected directly into the column

without heating. The column temperature is then increased, volatilizing the sample with as low a temperature as is practical.

12D.4 Temperature Control

Control of the column's temperature is critical to attaining a good separation in gas chromatography. For this reason the column is placed inside a thermostated oven (see Figure 12.22). In an ISOTHERMAL separation we maintain the column at a constant temperature. To increase the interaction between the solutes and the stationary phase, the temperature usually is set slightly below that of the lowest-boiling solute.

One difficulty with an isothermal separation is that a temperature favoring the separation of a low-boiling solute may lead to an unacceptably long retention time for a higher-boiling solute. TEMPERATURE PROGRAMMING provide a solution to this problem. At the beginning of the analysis we set the column's initial temperature below that for the lowest-boiling solute. As the separation progresses, we slowly increase the temperature at either a uniform rate or in a series of steps.

You may recall that we called this the general elution problem (see Figure 12.16).

12D.5 Detectors for Gas Chromatography

The final part of a gas chromatograph is the detector. The ideal detector has several desirable features, including: a low detection limit, a linear response over a wide range of solute concentrations (which makes quantitative work easier), sensitivity for all solutes or selectivity for a specific class of solutes, and an insensitivity to a change in flow rate or temperature.

THERMAL CONDUCTIVITY DETECTOR (TCD)

One of the earliest gas chromatography detectors takes advantage of the mobile phase's thermal conductivity. As the mobile phase exits the column it passes over a tungsten-rhenium wire filament (see Figure 12.31). The filament's electrical resistance depends on its temperature, which, in turn, depends on the thermal conductivity of the mobile phase. Because of its high thermal conductivity, helium is the mobile phase of choice when using a THERMAL CONDUCTIVITY DETECTOR (TCD).

When a solute elutes from the column, the thermal conductivity of the mobile phase in the TCD cell decreases and the temperature of the wire

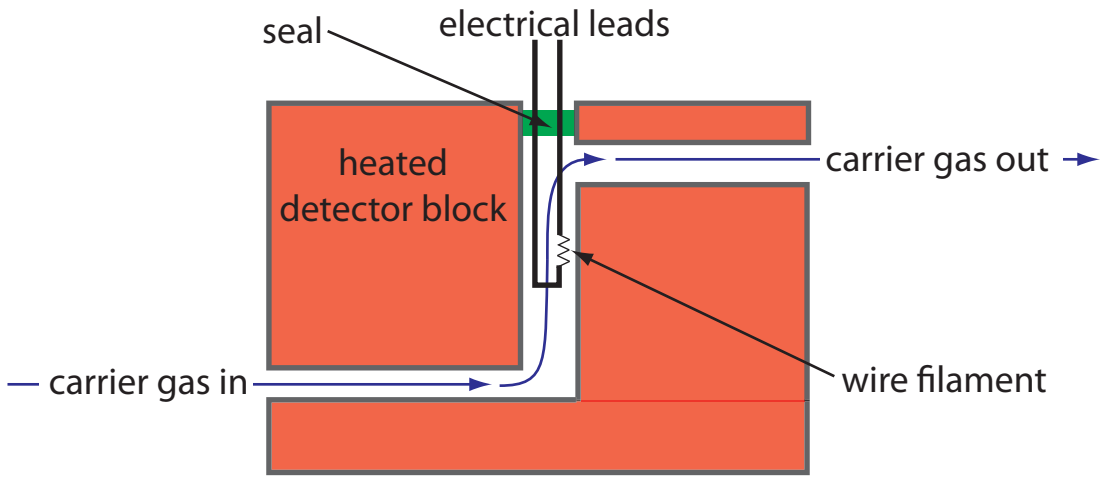


Figure 12.31 Schematic diagram showing a thermal conductivity detector. This is one cell of a matched pair. The sample cell takes the carrier gas as it elutes from the column. A source of carrier gas that bypasses the column passes through a reference cell.

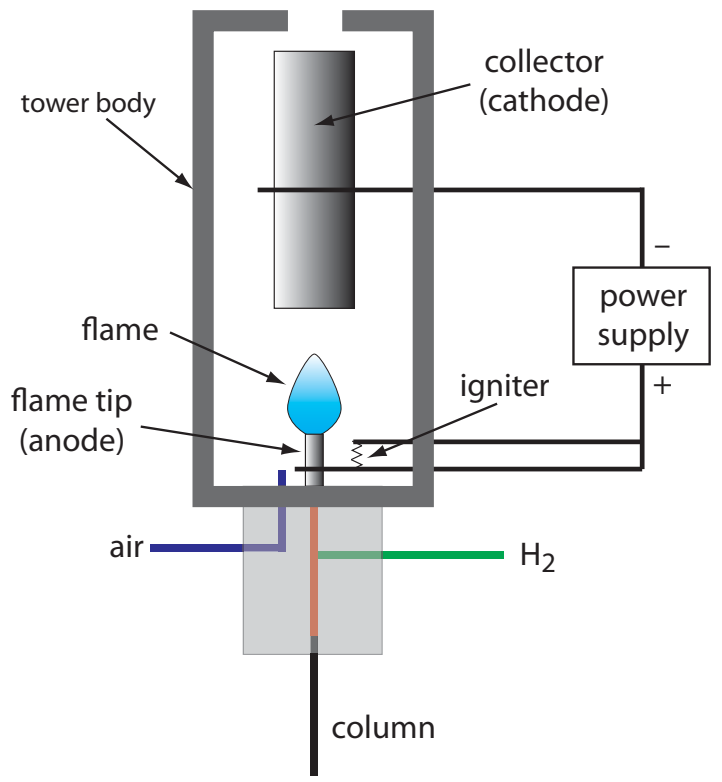


Figure 12.32 Schematic diagram showing a flame ionization detector. The eluent from the column mixes with H_2 and is burned in the presence of excess air. Combustion produces a flame containing electrons and the cation CHO^+. Applying a potential between the flame tip and the collector, a current that is proportional to the concentration of cations in the flame.

filament, and thus it resistance, increases. A reference cell, through which only the mobile phase passes, corrects for any time-dependent variations in flow rate, pressure, or electrical power, all of which may lead to a change in the filament's resistance.

Because all solutes affect the mobile phase's thermal conductivity, the thermal conductivity detector is a universal detector. Another advantage is the TCD's linear response over a concentration range spanning 10^4–10^5 orders of magnitude. The detector also is non-destructive, allowing us to isolate analytes using a postdetector cold trap. One significant disadvantage of the TCD detector is its poor detection limit for most analytes.

Flame Ionization Detector (FID)

The combustion of an organic compound in an H_2/air flame results in a flame containing electrons and organic cations, presumably CHO^+. Applying a potential of approximately 300 volts across the flame creates a small current of roughly 10^{-9} to 10^{-12} amps. When amplified, this current provides a useful analytical signal. This is the basis of the popular **flame ionization detector**, a schematic diagram of which is shown in Figure 12.32.

Most carbon atoms—except those in carbonyl and carboxylic groups—generate a signal, which makes the FID an almost universal detector for organic compounds. Most inorganic compounds and many gases, such as H_2O and CO_2, are not detected, which makes the FID detector a useful detector for the analysis of atmospheric and aqueous environmental samples. Advantages of the FID include a detection limit that is approximately two to three orders of magnitude smaller than that for a thermal conductivity detector, and a linear response over 10^6–10^7 orders of magnitude in the amount of analyte injected. The sample, of course, is destroyed when using a flame ionization detector.

Electron Capture Detector (ECD)

The **electron capture detector** is an example of a selective detector. As shown in Figure 12.33, the detector consists of a β-emitter, such as ^{63}Ni. The emitted electrons ionize the mobile phase, which is usually N_2, generating a standing current between a pair of electrodes. When a solute with a high affinity for capturing electrons elutes from the column, the current decreases. This decrease in current serves as the signal. The ECD is highly selective toward solutes with electronegative functional groups, such as halogens and nitro groups, and is relatively insensitive to amines, alcohols, and hydrocarbons. Although its detection limit is excellent, its linear range extends over only about two orders of magnitude.

A β-particle is an electron.

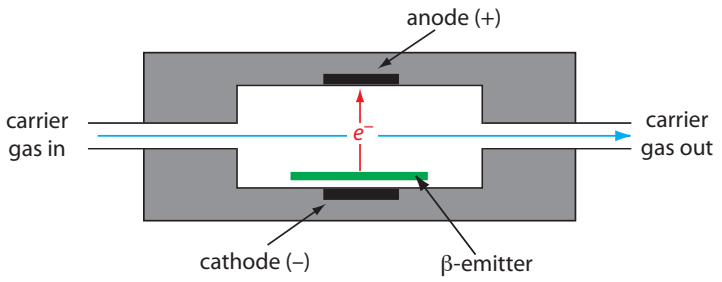


Figure 12.33 Schematic diagram showing an electron capture detector.

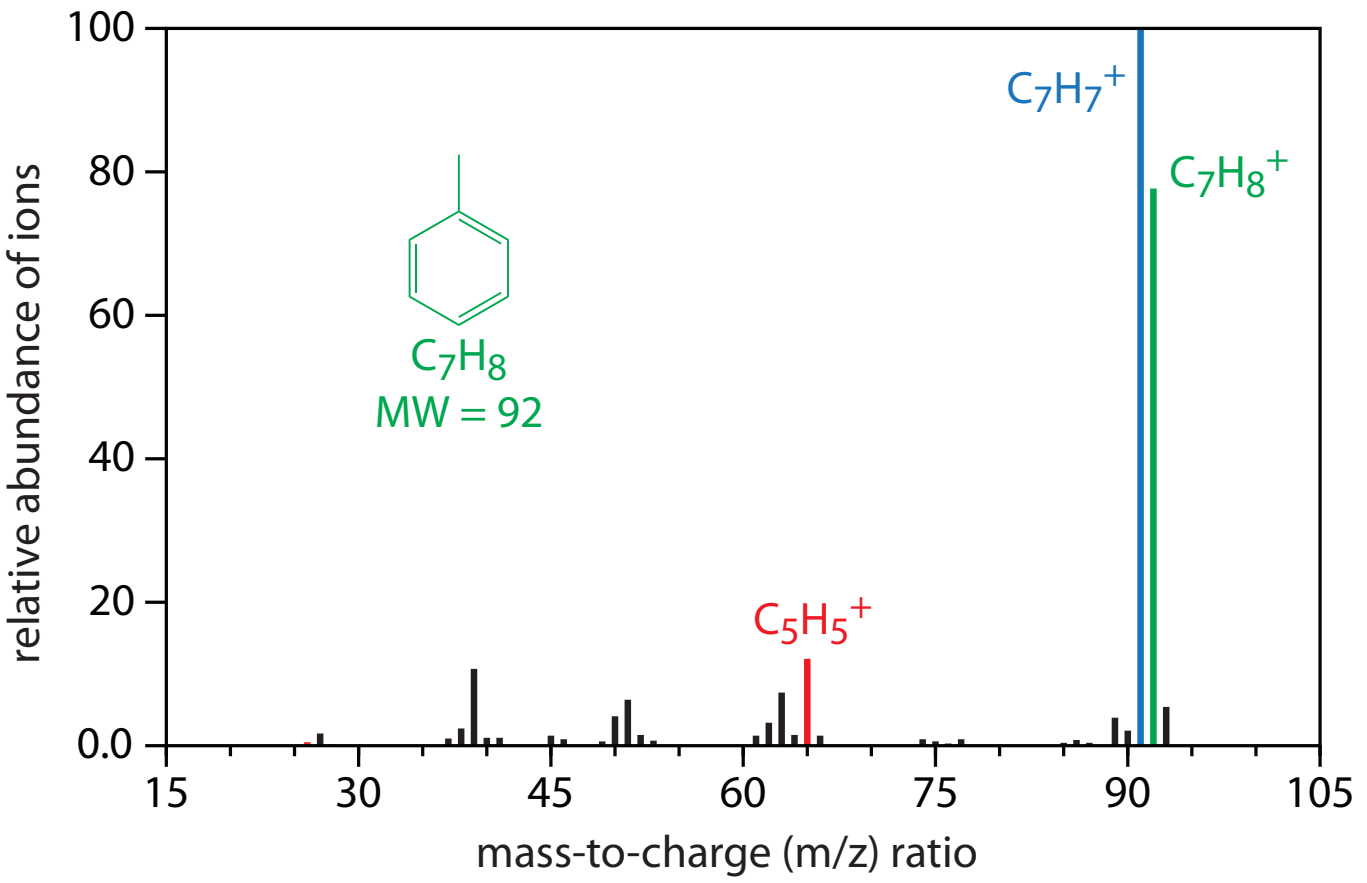


Figure 12.34 Mass spectrum for toluene highlighting the molecular ion in green (m/z = 92), and two fragment ions in blue (m/z = 91) and in red (m/z = 65). A mass spectrum provides both quantitative and qualitative information: the height of any peak is proportional to the amount of toluene in the mass spectrometer and the fragmentation pattern is unique to toluene.

Mass Spectrometer (MS)

A MASS SPECTROMETER is an instrument that ionizes a gaseous molecule using enough energy that the resulting ion breaks apart into smaller ions. Because these ions have different mass-to-charge ratios, it is possible to separate them using a magnetic field or an electrical field. The resulting MASS SPECTRUM contains both quantitative and qualitative information about the analyte. Figure 12.34 shows a mass spectrum for toluene.

Figure 12.35 shows a block diagram of a typical gas chromatography-mass spectrometer (GC–MS) instrument. The effluent from the column enters the mass spectrometer's ion source in a manner that eliminates the majority of the carrier gas. In the ionization chamber the remaining molecules—a mixture of carrier gas, solvent, and solutes—undergo ionization and fragmentation. The mass spectrometer's mass analyzer separates the ions by their mass-to-charge ratio. A detector counts the ions and displays the mass spectrum.

There are several options for monitoring the chromatogram when using a mass spectrometer as the detector. The most common method is to continuously scan the entire mass spectrum and report the total signal for all ions reaching the detector during each scan. This total ion scan provides universal detection for all analytes. We can achieve some degree of selectivity by monitoring only specific mass-to-charge ratios, a process called selective-ion monitoring. A mass spectrometer provides excellent detection limits, typically 25 fg to 100 pg, with a linear range of 10^5 orders of magnitude. Because we are continuously recording the mass spectrum of the

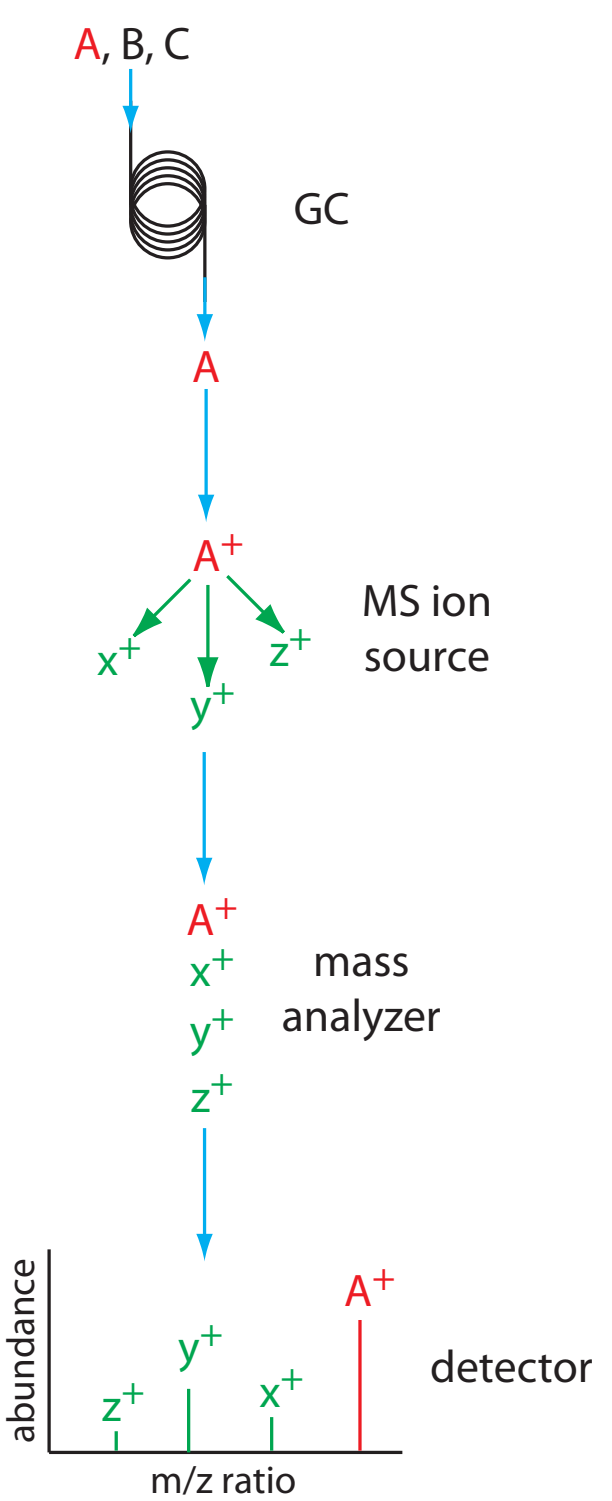


Figure 12.35 Block diagram of GC–MS. A three component mixture enters the GC. When component A elutes from the column, it enters the MS ion source and ionizes to form the parent ion and several fragment ions. The ions enter the mass analyzer, which separates them by their mass-to-charge ratio, providing the mass spectrum shown at the detector.

column's eluent, we can go back and examine the mass spectrum for any time increment. This is a distinct advantage for GC–MS because we can use the mass spectrum to help identify a mixture's components.

Other Detectors

Two additional detectors are similar in design to a flame ionization detector. In the flame photometric detector optical emission from phosphorous and sulfur provides a detector selective for compounds containing these elements. The thermionic detector responds to compounds containing nitrogen or phosphorous.

A Fourier transform infrared spectrophotometer (FT–IR) also can serve as a detector. In GC–FT–IR, effluent from the column flows through an optical cell constructed from a 10–40 cm Pyrex tube with an internal diameter of 1–3 mm. The cell's interior surface is coated with a reflecting layer of gold. Multiple reflections of the source radiation as it is transmitted through the cell increase the optical path length through the sample. As is the case with GC–MS, an FT–IR detector continuously records the column eluent's spectrum, which allows us to examine the IR spectrum for any time increment.

See Chapter 10C for a discussion of FT-IR spectroscopy and instrumentation.

12D.6 Quantitative Applications

Gas chromatography is widely used for the analysis of a diverse array of samples in environmental, clinical, pharmaceutical, biochemical, forensic, food science and petrochemical laboratories. Table 12.3 provides some representative examples of applications.

Table 12.3 Representative Applications of Gas Chromatography

area	applications
environmental analysis	green house gases (CO_2, CH_4, NO_x) in air pesticides in water, wastewater, and soil vehicle emissions trihalomethanes in drinking water
clinical analysis	drugs blood alcohols
forensic analysis	analysis of arson accelerants detection of explosives
consumer products	volatile organics in spices and fragrances trace organics in whiskey monomers in latex paint
petroleum and chemical industry	purity of solvents refinery gas composition of gasoline

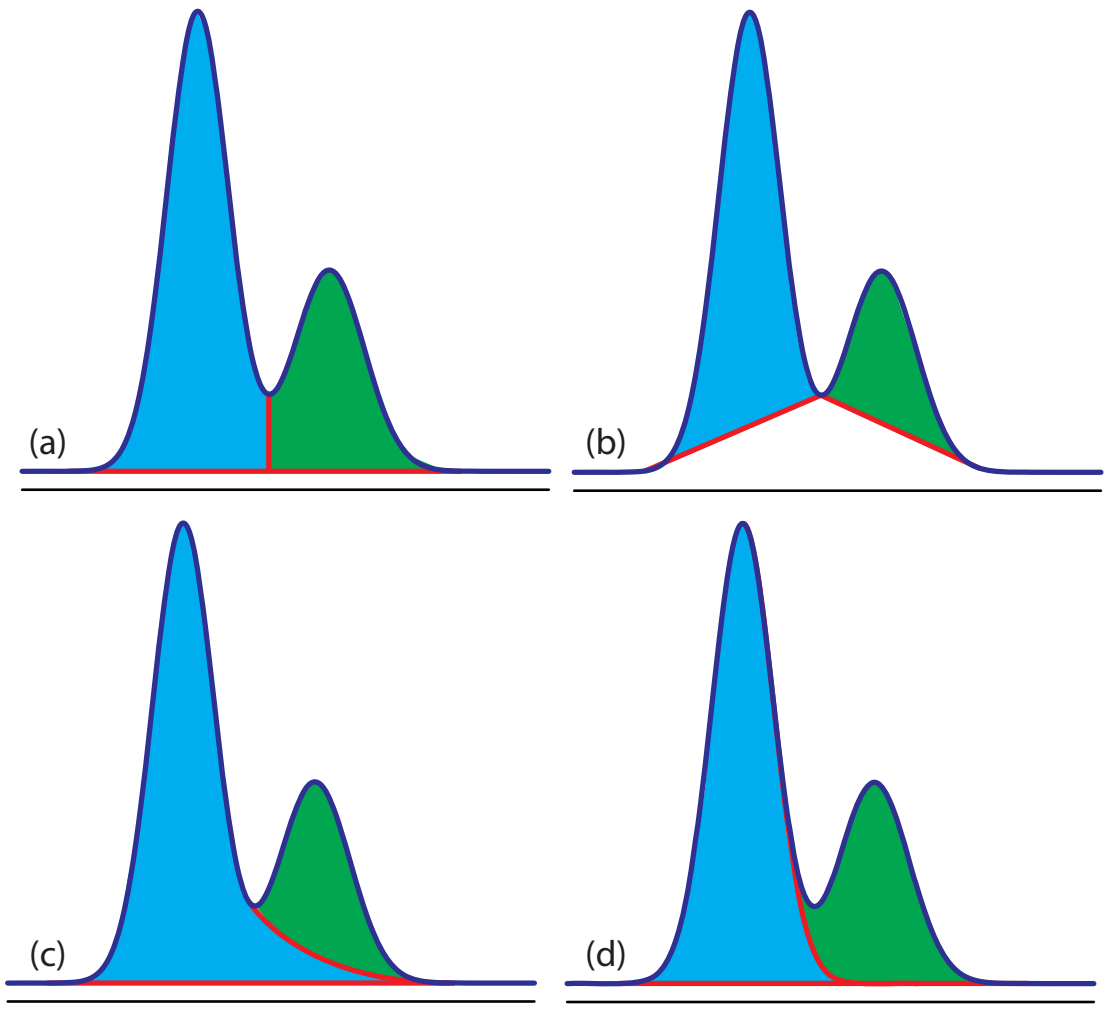


Figure 12.36 Four methods for determining the areas under two overlapping chromatographic peaks: (a) the drop method; (b) the valley method; (c) the exponential skim method; and (d) the Gaussian skim method. Other methods for determining areas also are available.

Quantitative Calculations

In a GC analysis the area under the peak is proportional to the amount of analyte injected onto the column. The peak's area is determined by integration, which usually is handled by the instrument's computer or by an electronic integrating recorder. If two peak are fully resolved, the determination of their respective areas is straightforward. Overlapping peaks, however, require a choice between one of several options for dividing up the area shared by the two peaks (Figure 12.36). Which method to use depends on the relative size of the two peaks and their resolution. In some cases, the use of peak heights provides more accurate results.[8]

For quantitative work we need to establish a calibration curve that relates the detector's response to the concentration of analyte. If the injection volume is identical for every standard and sample, then an external standardization provides both accurate and precise results. Unfortunately, even under the best conditions the relative precision for replicate injections may differ by 5%—often it is substantially worse. For quantitative work requiring high accuracy and precision, the use of internal standards is recommended.

Before electronic integrating recorders and computers, two methods were used to find the area under a curve. One method used a manual planimeter; as you use the planimeter to trace an object's perimeter, it records the area. A second approach for finding the area is the cut-and-weigh method. The chromatogram is recorded on a piece of paper. Each peak of interest is cut out and weighed. Assuming the paper is uniform in thickness and density of fibers, the ratio of weights for two peaks is the same as the ratio of areas. Of course, this approach destroys your chromatogram.

To review the method of internal standards, see Section 5C.4.

Example 12.5

Marriott and Carpenter report the following data for five replicate injections of a mixture consisting of 1% v/v methylisobutylketone and 1% v/v *p*-xylene in dichloromethane.[9]

8 (a) Bicking, M. K. L. Chromatography Online, April 2006; (b) Bicking, M. K. L. Chromatography Online, June 2006.

9 Marriott, P. J.; Carpenter, P. D. *J. Chem. Educ.* **1996**, *73*, 96–99.

injection	peak	peak area (arb. units)
I	1	49 075
	2	78 112
II	1	85 829
	2	135 404
III	1	84 136
	2	132 332
IV	1	71 681
	2	112 889
V	1	58 054
	2	91 287

Assume that *p*-xylene (peak 2) is the analyte, and that methylisobutylketone (peak 1) is the internal standard. Determine the 95% confidence interval for a single-point standardization, with and without using the internal standard.

Solution

For a single-point external standardization we ignore the internal standard and determine the relationship between the peak area for *p*-xylene, A_2, and the concentration, C_2, of *p*-xylene.

$$A_2 = kC_2$$

Substituting the known concentration for *p*-xylene and the appropriate peak areas, gives the following values for the constant *k*.

78 112 135 404 132 332 112 889 91 287

The average value for *k* is 110 000 with a standard deviation of 25 100 (a relative standard deviation of 22.8%). The 95% confidence interval is

$$\mu = \bar{X} \pm \frac{ts}{\sqrt{n}} = 111\,000 \pm \frac{(2.78)(25\,100)}{\sqrt{5}} = 111\,000 \pm 31\,200$$

For an internal standardization, the relationship between the analyte's peak area, A_2, the peak area for the internal standard, A_1, and their respective concentrations, C_2 and C_1, is

$$\frac{A_2}{A_1} = k\frac{C_2}{C_1}$$

Substituting the known concentrations and the appropriate peak areas gives the following values for the constant *k*.

1.5917 1.5776 1.5728 1.5749 1.5724

The average value for k is 1.5779 with a standard deviation of 0.0080 (a relative standard deviation of 0.507%). The 95% confidence interval is

$$\mu = \bar{X} \pm \frac{ts}{\sqrt{n}} = 1.5779 \pm \frac{(2.78)(0.0080)}{\sqrt{5}} = 1.5779 \pm 0.0099$$

Although there is a substantial variation in the individual peak areas for this set of replicate injections, the internal standard compensates for these variations, providing a more accurate and precise calibration.

Practice Exercise 12.5

Figure 12.37 shows chromatograms for five standards and one sample. Each standard and the sample contains the same concentration of an internal standard, which is 2.50 mg/mL. For the five standards, the concentrations of analyte are 0.20 mg/mL, 0.40 mg/mL, 0.60 mg/mL, 0.80 mg/mL, and 1.00 mg/mL, respectively. Determine the concentration of analyte in the sample by (a) ignoring the internal standards and creating an external standards calibration curve, and (b) creating an internal standard calibration curve. For each approach, report the analyte's concentration and the 95% confidence interval. Use peak heights instead of peak areas.

Click here to review your answer to this exercise.

You may wish to review our earlier coverage of linear regression in Chapter 5D.

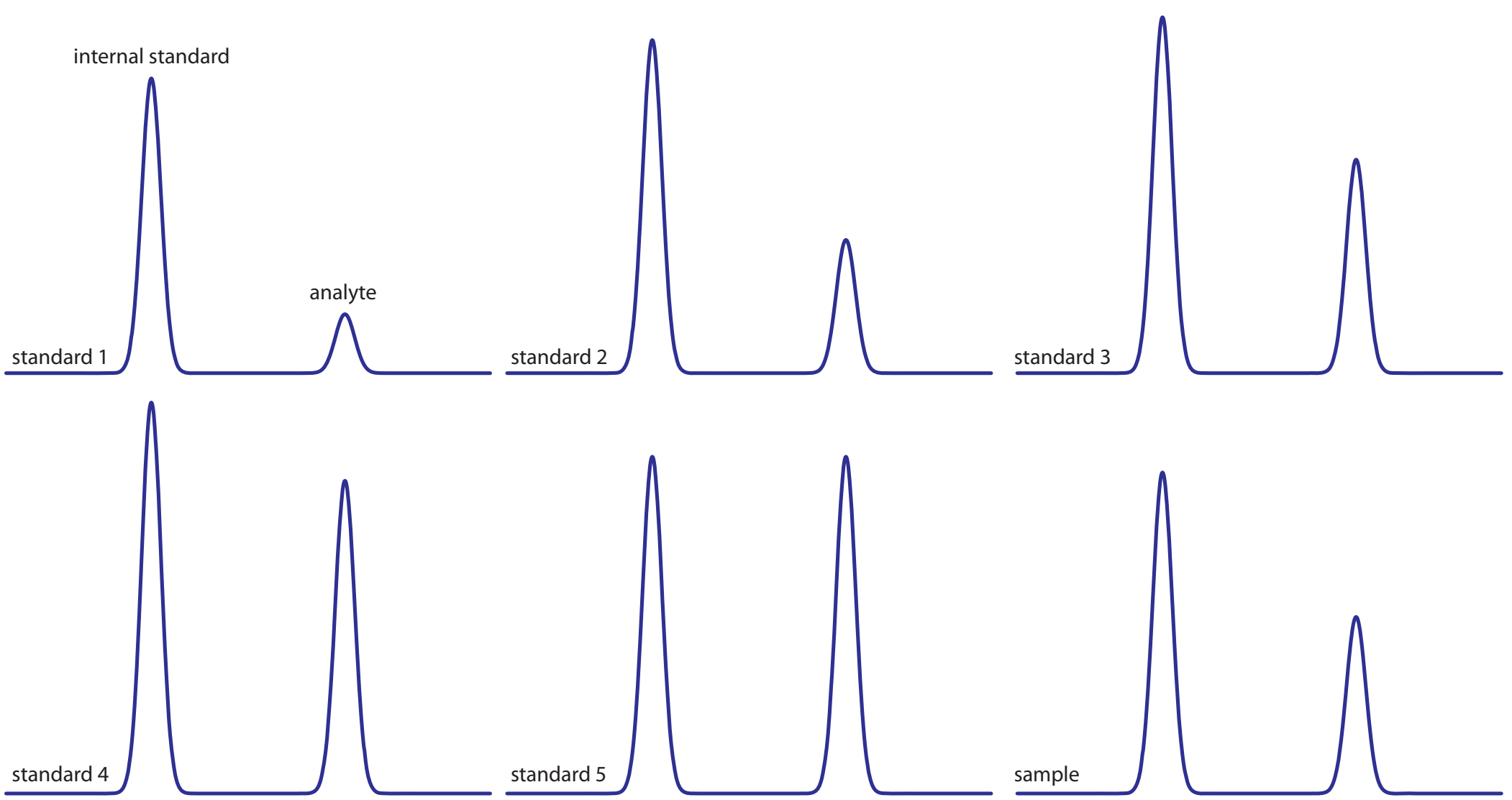


Figure 12.37 Chromatograms for Practice Exercise 12.5.

12D.7 Qualitative Applications

In addition to identifying the component responsible for a particular chromatographic peak, we also can use the saved spectra to evaluate peak purity. If only one component is responsible for a chromatographic peak, then the spectra should be identical throughout the peak's elution. If a spectrum at the beginning of the peak's elution is different from a spectrum taken near the end of the peak's elution, then there must be at least two components that are co-eluting.

In addition to a quantitative analysis, we also can use chromatography to identify the components of a mixture. As noted earlier, when using an FT–IR or a mass spectrometer as the detector we have access to the eluent's full spectrum for any retention time. By interpreting the spectrum or by searching against a library of spectra, we can identify the analyte responsible for each chromatographic peak.

When using a nonspectroscopic detector, such as a flame ionization detector, we must find another approach if we need to identify the components of a mixture. One approach is to spike the sample the suspected compound and looking for an increase in peak height. We also can compare a peak's retention time to the retention time for a known compound if we use identical operating conditions.

Because a compound's retention times on two identical columns are not likely to be the same—differences in packing efficiency, for example, will affect a solute's retention time on a packed column—a table of standard retention times is not useful. KOVAT'S RETENTION INDEX provides one solution to the problem of matching retention times. Under isothermal conditions, the adjusted retention times for normal alkanes increase logarithmically. Kovat defined the retention index, I, for a normal alkane as 100 times the number of carbon atoms. For example, the retention index is 400 for butane, C_4H_{10}, and 500 for pentane, C_5H_{12}. To determine the a compound's retention index, I_{cpd}, we use the following formula

$$I_{\text{cpd}} = 100 \times \frac{\log t'_{\text{r,cpd}} - \log t'_{\text{r},x}}{\log t'_{\text{r},x+1} - \log t'_{\text{r},x}} + I_x \qquad 12.27$$

where $t'_{\text{r,cpd}}$ is the compound's adjusted retention time, $t'_{\text{r},x}$ and $t'_{\text{r},x+1}$ are the adjusted retention times for the normal alkanes that elute immediately before the compound and after the compound, respectively, and I_x is the retention index for the normal alkane eluting immediately before the compound. A compound's retention index for a particular set of chromatographic conditions—stationary phase, mobile phase, column type, column length, temperature, etc.—should be reasonably consistent from day to day and between different columns and instruments.

Tables of Kovat's retention indices are available; see, for example, the NIST Chemistry Webbook. A search for toluene returns 341 values of I for over 20 different stationary phases, and for both packed columns and capillary columns.

Example 12.6

In a separation of a mixture of hydrocarbons the following adjusted retention times were measured: 2.23 min for propane, 5.71 min for isobutane, and 6.67 min for butane. What is the Kovat's retention index for each of these hydrocarbons?

SOLUTION

Kovat's retention index for a normal alkane is 100 times the number of carbons; thus, for propane, $I = 300$ and for butane, $I = 400$. To find Kovat's retention index for isobutane we use equation 12.27.

$$I_{cpd} = 100 \times \frac{\log(5.71) - \log(2.23)}{\log(6.67) - \log(2.23)} + 300 = 386$$

Practice Exercise 12.6

When using a column with the same stationary phase as in Example 12.6, you find that the retention times for propane and butane are 4.78 min and 6.86 min, respectively. What is the expected retention time for isobutane?

Click here to review your answer to this exercise.

Representative Method 12.1

Determination of Trihalomethanes in Drinking Water

The best way to appreciate the theoretical and practical details discussed in this section is to carefully examine a typical analytical method. Although each method is unique, the following description of the determination of trihalomethanes in drinking water provides an instructive example of a typical procedure. The description here is based on a Method 6232B in *Standard Methods for the Examination of Water and Wastewater*, 20th Ed., American Public Health Association: Washington, DC, 1998.

DESCRIPTION OF METHOD

Trihalomethanes, such as chloroform ,$CHCl_3$, and bromoform, $CHBr_3$, are found in most chlorinated waters. Because chloroform is a suspected carcinogen, the determination of trihalomethanes in public drinking water supplies is of considerable importance. In this method the trihalomethanes $CHCl_3$, $CHBrCl_2$, $CHBr_2Cl$, and $CHBr_3$ are isolated using a liquid–liquid extraction with pentane and determined using a gas chromatograph equipped with an electron capture detector.

PROCEDURE

Collect the sample in a 40-mL glass vial with a screw-cap lined with a TFE-faced septum. Fill the vial to overflowing, ensuring that there are no air bubbles. Add 25 mg of ascorbic acid as a reducing agent to quench the further production of trihalomethanes. Seal the vial and store the sample at 4 °C for no longer than 14 days.

TFE is tetrafluoroethylene. Teflon is a polymer formed from TFE.

Prepare a standard stock solution for each trihalomethane by placing 9.8 mL of methanol in a 10-mL volumetric flask. Let the flask stand for 10 min, or until all surfaces wetted with methanol are dry. Weigh the flask to the nearest ±0.1 mg. Using a 100-μL syringe, add 2 or more drops of trihalomethane to the volumetric flask, allowing each drop to fall directly into the methanol. Reweigh the flask before diluting to volume and mixing. Transfer the solution to a 40-mL glass vial with a TFE-lined screw-top and report the concentration in μg/mL. Store the stock solutions at –10 to –20 °C and away from the light.

Prepare a multicomponent working standard from the stock standards by making appropriate dilutions of the stock solution with methanol in a volumetric flask. Choose concentrations so that calibration standards (see below) require no more than 20 μL of working standard per 100 mL of water.

Using the multicomponent working standard, prepare at least three, but preferably 5–7 calibration standards. At least one standard must be near the detection limit and the standards must bracket the expected concentration of trihalomethanes in the samples. Using an appropriate volumetric flask, prepare the standards by injecting at least 10 μL of the working standard below the surface of the water and dilute to volume. Mix each standard gently three times only. Discard the solution in the neck of the volumetric flask and then transfer the remaining solution to a 40-mL glass vial with a TFE-lined screw-top. If the standard has a headspace, it must be analyzed within 1 hr; standards without any headspace may be held for up to 24 hr.

Prepare an internal standard by dissolving 1,2-dibromopentane in hexane. Add a sufficient amount of this solution to pentane to give a final concentration of 30 μg 1,2-dibromopentane/L.

To prepare the calibration standards and samples for analysis, open the screw top vial and remove 5 mL of the solution. Recap the vial and weigh to the nearest ±0.1 mg. Add 2.00 mL of pentane (with the internal standard) to each vial and shake vigorously for 1 min. Allow the two phases to separate for 2 min and then use a glass pipet to transfer at least 1 mL of the pentane (the upper phase) to a 1.8-mL screw top sample vial with a TFE septum, and store at 4 °C until you are ready to inject them into the GC. After emptying, rinsing, and drying the sample's original vial, weigh it to the nearest ±0.1 mg and calculate the sample's weight to ±0.1 g. If the density is 1.0 g/mL, then the sample's weight is equivalent to its volume.

Inject a 1–5 μL aliquot of the pentane extracts into a GC equipped with a 2-mm ID, 2-m long glass column packed with a stationary phase of 10% squalane on a packing material of 80/100 mesh Chromosorb WAW. Operate the column at 67 °C and a flow rate of 25 mL/min.

A variety of other columns can be used. Another option, for example, is a 30-m fused silica column with an internal diameter of 0.32 mm and a 1 μm coating of the stationary phase DB-1. A linear flow rate of 20 cm/s is used with the following temperature program: hold for 5 min at 35 °C; increase to 70 °C at 10 °C/min; increase to 200 °C at 20 °C.

Questions

1. A simple liquid–liquid extraction rarely extracts 100% of the analyte. How does this method account for incomplete extractions?

   Because the extraction procedure for the samples and the standards are identical, the ratio of analyte between any two samples or standards is unaffected by an incomplete extraction.

2. Water samples are likely to contain trace amounts of other organic compounds, many of which will extract into pentane along with the trihalomethanes. A short, packed column, such as the one used in this

method, generally does not do a particularly good job of resolving chromatographic peaks. Why do we not need to worry about these other compounds?

An electron capture detector responds only to compounds, such as the trihalomethanes, that have electronegative functional groups. Because an electron capture detector will not respond to most of the potential interfering compounds, the chromatogram will have relatively few peaks other than those for the trihalomethanes and the internal standard.

3. Predict the order in which the four analytes elute from the GC column.

   Retention time should follow the compound's boiling points, eluting from the lowest boiling point to the highest boiling points. The elution order is $CHCl_3$ (61.2 °C), $CHCl_2Br$ (90 °C), $CHClBr_2$ (119 °C), and $CHBr_3$ (149.1 °C).

4. Although chloroform is an analyte, it also is an interferent because it is present at trace levels in the air. Any chloroform present in the laboratory air, for example, may enter the sample by diffusing through the sample vial's silicon septum. How can we determine whether samples have been contaminated in this manner?

   A sample blank of trihalomethane-free water can be kept with the samples at all times. If the sample blank shows no evidence for chloroform, then we can safely assume that the samples also are free from contamination.

5. Why is it necessary to collect samples without any headspace (a layer of air overlying the liquid) in the sample vial?

   Because trihalomethanes are volatile, the presence of a headspace allows for the possible loss of analyte.

6. In preparing the stock solution for each trihalomethane, the procedure specifies that we can add the two or more drops of the pure compound by dropping them into the volumetric flask containing methanol. When preparing the calibration standards, however, the working standard must be injected below the surface of the water. Explain the reason for this difference.

   When preparing a stock solution, the potential loss of the volatile trihalomethane is unimportant because we determine its concentration by weight after adding it to the water. When we prepare the calibration standard, however, we must ensure that the addition of trihalomethane is quantitative.

12D.8 Evaluation

See Figure 3.5 to review the meaning of major, minor, and ultratrace analytes.

Scale of Operation

Gas chromatography is used to analyze analytes present at levels ranging from major to ultratrace components. Depending on the detector, samples with major and minor analytes may need to be diluted before analysis. The thermal conductivity and flame ionization detectors can handle larger amounts of analyte; other detectors, such as an electron capture detector or a mass spectrometer, require substantially smaller amounts of analyte. Although the injection volume for gas chromatography is quite small—typically about a microliter—the amount of available sample must be sufficient that the injection is a representative subsample. For a trace analyte, the actual amount of injected analyte is often in the picogram range. Using Representative Method 12.1 as an example, a 3.0-μL injection of 1 μg/L $CHCl_3$ is equivalent to 15 pg of $CHCl_3$, assuming a 100% extraction efficiency.

Accuracy

The accuracy of a gas chromatographic method varies substantially from sample to sample. For routine samples, accuracies of 1–5% are common. For analytes present at very low concentration levels, samples with complex matrices, or samples requiring significant processing before analysis, accuracy may be substantially poorer. In the analysis for trihalomethanes described in Representative Method 12.1, for example, determinate errors as large as ±25% are possible.

Precision

The precision of a gas chromatographic analysis includes contributions from sampling, sample preparation, and the instrument. The relative standard deviation due to the instrument is typically 1–5%, although it can be significantly higher. The principle limitations are detector noise, which affects the determination of peak area, and the reproducibility of injection volumes. In quantitative work, the use of an internal standard compensates for any variability in injection volumes.

Sensitivity

In a gas chromatographic analysis, sensitivity is determined by the detector's characteristics. Of particular importance for quantitative work is the detector's linear range; that is, the range of concentrations over which a calibration curve is linear. Detectors with a wide linear range, such as the thermal conductivity detector and the flame ionization detector, can be used to analyze samples over a wide range of concentrations without ad-

justing operating conditions. Other detectors, such as the electron capture detector, have a much narrower linear range.

Selectivity

Because it combines separation with analysis, chromatographic methods provide excellent selectivity. By adjusting conditions it is usually possible to design a separation so that the analytes elute by themselves, even when the mixture is complex. Additional selectivity is obtained by using a detector, such as the electron capture detector, that does not respond to all compounds.

Time, Cost, and Equipment

Analysis time can vary from several minutes for samples containing only a few constituents, to more than an hour for more complex samples. Preliminary sample preparation may substantially increase the analysis time. Instrumentation for gas chromatography ranges in price from inexpensive (a few thousand dollars) to expensive (>$50,000). The more expensive models are equipped for capillary columns, include a variety of injection options and more sophisticated detectors, such as a mass spectrometer. Packed columns typically cost <$200, and the cost of a capillary column is typically $300–$1000.

12E High-Performance Liquid Chromatography

In HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) we inject the sample, which is in solution form, into a liquid mobile phase. The mobile phase carries the sample through a packed or capillary column that separates the sample's components based on their ability to partition between the mobile phase and the stationary phase. Figure 12.38 shows an example of a typical HPLC instrument, which consists of several key components: reservoirs containing the mobile phase; a pump for pushing the mobile phase through the system; an injector for introducing the sample; a column; and a detector for monitoring the eluent as it comes off the column. Let's consider each of these components.

A solute's retention time in HPLC is determined by its interaction with the stationary phase and the mobile phase. There are a several different types of solute/stationary phase interactions, including liquid–solid adsorption, liquid–liquid partitioning, ion-exchange, and size-exclusion (see Figure 12.4). Section 12E deals exclusively with HPLC separations based on liquid–liquid partitioning. Other forms of liquid chromatography receive consideration in Section 12F.

12E.1 HPLC Columns

An HPLC typically includes two columns: an analytical column responsible for the separation and a guard column. The guard column is placed before the analytical column, protecting it from contamination.

Analytical Columns

The most common type of HPLC column is a stainless steel tube with an internal diameter between 2.1 mm and 4.6 mm and a length between 30 mm and 300 mm (Figure 12.39). The column is packed with 3–10 μm

Figure 12.39 Typical packed column for HPLC. This particular column has an internal diameter of 4.6 mm and a length of 150 mm, and is packed with 5 μm particles coated with stationary phase.

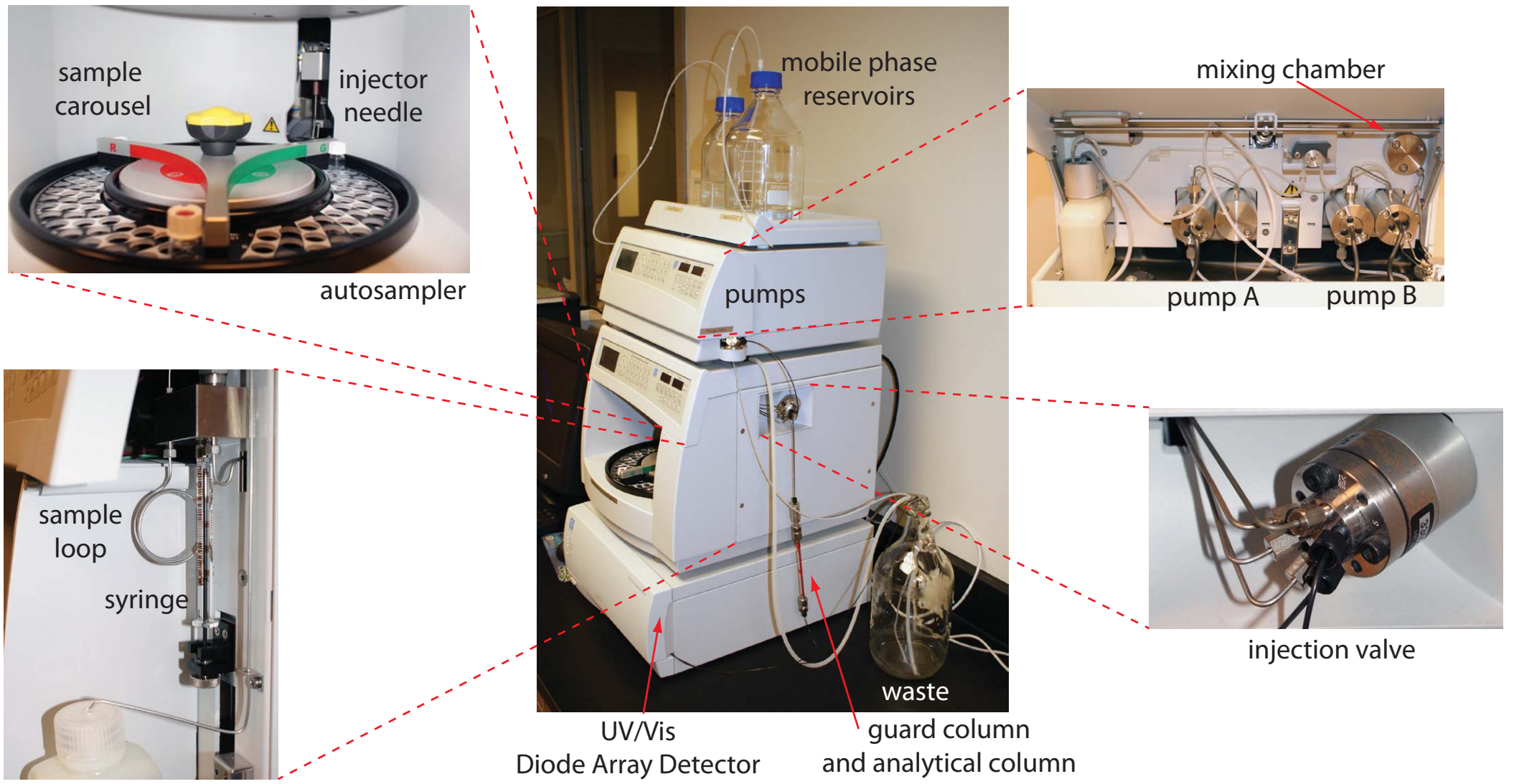


Figure 12.38 Example of a typical high-performance liquid chromatograph with insets showing the pumps that move the mobile phase through the system, and the plumbing used to inject the sample into the mobile phase. This particular instrument includes an autosampler. An instrument in which samples are injected manually does not include the features shown in the two left-most insets, and has a different style of loop injector (see Figure 12.45).

You can use equation 12.16 to estimate a column's peak capacity.

porous silica particles with either an irregular or a spherical shape. Typical column efficiencies are 40 000–60 000 theoretical plates/m. Assuming a V_{max}/V_{min} of approximately 50, a 25-cm column with 50 000 plates/m has 12 500 theoretical plates and a peak capacity of 110.

Capillary columns use less solvent and, because the sample is diluted to a lesser extent, produce larger signals at the detector. These columns are made from fused silica capillaries with internal diameters from 44–200 μm and lengths of 50–250 mm. Capillary columns packed with 3–5 μm particles have been prepared with column efficiencies of up to 250 000 theoretical plates.[10]

One limitation to a packed capillary column is the back pressure that develops when trying to move the mobile phase through the small interstitial spaces between the particulate micron-sized packing material (Figure 12.40). Because the tubing and fittings that carry the mobile phase have pressure limits, a higher back pressure requires a lower flow rate and a longer analysis time. MONOLITHIC COLUMNS, in which the solid support is a single, porous rod, offer column efficiencies equivalent to a packed capillary column while allowing for faster flow rates. A monolithic column—which usually is similar in size to a conventional packed column, although smaller, capillary columns also are available—is prepared by forming the monolithic rod in a mold and covering it with PTFE tubing or a polymer resin.

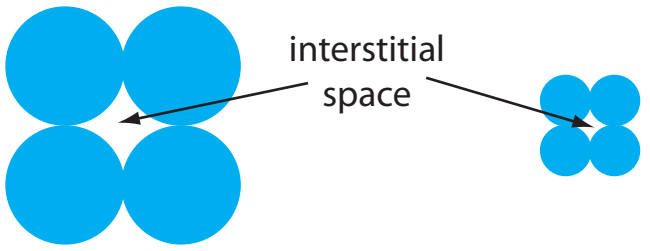


Figure 12.40 The packing of smaller particles creates smaller interstitial spaces than the packing of larger particles. Although reducing particle size by 2× increases efficiency by a factor of 1.4, it also produces a 4-fold increase in back pressure.

10 Novotony, M. *Science*, **1989**, *246*, 51–57.

Monolithic rods made of a silica-gel polymer typically have macropores with diameters of approximately 2 μm and mesopores—pores within the macropores—with diameters of approximately 13 nm.[11]

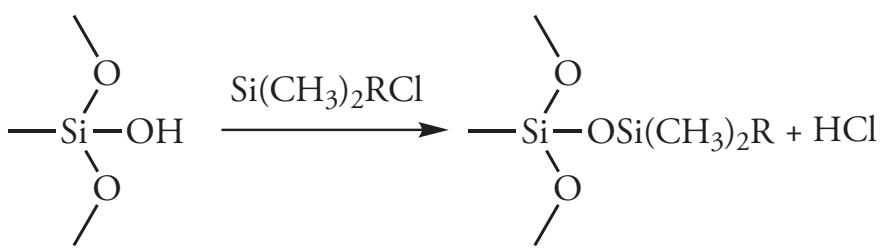
Guard Columns

Two problems tend to shorten the lifetime of an analytical column. First, solutes binding irreversibly to the stationary phase degrade the column's performance by decreasing the available stationary phase. Second, particulate material injected with the sample may clog the analytical column. To minimize these problems we place a guard column before the analytical column. GUARD COLUMNS usually contain the same particulate packing material and stationary phase as the analytical column, but are significantly shorter and less expensive—a length of 7.5 mm and a cost one-tenth of that for the corresponding analytical column is typical. Because they are intended to be sacrificial, guard columns are replaced regularly.

If you look closely at Figure 12.39, you will see the small guard column just above the analytical column.

Stationary Phases for Gas–Liquid Chromatography

In liquid–liquid chromatography the stationary phase is a liquid film coated on a packing material, typically 3–10 μm porous silica particles. Because the stationary phase may be partially soluble in the mobile phase, it may elute, or bleed from the column over time. To prevent the loss of stationary phase, which shortens the column's lifetime, it is covalently bound to the silica particles. BONDED STATIONARY PHASES are created by reacting the silica particles with an organochlorosilane of the general form $Si(CH_3)_2RCl$, where R is an alkyl, or substituted alkyl group.



To prevent unwanted interactions between the solutes and any remaining –SiOH groups, $Si(CH_3)_3Cl$ is added, converting the unreacted sites to $–SiOSi(CH_3)_3$; such columns are designated as end-capped.

The properties of a stationary phase depend on the organosilane's alkyl group. If R is a polar functional group, then the stationary phase is polar. Examples of polar stationary phases include those where R contains a cyano ($–C_2H_4CN$), a diol ($–C_3H_6OCH_2CHOHCH_2OH$), or an amino ($–C_3H_6NH_2$) functional group. Because the stationary phase is polar, the mobile phase is a nonpolar or moderately polar solvent. The combination of a polar stationary phase and a nonpolar mobile phase is called NORMAL-PHASE CHROMATOGRAPHY.

In REVERSED-PHASE CHROMATOGRAPHY, which is the more common form of HPLC, the stationary phase is nonpolar and the mobile phase

It might strike you as odd that the less common form of liquid chromatography is identified as normal-phase. You might recall that one of the earliest examples of chromatography was Mikhail Tswett's separation of plant pigments using a polar column of calcium carbonate and a nonpolar mobile phase of petroleum ether. The assignment of normal and reversed, therefore, is all about precedence.

11 Cabrera, K. Chromatography Online, April 1, 2008.

is polar. The most common nonpolar stationary phases use an organochlorosilane where the R group is an *n*-octyl (C_8) or *n*-octyldecyl (C_{18}) hydrocarbon chain. Most reversed-phase separations are carried out using a buffered aqueous solution as a polar mobile phase, or with other polar solvents, such as methanol and acetonitrile. Because the silica substrate may undergo hydrolysis in basic solutions, the pH of the mobile phase must be less than 7.5.

12E.2 Mobile Phases

The elution order of solutes in HPLC is governed by polarity. For a normal-phase separation, solutes of lower polarity spend proportionally less time in the polar stationary phase and are the first solutes to elute from the column. Given a particular stationary phase, retention times in normal-phase HPLC are controlled by adjusting the mobile phase's properties. For example, if the resolution between two solutes is poor, switching to a less polar mobile phase keeps the solutes on the column for a longer time and provides more opportunity for their separation. In reversed-phase HPLC the order of elution is the opposite of that in a normal-phase separation, with more polar solutes eluting first. Increasing the polarity of the mobile phase leads to longer retention times. Shorter retention times require a mobile phase of lower polarity.

Choosing a Mobile Phase–Using the Polarity Index

There are several indices that help in choosing a mobile phase, one of which is the POLARITY INDEX.[12] Table 12.4 provides values of the polarity index, P', for several common mobile phases, where larger values of P' correspond to more polar solvents. Mixing together two or more mobile phases—assuming they are miscible—creates a mobile phase of intermediate polarity. For example, a binary mobile phase made by combining solvents A and B has a polarity index, P'_{AB}, of

$$P'_{AB} = \Phi_A P'_A + \Phi_B P'_B \qquad 12.28$$

where P'_A and P'_B are the polarity indices for solvents A and B, and Φ_A and Φ_B are the volume fractions for the two solvents.

Example 12.7

A reversed-phase HPLC separation is carried out using a mobile phase of 60% v/v water and 40% v/v methanol. What is the mobile phase's polarity index?

Solution

Using equation 12.28 and the values in Table 12.4, the polarity index for a 60:40 water–methanol mixture is

12 Snyder, L. R.; Glajch, J. L.; Kirkland, J. J. *Practical HPLC Method Development*, Wiley-Interscience: New York, 1988.

Table 12.4 Properties of HPLC Mobile Phases

mobile phase	polarity index (P')	UV cutoff (nm)
cyclohexane	0.04	210
n-hexane	0.1	210
carbon tetrachloride	1.6	265
i-propyl ether	2.4	220
toluene	2.4	286
diethyl ether	2.8	218
tetrahydrofuran	4.0	220
ethanol	4.3	210
ethyl acetate	4.4	255
dioxane	4.8	215
methanol	5.1	210
acetonitrile	5.8	190
water	10.2	—

$$P'_{AB} = \Phi_{H_2O} P'_{H_2O} + \Phi_{CH_3OH} P'_{CH_3OH}$$

$$P'_{AB} = 0.60 \times 10.2 + 0.40 \times 5.1 = 8.2$$

Practice Exercise 12.7

Suppose you need a mobile phase with a polarity index of 7.5. Explain how you can prepare this mobile phase using methanol and water?

Click here to review your answer to this exercise.

As a general rule, a two unit change in the polarity index corresponds to approximately a 10-fold change in a solute's retention factor. Here is a simple example. If a solute's retention factor, k, is 22 when using water as a mobile phase ($P' = 10.2$), then switching to a mobile phase of 60:40 water–methanol ($P' = 8.2$) decreases k to approximately 2.2. Note that the retention factor becomes smaller because we are switching from a more polar mobile phase to a less polar mobile phase in a reversed-phase separation.

Choosing a Mobile Phase–Adjusting Selectivity

Changing the mobile phase's polarity index changes a solute's retention factor. As we learned in Section 12C.1, however, a change in k is not an effective method for improving resolution when its initial value is greater than 10. To effect a better separation between two solutes we must improve the selectivity factor, α. There are two commonly used approaches for increasing α: add a reagent to the mobile phase that reacts with the solutes in a secondary equilibrium reaction, or try a different mobile phase.

Acid–base chemistry is not the only example of a secondary equilibrium reaction. Other examples include ion-pairing, complexation, and the interaction of solutes with micelles. We will consider the last of these in Section 12G.3 when we discuss micellar electrokinetic capillary chromatography.

Taking advantage of a secondary equilibrium reaction is a useful strategy for improving a separation.[13] Figure 12.17, which we considered earlier in this chapter, shows the reversed-phase separation of four weak acids—benzoic acid, terephthalic acid, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid—on a nonpolar C_{18} column using a aqueous mobile phase that includes a buffer of acetic acid and sodium acetate. Retention times are shorter for less acidic mobile phases because each solute is present in an anionic, weak base form that is less soluble in the nonpolar stationary phase. If the mobile phase's pH is sufficiently acidic, the solutes are present as neutral weak acids that partition into the stationary phase and take longer to elute. Because these solutes do not have identical pK_a values, the pH of the mobile phase affects each solute's retention time differently, allowing us to find the optimum pH for effecting a complete separation of the four solutes.

In Example 12.7 we learned how to adjust the mobile phase's polarity by blending together two solvents. A polarity index, however, is just a guide, and binary mobile phase mixtures with identical polarity indices may not equally resolve a pair of solutes. Table 12.5, for example, shows retention times for four weak acids in two mobile phases with nearly identical values for P'. Although the order of elution is the same for both mobile phases, each solute's retention time is affected differently by the choice of organic solvent. If we switch from using acetonitrile to tetrahydrofuran, for example, benzoic acid elutes more quickly and *p*-hydroxybenzoic acid elutes more slowly. Although we can resolve these two solutes using a mobile phase that is 16% v/v acetonitrile, we cannot resolve them if the mobile phase is 10% tetrahydrofuran.

The choice to start with acetonitrile is arbitrary—we can just as easily choose to begin with methanol or with tetrahydrofuran.

One strategy for finding the best mobile phase is to use the solvent triangle shown in Figure 12.41, which allows us to explore a broad range of mobile phases with only seven experiments. We begin by adjusting the amount of acetonitrile in the mobile phase, producing the best possible separation within the desired analysis time. Next, we use Table 12.6 to es-

13 (a) Foley, J. P. *Chromatography*, **1987**, *7*, 118–128; (b) Foley, J. P.; May, W. E. Anal. Chem. 1987, 59, 102–109; (c) Foley, J. P.; May, W. E. Anal. Chem. 1987, 59, 110–115.

Table 12.5 Retention Times for Four Weak Acids in Mobile Phases With Similar Polarity Indexes

retention time (min)	16% acetonitrile (CH_3CN) 84% pH 4.11 aqueous buffer ($P' = 9.5$)	10% tetrahydrofuran (THF) 90% pH 4.11 aqueous buffer ($P' = 9.6$)
$t_{r,BA}$	5.18	4.01
$t_{r,PH}$	1.67	2.91
$t_{r,PA}$	1.21	1.05
$t_{r,TP}$	0.23	0.54

Key: BA is benzoic acid; PH is *p*-hydroxybenzoic acid; PA is *p*-aminobenzoic acid; TP is terephthalic acid
Source: Harvey, D. T.; Byerly, S.; Bowman, A.; Tomlin, J. "Optimization of HPLC and GC Separations Using Response Surfaces," *J. Chem. Educ.* **1991**, *68*, 162–168.

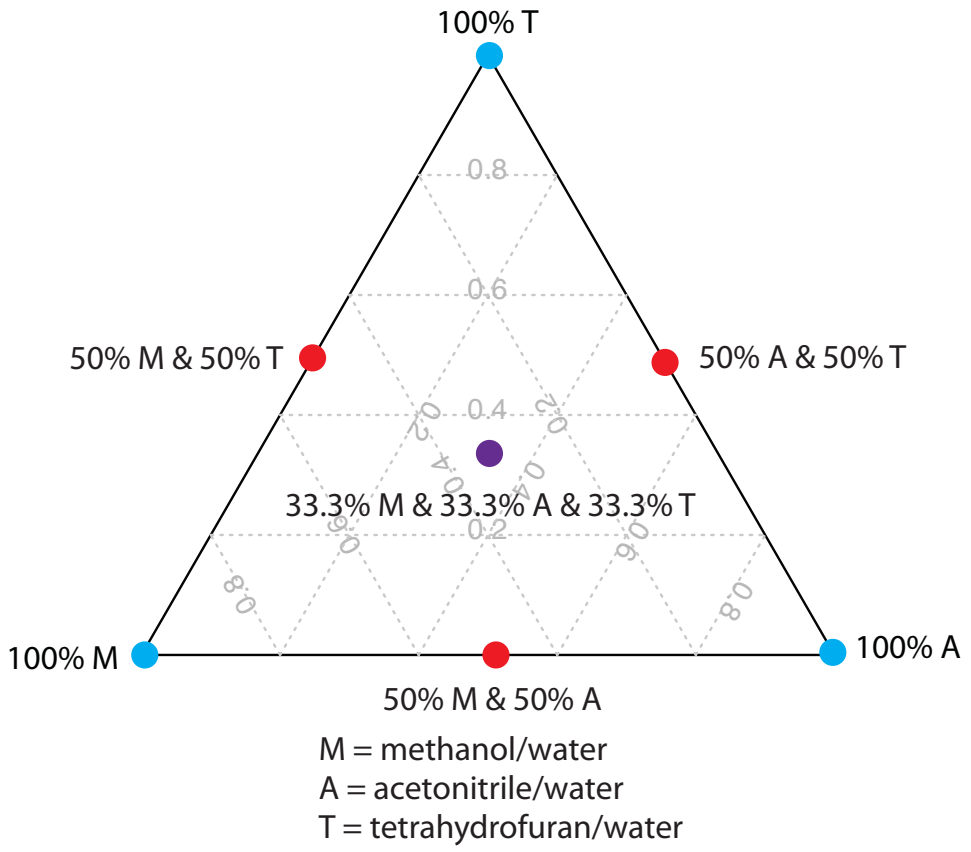


Figure 12.41 Solvent triangle for optimizing a reversed-phase HPLC separation. The three blue circles show mobile phases consisting of an organic solvent and water. The three red circles are binary mobile phases created by combining equal volumes of the pure mobile phases. The ternary mobile phase shown by the purple circle contains all three of the pure mobile phases.

timate the composition of methanol/H_2O and tetrahydrofuran/H_2O mobile phases that will produce similar analysis times. Four additional mobile phases are prepared using the binary and ternary mobile phases shown in Figure 12.41. By evaluating the chromatograms from these seven mobile phases, we may find that one or more provides an adequate separation, or identify a region within the solvent triangle where a separation if feasible. Figure 12.42 shows results for the reversed-phase separation of benzoic acid, terephthalic acid, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid on a nonpolar C_{18} column in which the maximum analysis time is set to 6 min.[14] The areas in blue, green, and red show mobile phase compositions that do not provide baseline resolution. The unshaded area represents mobile phase compositions where a separation is possible.

14 Harvey, D. T.; Byerly, S.; Bowman, A.; Tomlin, J. *J. Chem. Educ.* **1991**, *68*, 162–168

Table 12.6 Compositions of Mobile Phases with Approximately Equal Solvent Strengths

%v/v CH_3OH	%v/v CH_3CN	%v/v THF
0	0	0
10	6	4
20	14	10
30	22	16
40	32	24
50	40	30
60	50	36
70	60	44
80	72	52
90	87	62
100	99	71

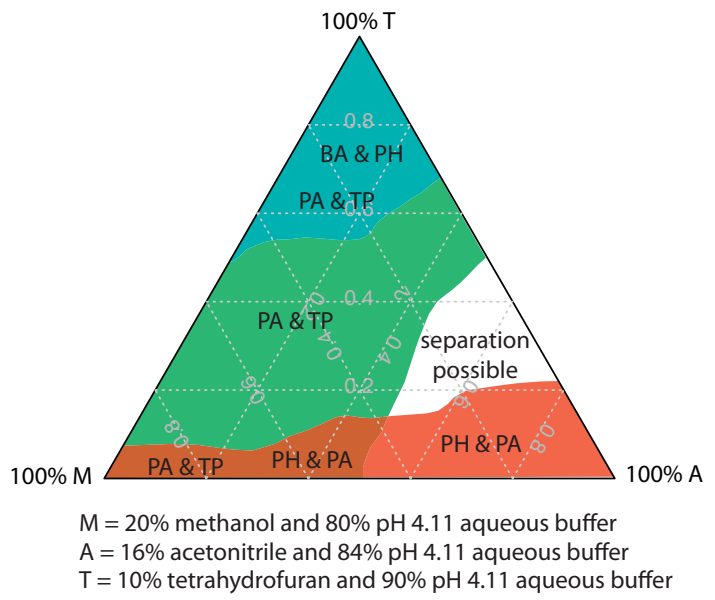


Figure 12.42 Resolution map for the separation of benzoic acid (BA), terephthalic acid (TP), *p*-aminobenzoic acid (PA), and *p*-hydroxybenzoic acid (PH) on a nonpolar C_{18} column subject to a maximum analysis time of 6 min. The shaded areas represent regions where a separation is not possible, with the unresolved solutes identified. A separation is possible in the unshaded area. See Chapter 14 for a discussion of how we can develop a mathematical model for optimizing separations.

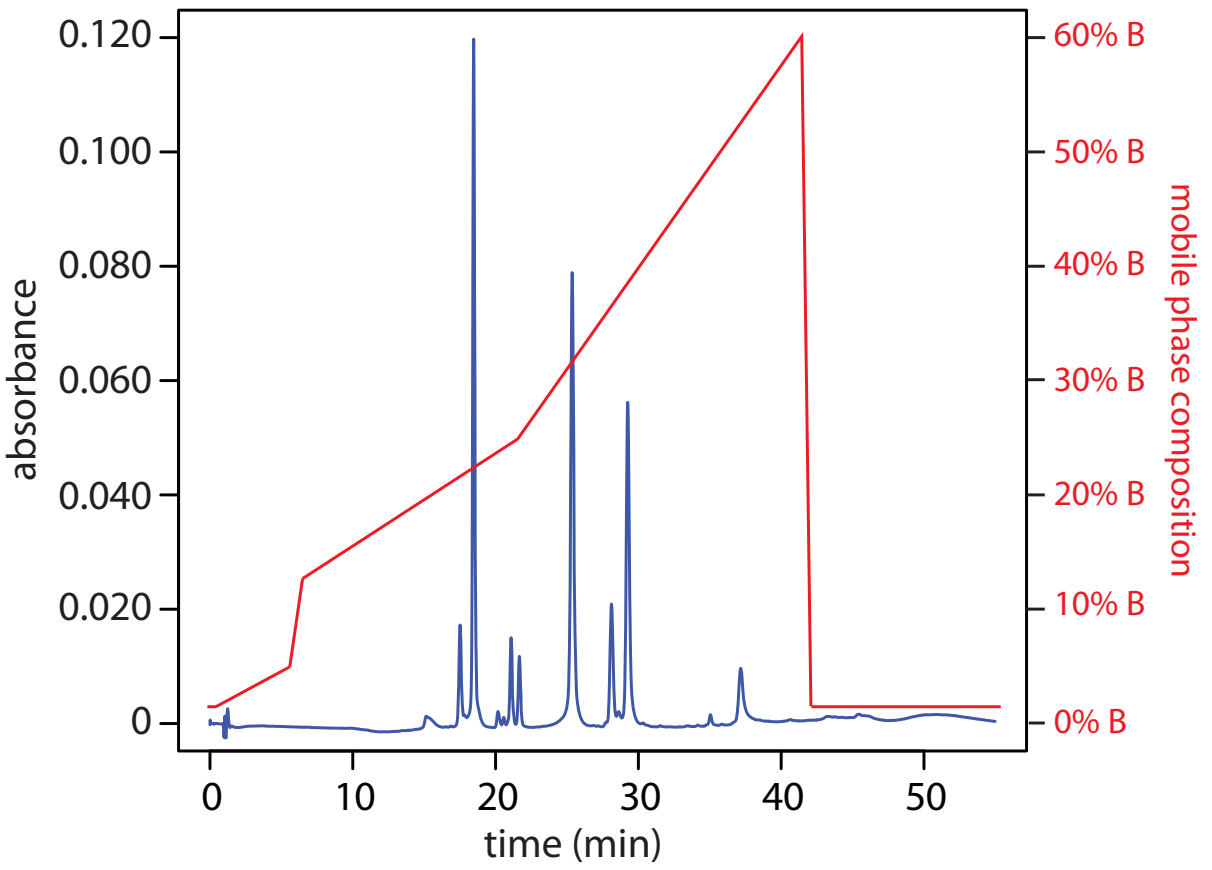


Figure 12.43 Gradient elution separation of a mixture of flavonoids. Mobile phase A is an aqueous solution of 0.1% formic acid and mobile phase B is 0.1% formic acid in acetonitrile. The initial mobile phase is 98% A and 2% B. The percentage of mobile phase B increases in four steps: from 2% to 5% over 5 min, beginning at 0.5 min; from 5% to 12% over 1 min, beginning at 5.5 min; from 12% to 25% over 15 min, beginning at 6.5 min; and from 25% to 60% over 20 min, beginning at 21.5 min. Data provided by Chistopher Schardon, Kyle Meinhardt, and Michelle Bushey, Department of Chemistry, Trinty University.

Choosing a Mobile Phase–Isocratic and Gradient Elutions

You may recall that we called this the general elution problem (see Figure 12.16).

A separation using a mobile phase that has a fixed composition is an ISOCRATIC ELUTION. One difficulty with an isocratic elution is that an appropriate mobile phase strength for early eluting solutes may lead to unacceptably long retention times for later eluting solutes. Optimizing the mobile phase for late eluting solutes, on the other hand, may provide an inadequate separation of early eluting solutes. Changing the mobile phase's composition as the separation progresses is one solution to this problem. For a reversed-phase separation we use an initial mobile phase that is more polar. As the separation progresses, we adjust the composition of mobile phase so that it becomes less polar (see Figure 12.43). Such separations are called GRADIENT ELUTIONS.

12E.3 HPLC Plumbing

In a gas chromatograph the pressure of the compressed gas cylinder containing the mobile phase is sufficient to push it through the column. Pushing a liquid mobile phase through a column takes a great deal more effort, generating pressures in excess of several hundred atmospheres. In this section we consider the basic plumbing needed to move the mobile phase through the column and to inject the sample into the mobile phase.

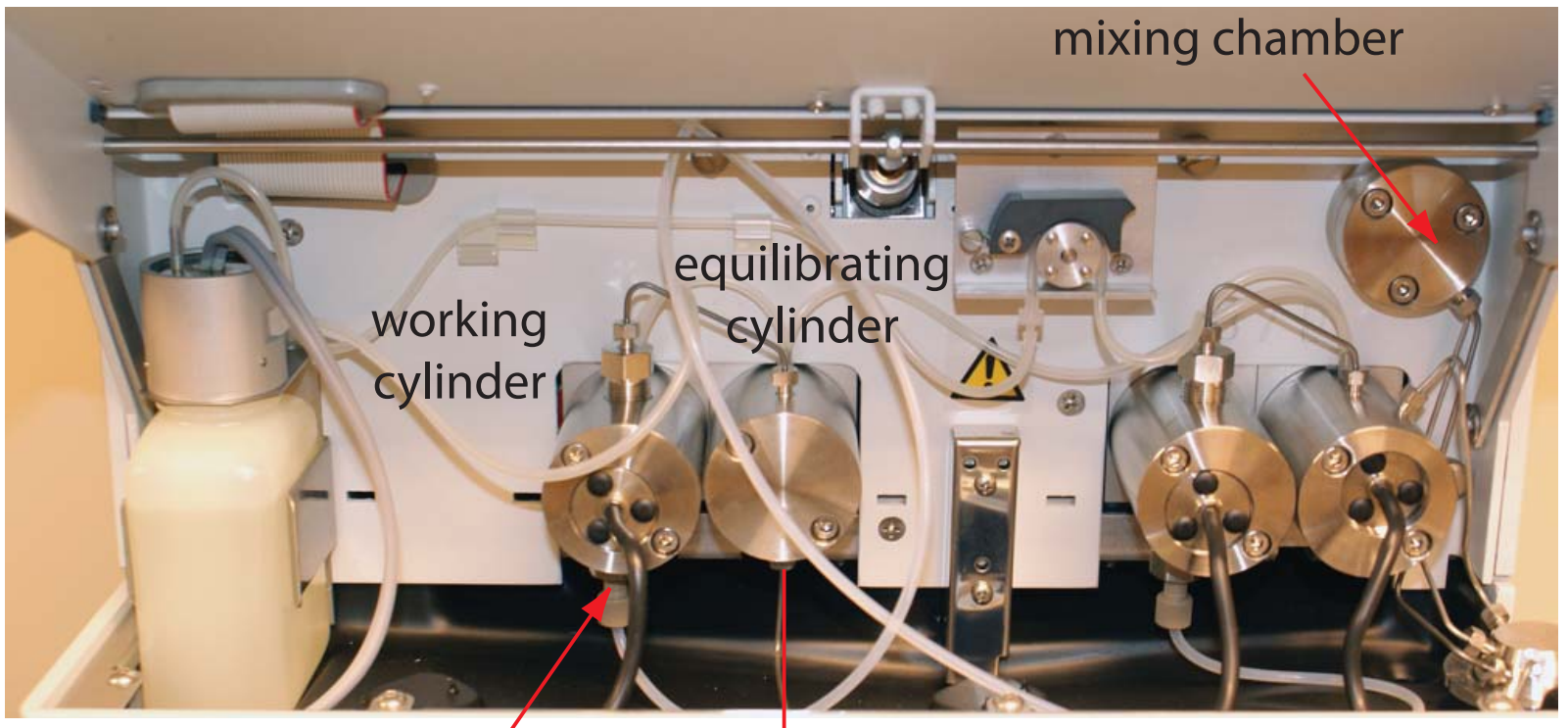


Figure 12.44 Close-up view of the pumps for the instrument shown in Figure 12.38. The working cylinder and equilibrating cylinder for the pump on the left take solvent from reservoir A and send it to the mixing chamber. The pump on the right moves solvent from reservoir B to the mixing chamber. The mobile phase's flow rate is determined by the combined speeds of the two pumps. By changing the relative speeds of the two pumps, different binary mobile phases can be prepared.

Moving the Mobile Phase

A typical HPLC includes between 1–4 reservoirs for storing mobile phase solvents. The instrument in Figure 12.38, for example, has two mobile phase reservoirs that can be used for an isocratic or a gradient elution by drawing solvents from one or both reservoirs.

Before using a mobile phase solvent we must remove dissolved gases, such as N_2 and O_2, and small particulate matter, such as dust. Because there is a large drop in pressure across the column—the pressure at the column's entrance may be several hundred atmospheres, but it is atmospheric pressure at its exit—any dissolved gases in the mobile phase are released as gas bubbles that may interfere with the detector's response. Degassing is accomplished in several ways, but the most common are the use of a vacuum pump or sparging with an inert gas, such as He, which has a low solubility in the mobile phase. Particulate material, which may clog the HPLC tubing or column, is removed by filtering the solvents.

Bubbling an inert gas through the mobile phase releases volatile dissolved gases. This process is called sparging.

The mobile phase solvents are pulled from their reservoirs by the action of one or more pumps. Figure 12.44 shows a close-up view of the pumps for the instrument in Figure 12.38. The working pump and equilibrating pump each have a piston whose back and forth movement is capable both of maintaining a constant flow rate of up to several mL/min and of obtaining the high output pressure needed to push the mobile phase through the chromatographic column. In this particular instrument, each pump sends

There are other possible ways to control the mobile phase's composition and flow rate. For example, instead of the two pumps in Figure 12.45, we can place a solvent proportioning valve before a single pump. The solvent proportioning value connects two or more solvent reservoirs to the pump and determines how much of each solvent is pulled during each of the pump's cycles.

Another approach for eliminating a pulsed flow is to include a pulse damper between the pump and the column. A pulse damper is a chamber filled with an easily compressed fluid and a flexible diaphragm. During the piston's forward stroke the fluid in the pulse damper is compressed. When the piston withdraws to refill the pump, pressure from the expanding fluid in the pulse damper maintains the flow rate.

its mobile phase to a mixing chamber where they combine to form the final mobile phase. The relative speed of the two pumps determines the mobile phase's final composition.

The back and forth movement of a reciprocating pump creates a pulsed flow that contributes noise to the chromatogram. To minimize these pulses, each pump in Figure 12.44 has two cylinders. During the working cylinder's forward stoke it fills the equilibrating cylinder and establishes flow through the column. When the working cylinder is on its reverse stroke, the flow is maintained by the piston in the equilibrating cylinder. The result is a pulse-free flow.

Injecting the Sample

The operating pressure within an HPLC is sufficiently high that we cannot inject the sample into the mobile phase by inserting a syringe through a septum. Instead, we inject the sample using a LOOP INJECTOR, a diagram of which is shown in Figure 12.45.

The instrument in Figure 12.39 uses an autosampler to inject samples. Instead of using a syringe to push the sample into the sample loop, the syringe draws sample into the sample loop.

In the load position a sample loop—which is available in a variety of sizes ranging from 0.5 μL to 5 mL—is isolated from the mobile phase and open to the atmosphere. The sample loop is filled using a syringe with a capacity several times that of the sample loop, with the excess sample exiting through the waste line. After loading the sample, the injector is turned to the inject position, directing the mobile phase through the sample loop and onto the column.

12E.4 Detectors for HPLC

Many different types of detectors have been use to monitor HPLC separations, most of which use the spectroscopic techniques from Chapter 10 or the electrochemical techniques from Chapter 11.

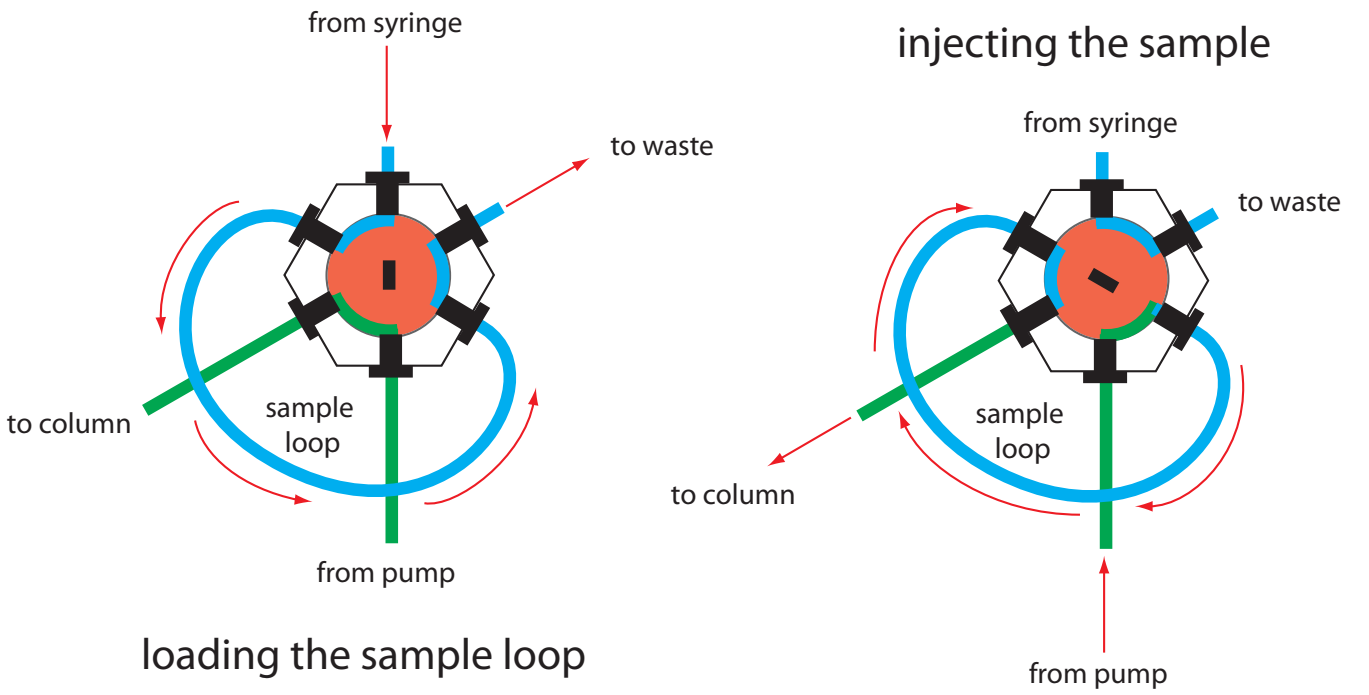


Figure 12.45 Schematic diagram showing a manual loop injector. In the load position the flow of mobile phase from the pump to the column (shown in green) is isolated from the sample loop, which is filled using a syringe (shown in blue). Rotating the inner valve (shown in red) to the inject position directs the mobile phase through the sample loop and onto the column.

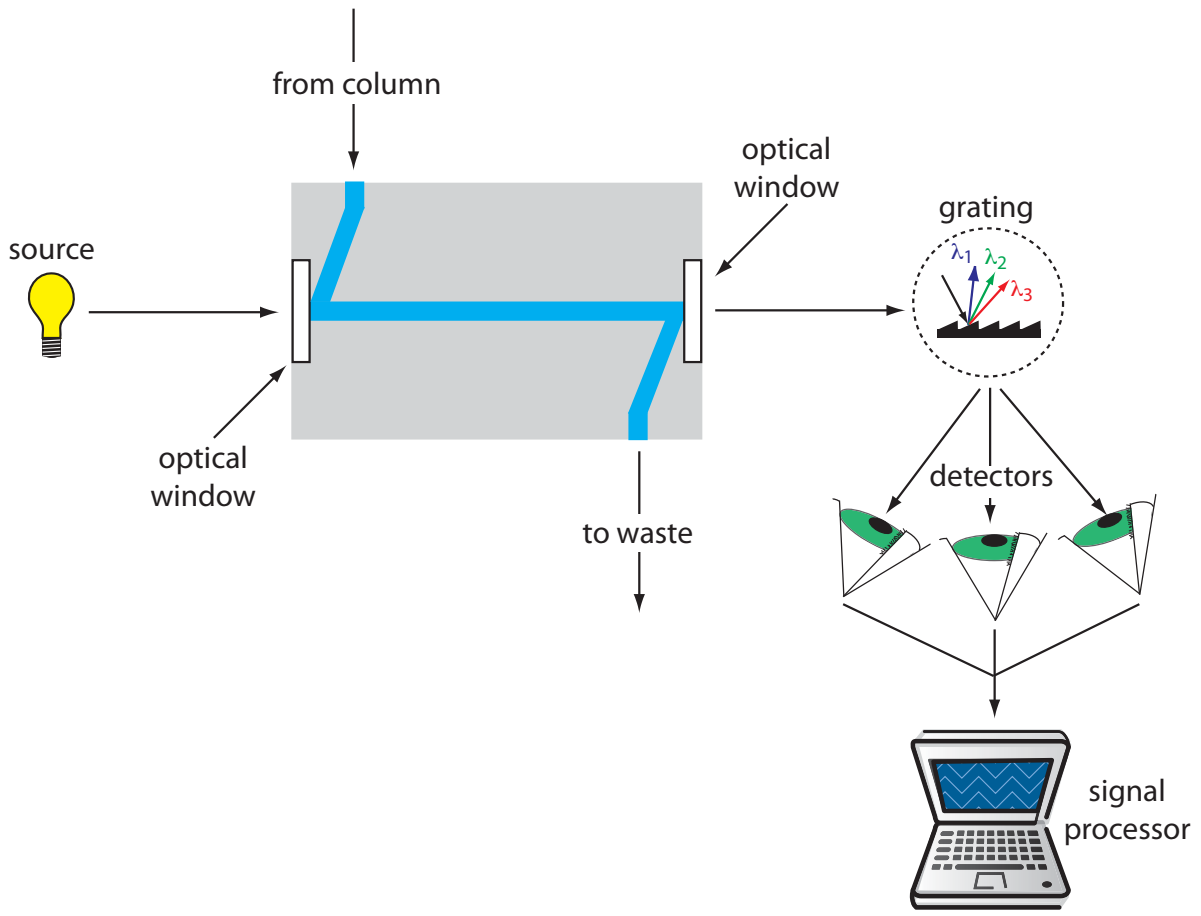


Figure 12.46 Schematic diagram showing a flow cell for a detector using a diode array spectrometer.

Spectroscopic Detectors

The most popular HPLC detectors take advantage of an analyte's UV/Vis absorption spectrum. These detectors range from simple designs, in which the analytical wavelength is selected using appropriate filters, to a modified spectrophotometer in which the sample compartment includes a flow cell. Figure 12.46 shows the design of a typical flow cell for a detector using a diode array spectrometer. The flow cell has a volume of 1–10 μL and a path length of 0.2–1 cm.

To review the details of how we measure absorbance, see Chapter 10B. More information about different types of instruments, including the diode array spectrometer, is in Chapter 10C.

When using a UV/Vis detector the resulting chromatogram is a plot of absorbance as a function of elution time (see Figure 12.47). If the detector is a diode array spectrometer, then we also can display the result as a three-dimensional chromatogram showing absorbance as a function of

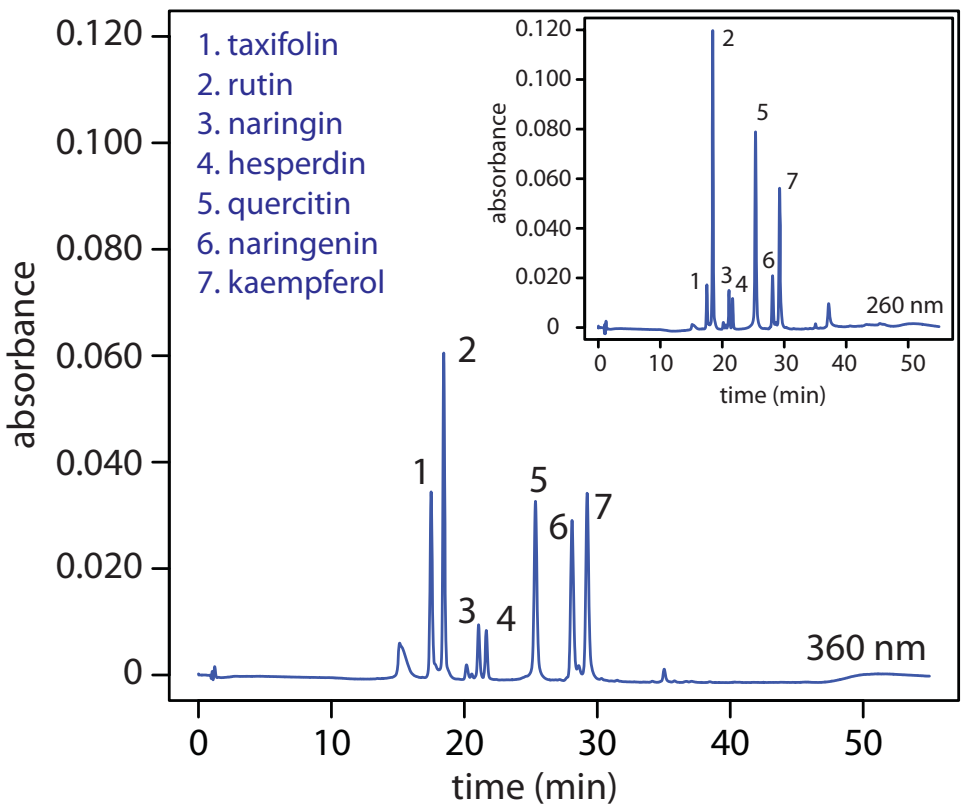


Figure 12.47 HPLC separation of a mixture of flavonoids with UV/Vis detection at 360 nm and 260 nm. The choice of wavelength affects each analyte's signal. By carefully choosing the wavelength, we can enhance the signal for the analytes of greatest interest. Data provided by Chistopher Schardon, Kyle Meinhardt, and Michelle Bushey, Department of Chemistry, Trinty University.

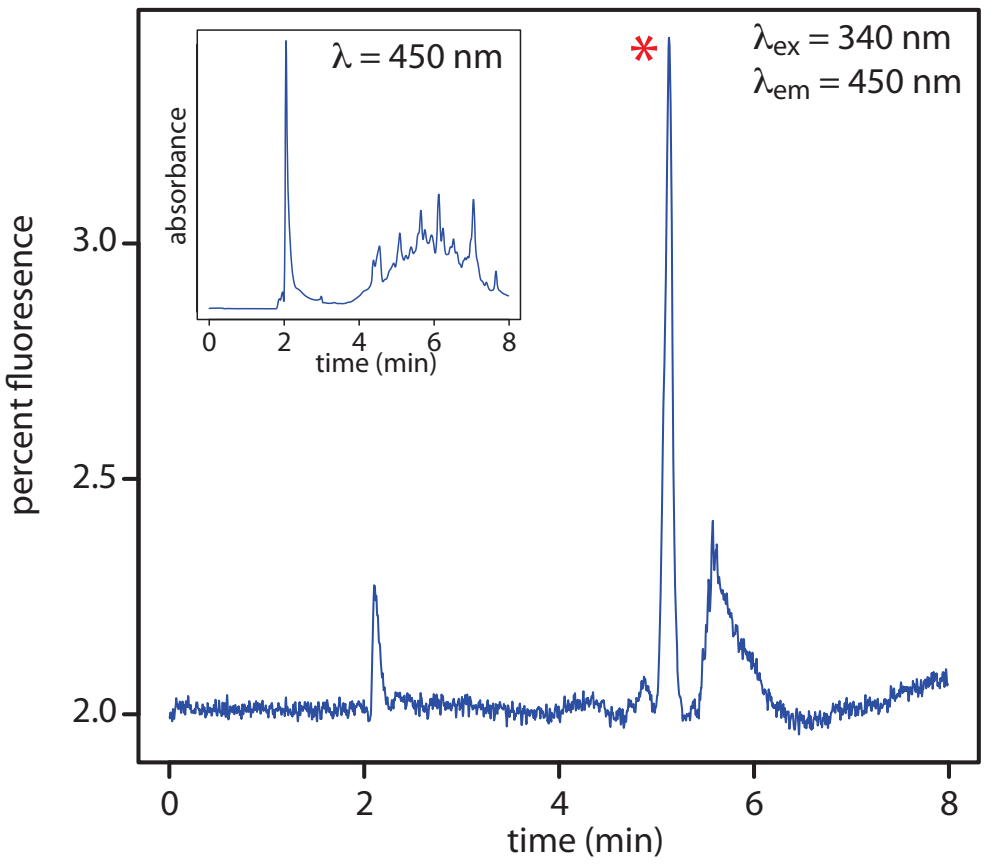


Figure 12.48 HPLC chromatogram for the determination of riboflavin in urine using fluorescence detection with excitation at a wavelength of 340 nm and detection at 450 nm. The peak corresponding to riboflavin is marked with a red asterisk (*). The inset shows the same chromatogram when using a less-selective UV/Vis detector at a wavelength of 450 nm. Data provided by Jason Schultz, Jonna Berry, Kaelene Lundstrom, and Dwight Stoll, Department of Chemistry, Gustavus Adolphus College.

wavelength and elution time. One limitation to using absorbance is that the mobile phase cannot absorb at the wavelengths we wish to monitor. Table 12.4 lists the minimum useful UV wavelength for several common HPLC solvents. Absorbance detectors provide detection limits of as little as 100 pg–1 ng of injected analyte.

See Chapter 10F for a review of fluorescence spectroscopy and spectrofluorimeters.

If an analyte is fluorescent, we can place the flow cell in a spectrofluorimeter. As shown in Figure 12.48, a fluorescence detector provides additional selectivity because only a few of a sample's components are fluorescent. Detection limits are as little as 1–10 pg of injected analyte.

Electrochemical Detectors

See Chapter 11D.5 for a review of amperometry.

Another common group of HPLC detectors are those based on electrochemical measurements such as amperometry, voltammetry, coulometry, and conductivity. Figure 12.49, for example, shows an amperometric flow cell. Effluent from the column passes over the working electrode, which is held at a constant potential—relative to a downstream reference electrode—that completely oxidizes or reduces the analytes. The current flow-

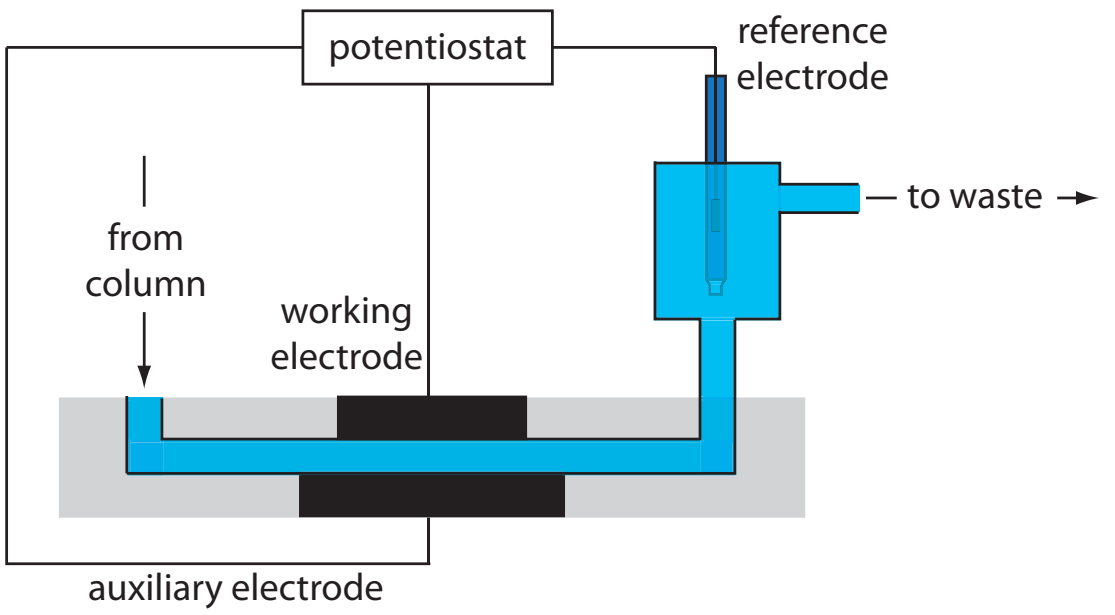


Figure 12.49 Schematic diagram showing a flow cell for an amperometric electrochemical detector.

ing between the working electrode and the auxiliary electrode serves as the analytical signal. Detection limits for amperometric electrochemical detection are from 10 pg–1 ng of injected analyte.

Other Detectors

Several other detectors have been used in HPLC. Measuring a change in the mobile phase's refractive index is analogous to monitoring the mobile phase's thermal conductivity in gas chromatography. A refractive index detector is nearly universal, responding to almost all compounds, but has a relatively poor detection limit of 100 ng–1 μg of injected analyte. An additional limitation of a refractive index detector is that it cannot be used for gradient elution unless the mobile phase components have identical refractive indexes.

Another useful detector is a mass spectrometer. Figure 12.50 shows a block diagram of a typical HPLC–MS instrument. The effluent from the column enters the mass spectrometer's ion source using an interface the removes most of the mobile phase, an essential need because of the incompatibility between the liquid mobile phase and the mass spectrometer's high vacuum environment. In the ionization chamber the remaining molecules—a mixture of carrier gas, solvent, and solutes—undergo ionization and fragmentation. The mass spectrometer's mass analyzer separates the ions by their mass-to-charge ratio (m/z). A detector counts the ions and displays the mass spectrum.

There are several options for monitoring the chromatogram when using a mass spectrometer as the detector. The most common method is to continuously scan the entire mass spectrum and report the total signal for all ions reaching the detector during each scan. This total ion scan provides universal detection for all analytes. As seen in Figure 12.51, we can achieve some degree of selectivity by monitoring only specific mass-to-charge ratios, a process called selective-ion monitoring).

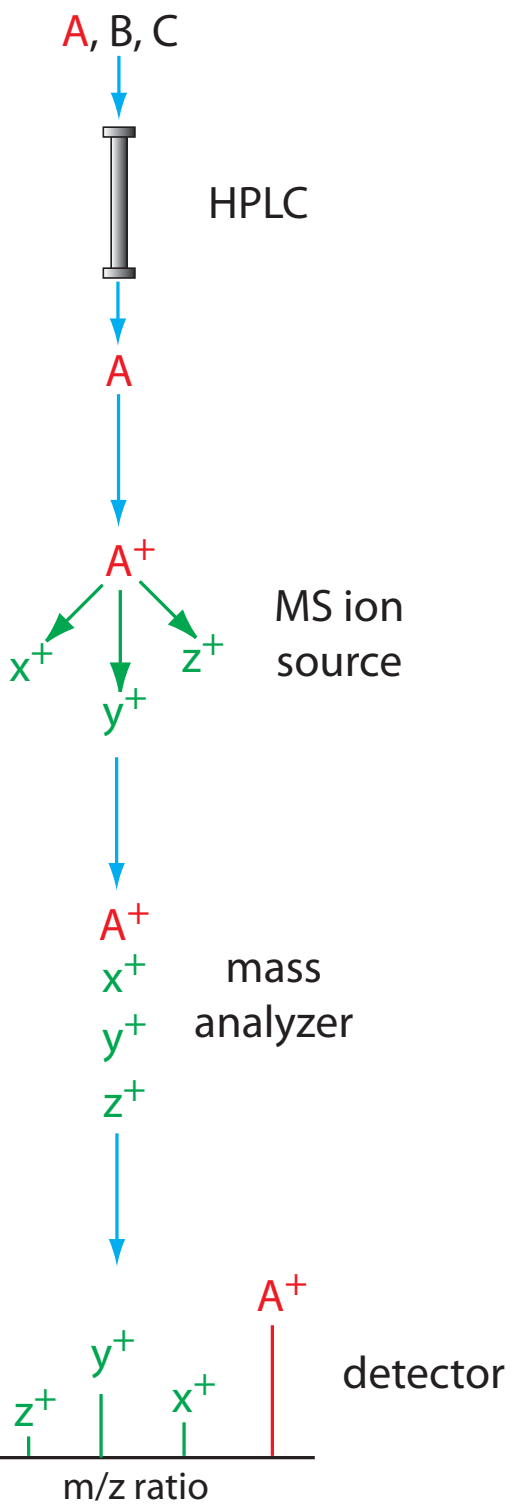


Figure 12.50 Block diagram of an HPLC–MS. A three component mixture enters the HPLC. When component A elutes from the column, it enters the MS ion source and ionizes to form the parent ion (A^+) and several fragment ions. The ions enter the mass analyzer, which separates them by their mass-to-charge ratio, providing the mass spectrum shown at the detector.

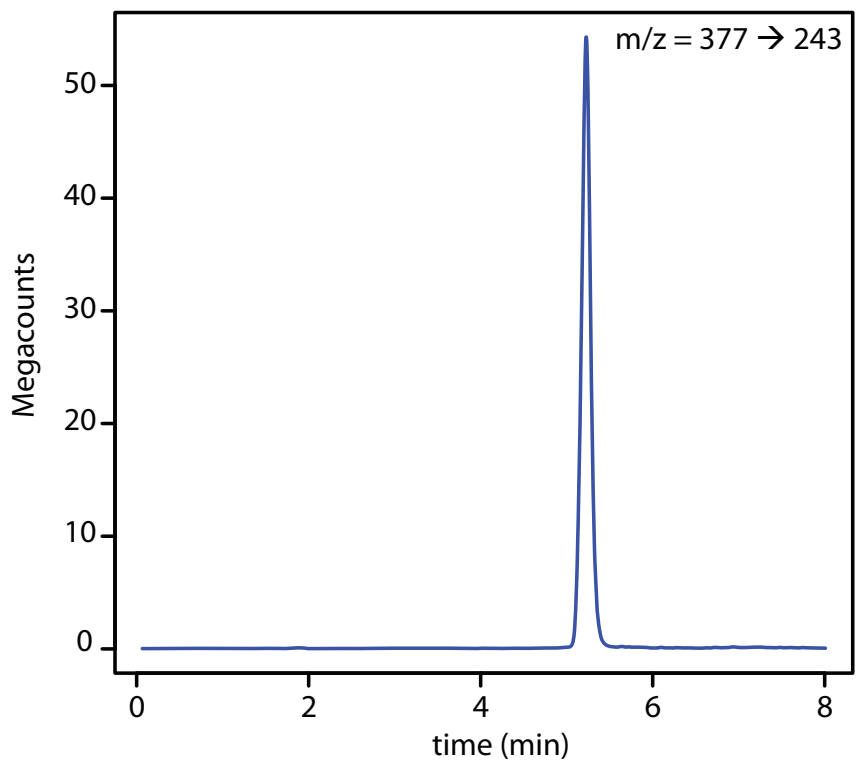


Figure 12.51 HPLC–MS/MS chromatogram for the determination of riboflavin in urine. An initial parent ion with an m/z ratio of 377 enters a second mass spectrometer where it undergoes additional ionization; the fragment ion with an m/z ratio of 243 provides the signal. The selectivity of this detector is evident when you compare this chromatogram to the one in Figure 12.48, which uses fluoresence deterction. Data provided by Jason Schultz, Jonna Berry, Kaelene Lundstrom, and Dwight Stoll, Department of Chemistry, Gustavus Adolphus College.

The advantages of using a mass spectrometer in HPLC are the same as for gas chromatography. Detection limits are very good, typically 100 pg–1 ng of injected analyte, with values as low as 1–10 pg for some samples. In addition, a mass spectrometer provides qualitative, structural information that can help in identifying the analytes. The interface between the HPLC and mass spectrometer is technically more difficult than that in a GC–MS because of the incompatibility of a liquid mobile phase with the mass spectrometer's high vacuum requirement.

12E.5 Quantitative Applications

High-performance liquid chromatography is routinely used for both qualitative and quantitative analyses of environmental, pharmaceutical, industrial, forensic, clinical, and consumer product samples.

Preparing Samples for Analysis

Samples in liquid form are injected into the HPLC after a suitable clean-up to remove any particulate materials, or after a suitable extraction to remove matrix interferents. In determining polyaromatic hydrocarbons (PAH) in wastewater, for example, an extraction with CH_2Cl_2 serves the dual purpose of concentrating the analytes and isolating them from matrix interferents. Solid samples are first dissolved in a suitable solvent, or the analytes of interest brought into solution by extraction. For example, an HPLC analysis for the active ingredients and degradation products in a pharmaceutical tablet often begins by extracting the powdered tablet with a portion of mobile phase. Gas samples are collected by bubbling them through a trap containing a suitable solvent. Organic isocyanates in industrial atmospheres are collected by bubbling the air through a solution of 1-(2-methoxyphenyl)piperazine in toluene. The reaction between the isocyanates and 1-(2-methoxyphenyl)piperazine both stabilizes them against degradation before the HPLC analysis and converts them to a chemical form that can be monitored by UV absorption.

Quantitative Calculations

An internal standard is necessary when using HPLC–MS because the interface between the HPLC and the mass spectrometer does not allow for a reproducible transfer of the column's eluent into the MS's ionization chamber.

A quantitative HPLC analysis is often easier than a quantitative GC analysis because a fixed volume sample loop provides a more precise and accurate injection. As a result, most quantitative HPLC methods do not need an internal standard and, instead, use external standards and a normal calibration curve.

Example 12.8

The concentration of polynuclear aromatic hydrocarbons (PAH) in soil are determined by first extracting the PAHs with methylene chloride. The extract is diluted, if necessary, and the PAHs separated by HPLC using a UV/Vis or fluorescence detector. Calibration is achieved using one or more

external standards. In a typical analysis a 2.013-g sample of dried soil is extracted with 20.00 mL of methylene chloride. After filtering to remove the soil, a 1.00-mL portion of the extract is removed and diluted to 10.00 mL with acetonitrile. Injecting 5 μL of the diluted extract into an HPLC gives a signal of 0.217 (arbitrary units) for the PAH fluoranthene. When 5 μL of a 20.0-ppm fluoranthene standard is analyzed using the same conditions, a signal of 0.258 is measured. Report the parts per million of fluoranthene in the soil.

SOLUTION

For a single-point external standard, the relationship between the signal, S, and the concentration, C, of fluoranthene is

$$S = kC$$

Substituting in values for the standard's signal and concentration gives the value of k as

$$k = \frac{S}{C} = \frac{0.258}{20.0 \text{ ppm}} = 0.0129 \text{ ppm}^{-1}$$

Using this value for k and the sample's HPLC signal gives a fluoranthene concentration of

$$C = \frac{S}{k} = \frac{0.217}{0.0129 \text{ ppm}^{-1}} = 16.8 \text{ ppm}$$

for the extracted and diluted soil sample. The concentration of fluoranthene in the soil is

$$\frac{16.8 \text{ g/mL} \times \dfrac{10.00 \text{ mL}}{1.00 \text{ mL}} \times 20.00 \text{ mL}}{2.013 \text{ g sample}} = 1670 \text{ ppm fluoranthene}$$

Practice Exercise 12.8

The concentration of caffeine in beverages can be determined by a reversed-phase HPLC separation using a mobile phase of 20% acetonitrile and 80% water and a nonpolar C_8 column. Results for a series of 10-μL injections of caffeine standards are in the following table.

[caffeine] (mg/L)	peak area (arb. units)
50.0	226 724
100.0	453 762
125.0	559 443
250.0	1 093 637

The data in this problem comes from Kusch, P.; Knupp, G. "Simultaneous Determination of Caffeine in Cola Drinks and Other Beverages by Reversed-Phase HPTLC and Reversed-Phase HPLC," *Chem. Educator*, **2003**, *8*, 201–205.

What is the concentration of caffeine in a sample if a 10-μL injection gives a peak area of 424 195?

Click here to review your answer to this exercise.

The best way to appreciate the theoretical and practical details discussed in this section is to carefully examine a typical analytical method. Although each method is unique, the following description of the determination of fluoxetine in serum provides an instructive example of a typical procedure. The description here is based on Smyth, W. F. *Analytical Chemistry of Complex Matricies*, Wiley Teubner: Chichester, England, 1996, pp. 187–189.

For a review of solid-phase extraction (SPE), see Section 7F.5. Table 7.8 describes the properties of several different types of SPE cartridges. Figure 7.22 shows a photo of SPE cartridges, and Figure 7.23 illustrates the steps in completing a solid-phase extraction.

Representative Method 12.2

Determination of Fluoxetine in Serum

Description of Method

Fluoxetine is another name for the antidepressant drug Prozac. The determination of fluoxetine in serum is an important part of monitoring its therapeutic use. The analysis is complicated by the complex matrix of serum samples. A solid-phase extraction followed by an HPLC analysis using a fluorescence detector provides the necessary selectivity and detection limits.

Procedure

Add a known amount of the antidepressant protriptyline, which serves as an internal standard, to each serum sample and to each external standard. To remove matrix interferents, pass a 0.5-mL aliquot of each serum sample or standard through a C18 solid-phase extraction cartridge. After washing the cartridge to remove the interferents, elute the remaining constituents, including the analyte and the internal standard, by washing the cartridge with 0.25 mL of a 25:75 v/v mixture of 0.1 M $HClO_4$ and acetonitrile. Inject a 20-μL aliquot onto a 15-cm × 4.6-mm column packed with a 5 μm C8-bonded stationary phase. The isocratic mobile phase is 37.5:62.5 v/v acetonitrile and water (containing 1.5 g of tetramethylammonium perchlorate and 0.1 mL of 70% v/v $HClO_4$). Monitor the chromatogram using a fluorescence detector set to an excitation wavelength of 235 nm and an emission wavelength of 310 nm.

Questions

1. The solid-phase extraction is important because it removes constitutions in the serum that might interfere with the analysis. What types of interferences are possible?

   Blood serum, which is a complex mixture of compounds, is approximately 92% water, 6–8% soluble proteins, and less than 1% each of various salts, lipids, and glucose. A direct injection of serum is not advisable for three reasons. First, any particulate materials in the serum will clog the column and restrict the flow of mobile phase. Second, some of the compounds in the serum may absorb too strongly to the stationary phase, degrading the column's performance. Finally, although an HPLC is capable of separating and analyzing complex mixtures, an analysis may still be difficult if the number of constituents exceeds the column's peak capacity.

2. One advantage of an HPLC analysis is that a loop injector often eliminates the need for an internal standard. Why is an internal standard used in this analysis? What assumption(s) must we make when using the internal standard?

An internal standard is necessary because of uncertainties introduced during the solid-phase extraction. For example, the volume of serum transferred to the solid-phase extraction cartridge, 0.5 mL, and the volume of solvent used to remove the analyte and internal standard, 0.25 mL, are very small. The precision and accuracy with which we can measure these volumes is not as good as when using larger volumes. In addition, the concentration of eluted analytes may vary from trial to trial due to variations in the amount of solution held up by the cartridge. Using an internal standard compensates for these variation. To be useful we must assume that the analyte and the internal standard are completely retained during the initial loading, that they are not lost when the cartridge is washed, and that they are completely extracted during the final elution.

If we extract all of the analyte into a volume of 0.24 mL instead of 025 mL, the concentration of the analyte increases by slightly more than 4%.

3. Why does the procedure monitor fluorescence instead of monitoring UV absorption?

   Fluorescence is a more selective technique for detecting analytes. Many other commonly prescribed antidepressants (and their metabolites) elute with retention times similar to that of fluoxetine. These compounds, however, either do not fluoresce or are only weakly fluorescent.

4. If the peaks for fluoxetine and protriptyline are insufficiently resolved, how might you alter the mobile phase to improve their separation?

   Decreasing the amount of acetonitrile and increasing the amount of water in the mobile will increase retention times, providing a better resolution.

12E.6 Evaluation

With a few exceptions, the scale of operation, accuracy, precision, sensitivity, selectivity, analysis time, and cost for an HPLC method are similar to GC methods. Injection volumes for an HPLC method are usually larger than for a GC method because HPLC columns have a greater capacity. Because it uses a loop injection, the precision of an HPLC method is often better. HPLC is not limited to volatile analytes, which means that we can analyze a broader range of compounds. Capillary GC columns, on the other hand, have more theoretical plates, and can separate more complex mixtures.

See Section 12D.8 for an evaluation of GC methods.

12F Other Forms of Liquid Chromatography

At the beginning of Section 12E, we noted that there are several different types of solute/stationary phase interactions in liquid chromatography. In Section 12E we limited our discussion to liquid–liquid chromatography. In this section we turn our attention to liquid chromatography techniques in

which partitioning occurs by liquid–solid adsorption, ion-exchange, and size exclusion.

12F.1 Liquid-Solid Adsorption Chromatography

In LIQUID–SOLID ADSORPTION CHROMATOGRAPHY (LSC) the column packing also serves as the stationary phase. In Tswett's original work the stationary phase was finely divided $CaCO_3$, but modern columns employ porous 3–10 μm particles of silica or alumina. Because the stationary phase is polar, the mobile phase is usually a nonpolar or moderately polar solvent. Typical mobile phases include hexane, isooctane, and methylene chloride. The usual order of elution—from shorter to longer retention times—is

$$\text{olefins} < \text{aromatic hydrocarbons} < \text{ethers} < \text{esters, aldehydes, ketones} < \text{alcohols, amines} < \text{amide} < \text{carboxylic acids}$$

Nonpolar stationary phases, such as charcoal-based absorbents, also may be used. For most samples, liquid–solid chromatography does not offer any special advantages over liquid–liquid chromatography. One exception is the analysis of isomers, where LLC excels.

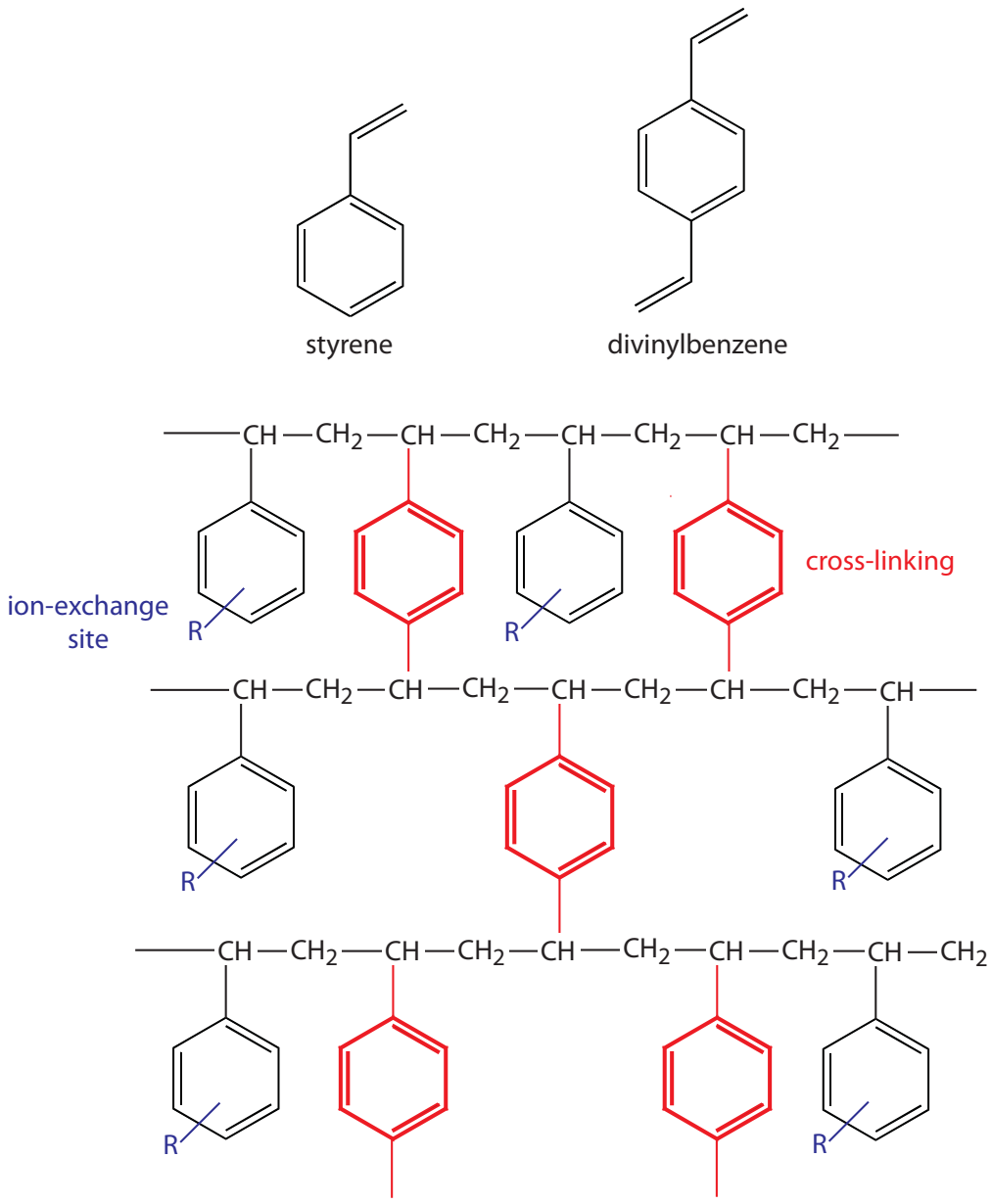
12F.2 Ion-Exchange Chromatography

In ION-EXCHANGE CHROMATOGRAPHY (IEC) the stationary phase is a cross-linked polymer resin, usually divinylbenzene cross-linked polystyrene, with covalently attached ionic functional groups (see Figure 12.52 and Table 12.7). The counterions to these fixed charges are mobile and can be displaced by ions that compete more favorably for the exchange sites. Ion-exchange resins are divided into four categories: strong acid cation exchangers; weak acid cation exchangers; strong base anion exchangers; and weak base anion exchangers.

Strong acid cation exchangers include a sulfonic acid functional group that retains it anionic form—and thus its capacity for ion-exchange—in strongly acidic solutions. The functional groups for a weak acid cation exchanger, however, become fully protonated at pH levels less then 4 and lose their exchange capacity. The strong base anion exchangers include a

Table 12.7 Examples of Common Ion-Exchange Resins

type	functional group (R)	examples
strong acid cation exchanger	sulfonic acid	$-SO_3^-$ $-CH_2CH_2SO_3^-$
weak acid cation exchanger	carboxylic acid	$-COO^-$ $-CH_2COO^-$
strong base anion exchanger	quaternary amine	$-CH_2N(CH_3)_3^+$ $-CH_2CH_2N(CH_2CH_3)_3^+$
weak base anion exchanger	amine	$-NH_3^+$ $-CH_2CH_2NH(CH_2CH_3)_2^+$



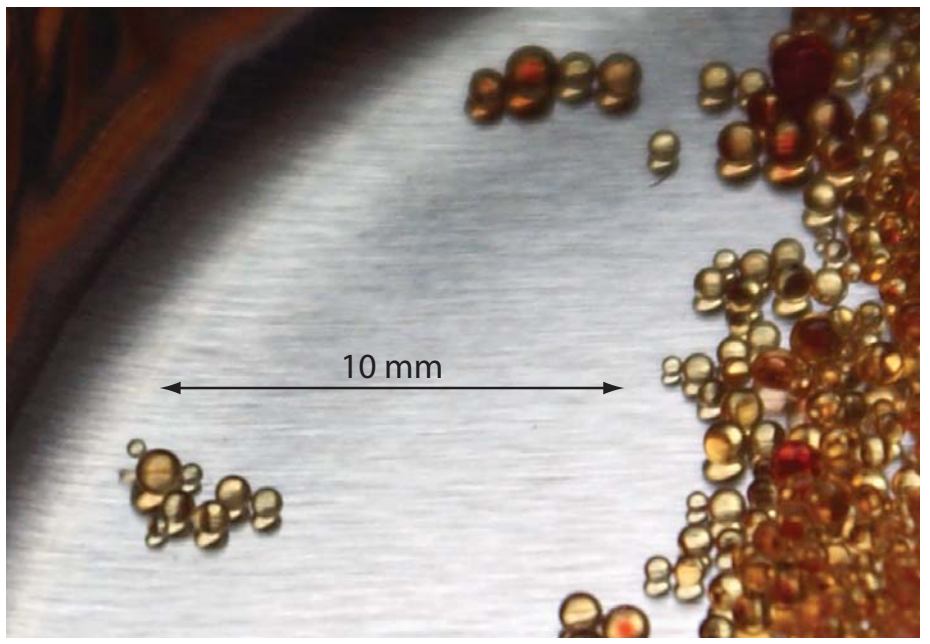


Figure 12.52 Structures of styrene, divinylbenzene, and a styrene–divinylbenzene co-polymer modified for use as an ion-exchange resin. The ion-exchange sites, indicated by R and shown in blue, are mostly in the *para* position and are not necessarily bound to all styrene units. The cross-linking is shown in red. The photo shows an example of the polymer beads. These beads are approximately 0.30–0.85 mm in diameter. Resins for use in ion-exchange chromatography are typically 5–11 μm in diameter.

quaternary amine, which retains a positive charge even in strongly basic solutions. Weak base anion exchangers remain protonated only at pH levels that are moderately basic. Under more basic conditions a weak base anion exchanger loses a proton and its exchange capacity.

The ion-exchange reaction of a monovalent cation, M^+, at a strong acid exchange site is

$$-SO_3^-H^+(s) + M^+(aq) \rightleftharpoons -SO_3^-M^+(s) + H^+(aq)$$

The equilibrium constant for this ion-exchange reaction, which we call the selectivity coefficient, K, is

$$K = \frac{\{-SO_3^-M^+\}[H^+]}{\{-SO_3^-H^+\}[M^+]} \qquad 12.29$$

where we use brackets { } to indicate a surface concentration instead of a solution concentration. Rearranging equation 12.29 shows us that the distribution ratio, D, for the exchange reaction

$$D = \frac{\text{amount of } M^+ \text{ in the stationary phase}}{\text{amount of } M^+ \text{ in the mobile phase}}$$

We don't usually think about a solid's concentration. There is a good reason for this. In most cases, a solid's concentration is a constant. If you break a piece of chalk into two parts, for example, the mass and the volume retain the same proportion. When we consider an ion binding to a reactive site on the solid's surface, however, the fraction of sites that are bound, and thus the concentration of bound sites, can take on any value between 0 and some maximum value that is proportional to the density of reactive sites.

$$D = \frac{\{-SO_3^-M^+\}}{[M^+]} = K \times \frac{\{-SO_3^-H^+\}}{[H^+]} \qquad 12.30$$

is a function of the concentration of H^+ and, therefore, the pH of the mobile phase.

An ion-exchange resin's selectivity is somewhat dependent on whether it includes strong or weak exchange sites and on the extent of cross-linking. The latter is particularly important as it controls the resin's permeability, and, therefore, the accessibility of exchange sites. An approximate order of selectivity for a typical strong acid cation exchange resin, in order of decreasing D, is

$$Al^{3+} > Ba^{2+} > Pb^{2+} > Ca^{2+} > Ni^{2+} > Cd^{2+} > Cu^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} > Ag^+ > K^+ > NH_4^+ > Na^+ > H^+ > Li^+$$

Note that highly charged ions bind more strongly than ions of lower charge, and that for ions of similar charge, those with a smaller hydrated radius (see Table 6.2 in Chapter 6), or that are more polarizable, bind more strongly. For a strong base anion exchanger the general elution order is

$$SO_4^{2-} > I^- > HSO_4^- > NO_3^- > > Br^- > NO_2^- > Cl^- > HCO_3^- > CH_3COO^- > OH^- > F^-$$

Again, ions of higher charge and of smaller hydrated radius bind more strongly than ions with a lower charge and a larger hydrated radius.

From equation 12.30, a cation's distribution ratio, D, becomes smaller when the concentration of H^+ in the mobile phase increases.

The mobile phase in IEC is usually an aqueous buffer, the pH and ionic composition of which determines a solute's retention time. Gradient elutions are possible in which the mobile phase's ionic strength or pH is changed with time. For example, an IEC separation of cations might use a dilute solution of HCl as the mobile phase. Increasing the concentration of HCl speeds the elution rate for more strongly retained cations because the higher concentration of H^+ allows it to compete more successfully for the ion-exchange sites.

An ion-exchange resin is incorporated into an HPLC column either as 5–11 μm porous polymer beads or by coating the resin on porous silica particles. Columns typically are 250 mm in length with internal diameters ranging from 2–5 mm.

Measuring the conductivity of the mobile phase as it elutes from the column is a universal detector for cationic and anionic analytes. Because the mobile phase contains a high concentration of ions—a mobile phase of dilute HCl, for example, contains significant concentrations of H^+ and Cl^-—we need a method for detecting the analytes in the presence of a significant background conductivity.

To minimize the mobile phase's contribution to conductivity an ION-SUPPRESSOR COLUMN is placed between the analytical column and the detector. This column selectively removes mobile phase ions without removing solute ions. For example, in cation-exchange chromatography using a

dilute solution of HCl as the mobile phase, the suppressor column contains a strong base anion-exchange resin. The exchange reaction

$$H^+(aq) + Cl^-(aq) + \text{Resin}^+OH^-(s) \rightleftharpoons \text{Resin}^+Cl^-(s) + H_2O(l)$$

replaces the mobile phase ions H^+ and Cl^- with H_2O. A similar process is used in anion-exchange chromatography where the suppressor column contains a cation-exchange resin. If the mobile phase is a solution of Na_2CO_3, the exchange reaction

$$2Na^+(aq) + CO_3^{2-}(aq) + 2\text{Resin}^-H^+(s) \rightleftharpoons 2\text{Resin}^-Na^+(s) + H_2CO_3(aq)$$

replaces a strong electrolyte, Na_2CO_3, with a weak electrolyte, H_2CO_3.

Ion-suppression is necessary when the mobile phase contains a high concentration of ions. SINGLE-COLUMN ION CHROMATOGRAPHY, in which an ion-suppressor column is not needed, is possible if the concentration of ions in the mobile phase is small. Typically the stationary phase is a resin with a low capacity for ion-exchange and the mobile phase is a very dilute solution of methane sulfonic acid for cationic analytes, or potassium benzoate or potassium hydrogen phthalate for anionic analytes. Because the background conductivity is sufficiently small, it is possible to monitor a change in conductivity as the analytes elute from the column.

A UV/Vis absorbance detector can also be used if the analytes absorb ultraviolet or visible radiation. Alternatively, we can detect indirectly analytes that do not absorb in the UV/Vis if the mobile phase contains a UV/Vis absorbing species. In this case, when a solute band passes through the detector, a decrease in absorbance is measured at the detector.

Ion-exchange chromatography is an important technique for the analysis of anions and cations in water. For example, an ion-exchange chromatographic analysis for the anions F^-, Cl^-, Br^-, NO_2^-, NO_3^-, PO_4^{3-}, and SO_4^{2-} takes approximately 15 minutes (Figure 12.53). A complete analysis of the same set of anions by a combination of potentiometry and spectrophotometry requires 1–2 days. Ion-exchange chromatography also

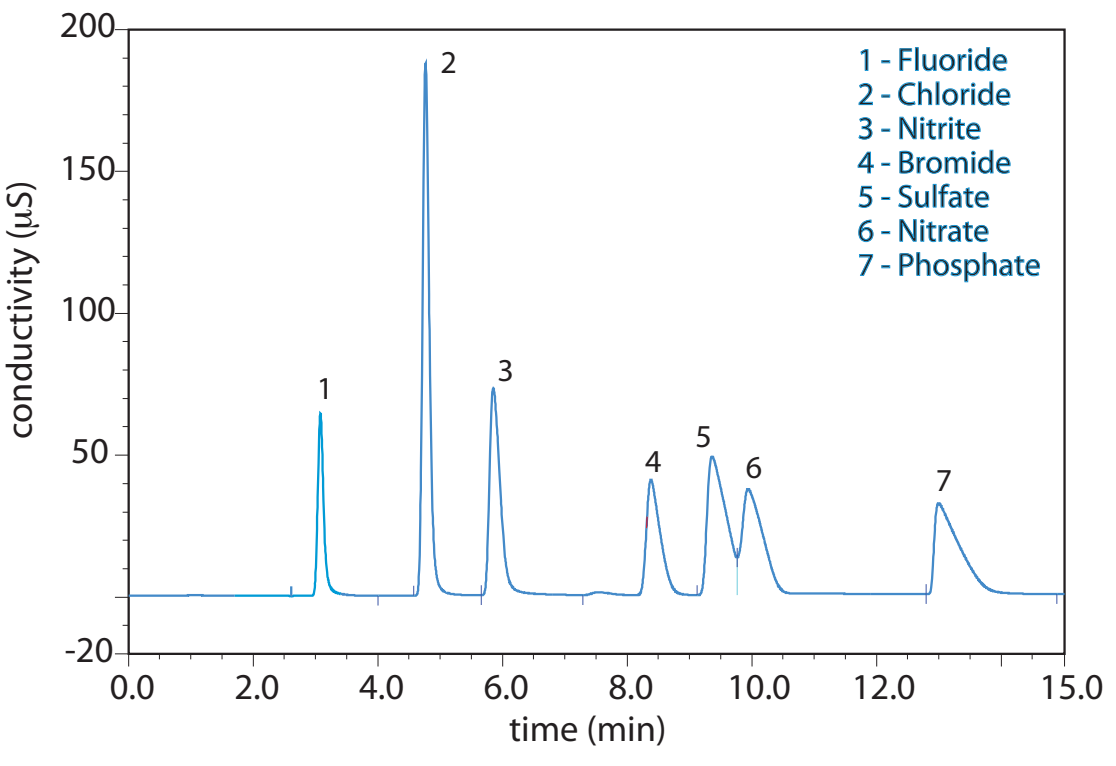


Figure 12.53 Ion-exchange chromatographic analysis of water from Big Walnut Creek in Putnam County, Indiana. Data provided by Jeanette Pope, Department of Geosciences, DePauw University.

has been used for the analysis of proteins, amino acids, sugars, nucleotides, pharmaceuticals, consumer products, and clinical samples.

12F.3 Size-Exclusion Chromatography

We have considered two classes of micron-sized stationary phases in this section: silica particles and cross-linked polymer resin beads. Both materials are porous, with pore sizes ranging from approximately 5–400 nm for silica particles, and from 5 nm to 100 μm for divinylbenzene cross-linked polystyrene resins. In SIZE-EXCLUSION CHROMATOGRAPHY—which also is known by the terms molecular-exclusion or gel permeation chromatography—the separation of solutes depends upon their ability to enter into the pores of the stationary phase. Smaller solutes spend proportionally more time within the pores and take longer to elute from the column.

A stationary phase's size selectivity extends over a finite range. All solutes significantly smaller than the pores move through the column's entire volume and elute simultaneously, with a retention volume, V_r, of

$$V_r = V_i + V_o \qquad 12.31$$

where V_i is the volume of mobile phase occupying the stationary phase's pore space, and V_o is volume of mobile phase in the remainder of the column. The largest solute for which equation 12.31 holds is the column's INCLUSION LIMIT, or permeation limit. Those solutes that are too large to enter the pores elute simultaneously with an retention volume of

$$V_r = V_o \qquad 12.32$$

Equation 12.32 defines the column's EXCLUSION LIMIT.

For a solute whose size is between the inclusion limit and the exclusion limit, the amount of time it spends in the stationary phase's pore space is proportional to its size. The retention volume for these solutes is

$$V_r = V_o + DV_i \qquad 12.33$$

where D is the solute's distribution ratio, which ranges from 0 at the exclusion limit, to 1 at the inclusion limit. Equation 12.33 assumes that size-exclusion is the only interaction between the solute and the stationary phase that affects the separation. For this reason, stationary phases using silica particles are deactivated as described earlier, and polymer resins are synthesized without exchange sites.

Size-exclusion chromatography provides a rapid means for separating larger molecules, including polymers and biomolecules. A stationary phase for proteins consisting of particles with 30 nm pores has an inclusion limit of 7500 g/mol and an exclusion limit of 1.2×10^6 g/mol. Mixtures containing proteins spanning a wider range of molecular weights can be separated by joining together in series several columns with different inclusion and exclusion limits.

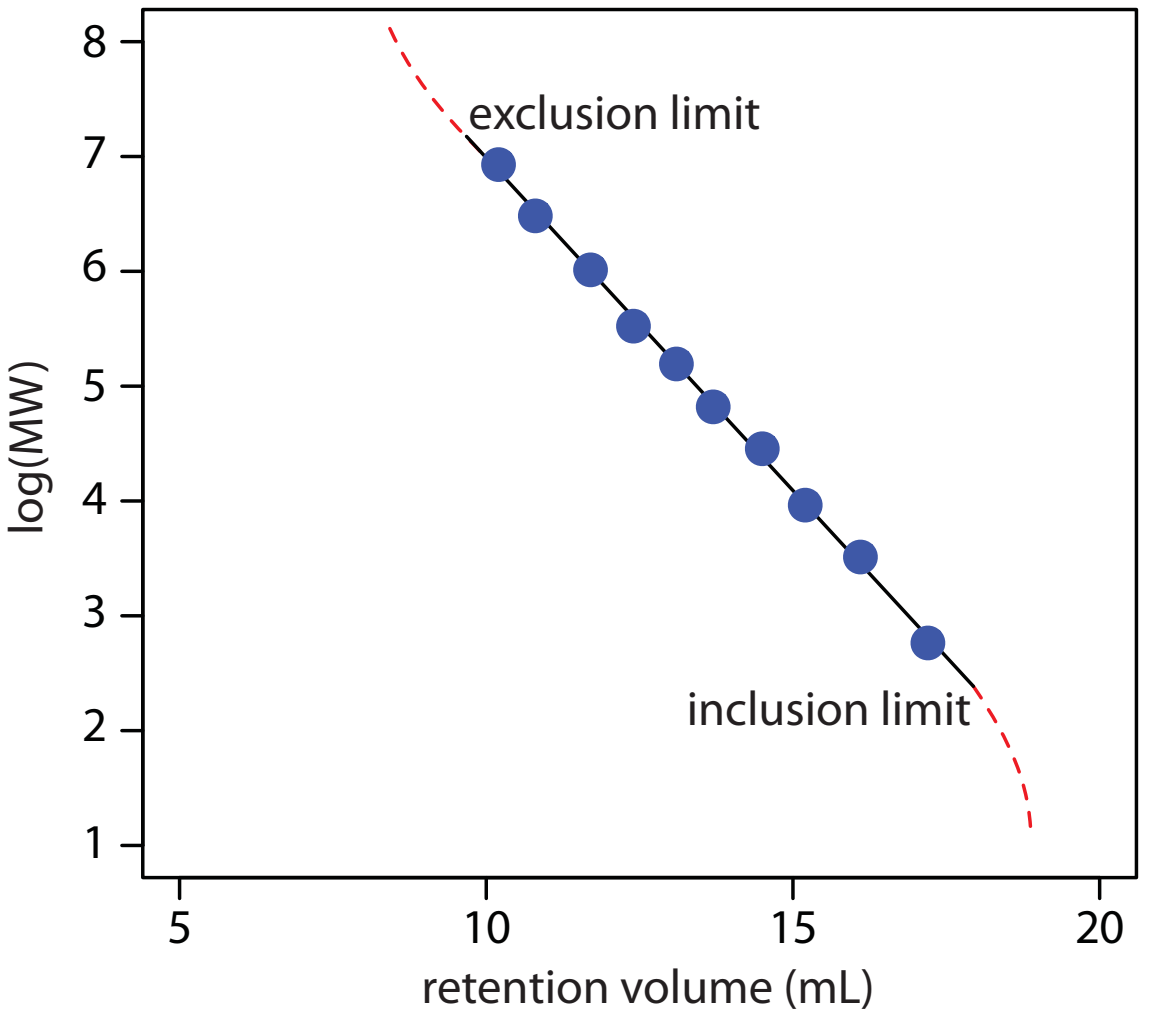


Figure 12.54 Calibration curve for the determination of molecular weight by size-exclusion chromatography. The data shown here are adapted from Rouessac, F.; Rouessac, A. *Chemical Analysis: Modern Instrumentation Methods and Techniques*, Wiley: Chichester, England, 2004, p 141.

Another important application of size-exclusion chromatography is the estimation of a solute's molecular weight (MW). Calibration curves are prepared using a series of standards of known molecular weight and measuring each standard's retention volume. As shown in Figure 12.54, a plot of log(MW) versus V_r is roughly linear between the exclusion limit and the inclusion limit. Because a solute's retention volume is influenced by both its size and its shape, a reasonably accurate estimation of molecular weight is possible only if the standards are carefully chosen to minimize the effect of shape.

Size-exclusion chromatography can be carried out using conventional HPLC instrumentation, replacing the HPLC column with an appropriate size-exclusion column. A UV/Vis detector is the most common means for obtaining the chromatogram.

12F.4 Supercritical Fluid Chromatography

Although there are many analytical applications of gas chromatography and liquid chromatography, they can not separate and analyze all types of samples. Capillary column GC can separate complex mixtures with excellent resolution and short analysis times. Its application is limited, however, to volatile analytes or to analytes made volatile by a suitable derivatization reaction. Liquid chromatography can separate a wider range of solutes than GC, but the most commonly used detectors—UV, fluorescence, and electrochemical—have poorer detection limits and smaller linear ranges than GC detectors, and are not as nearly universal in their selectivity.

For some samples, SUPERCRITICAL FLUID CHROMATOGRAPHY (SFC) provides a useful alternative to gas chromatography and liquid chromatogra-

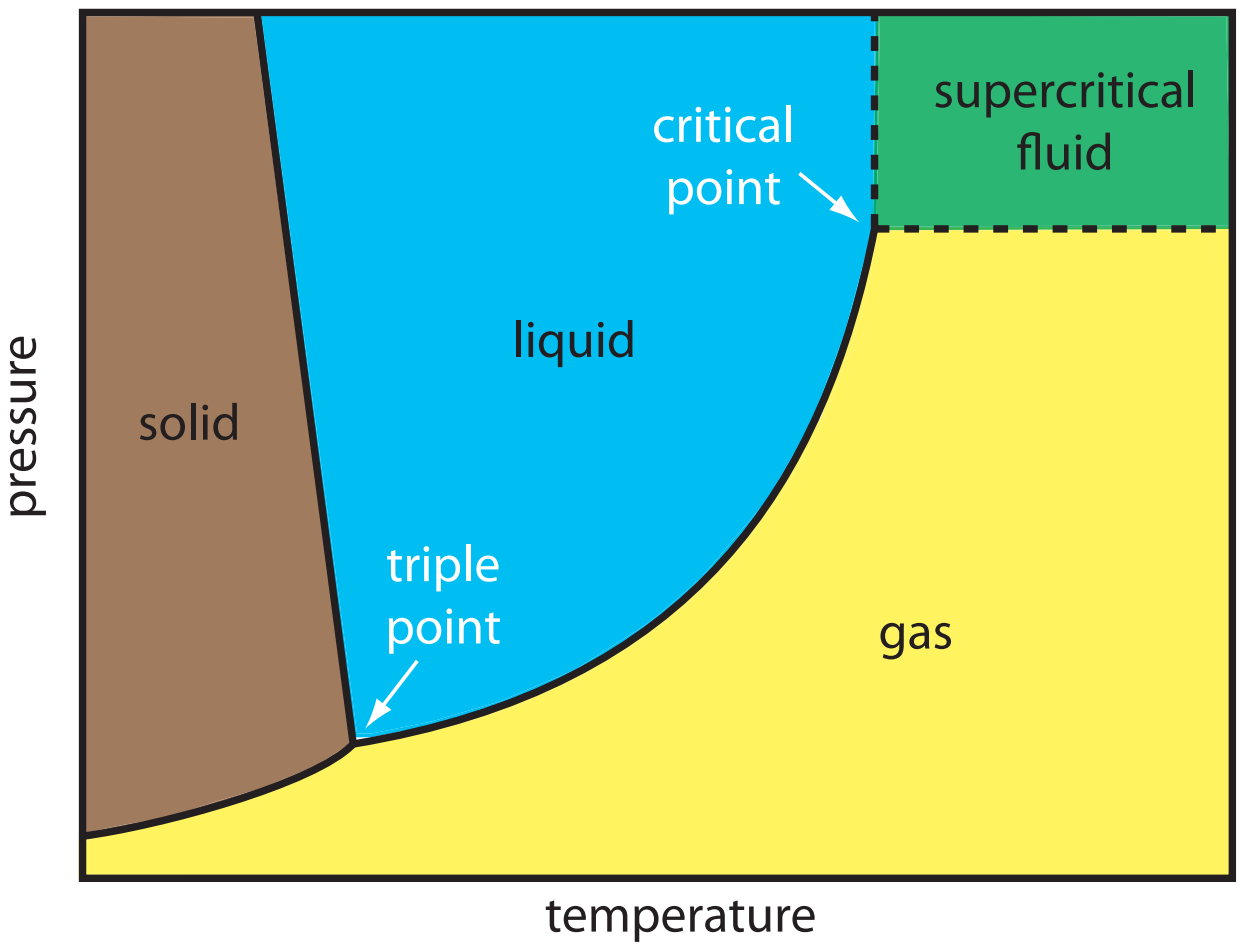


Figure 12.55 Phase diagram showing the combinations of temperature and pressure for which a compound is in its solid state, its liquid state, and its gas state. For pressures and temperatures above the compound's critical point, the compound is a supercritical fluid with properties intermediate between a gas and a liquid.

Click here to see an interesting video demonstration of supercritical fluids from Nottingham Science City at the University of Nottingham.

phy. The mobile phase in supercritical fluid chromatography is a gas held at a temperature and pressure that exceeds its critical point (Figure 12.55). Under these conditions the mobile phase is neither a gas nor a liquid. Instead, the mobile phase is a supercritical fluid.

The properties of a supercritical fluid, as shown in Table 12.8, are intermediate between those of a gas and a liquid. Specifically, the viscosity of a supercritical fluid is similar to that for a gas, which means we can move a supercritical fluid can move through a capillary or a packed column without the high pressures needed in HPLC. Analysis time and resolution, although not as good as in GC, are usually better than in conventional HPLC. The density of a supercritical fluid is much closer to that of a liquid, which explains why supercritical fluids are good solvents. The mobile phase in SFC, therefore, behaves more like the liquid mobile phase in HPLC than the gaseous mobile phase in GC.

The most common mobile phase for supercritical fluid chromatography is CO_2. Its low critical temperature, 3 °C, and critical pressure, 72.9 atm,

Table 12.8 Typical Properties of Gases, Liquids, and Supercritical Fluids

phase	density (g cm^{-3})	viscosity (g cm^{-1} s^{-1})	diffusion coefficient (cm^2 s^{-1})
gas	$\approx 10^{-3}$	$\approx 10^{-4}$	≈ 0.1
supercritical fluid	≈ 0.1–1	$\approx 10^{-4}$–10^{-3}	$\approx 10^{-4}$–10^{-3}
liquid	≈ 1	$\approx 10^{-2}$	$< 10^{-5}$

Table 12.9 Critical Points for Selected Supercritical Fluids

compound	critical temperature (°C)	critical pressure (atm)
carbon dioxide	31.3	72.9
ethane	32.4	48.3
nitrous oxide	36.5	71.4
ammonia	132.3	111.3
diethyl ether	193.6	36.3
isopropanol	235.3	47.0
methanol	240.5	78.9
ethanol	243.4	63.0
water	374.4	226.8

are relatively easy to achieve and maintain. Although supercritical CO_2 is a good solvent for nonpolar organics, it is less useful for polar solutes. The addition of an organic modifier, such as methanol, improves the mobile phase's elution strength. Other common mobile phases and their critical temperatures and pressures are listed in Table 12.9.

The instrumentation for supercritical fluid chromatography is essentially the same as that for a standard HPLC. The only important additions are a heated oven for the column and a pressure restrictor downstream from the column to maintain the critical pressure. Gradient elutions are accomplished by changing the applied pressure over time. The resulting change in the mobile phase's density affects its solvent strength. Detection can be accomplished using standard GC detectors or HPLC detectors. Supercritical fluid chromatography has found many applications in the analysis of polymers, fossil fuels, waxes, drugs, and food products.

12G Electrophoresis

Electrophoresis is a class of separation techniques in which we separate analytes by their ability to move through a conductive medium—usually an aqueous buffer—in response to an applied electric field. In the absence of other effects, cations migrate toward the electric field's negatively charged cathode. Cations with larger charge-to-size ratios—which favors ions of larger charge and of smaller size—migrate at a faster rate than larger cations with smaller charges. Anions migrate toward the positively charged anode and neutral species do not experience the electrical field and remain stationary.

As we will see shortly, under normal conditions even neutral species and anions migrate toward the cathode.

There are several forms of electrophoresis. In slab gel electrophoresis the conducting buffer is retained within a porous gel of agarose or polyacrylamide. Slabs are formed by pouring the gel between two glass plates separated by spacers. Typical thicknesses are 0.25–1 mm. Gel electrophoresis is an important technique in biochemistry where it is frequently used for separating DNA fragments and proteins. Although it is a powerful tool for the

qualitative analysis of complex mixtures, it is less useful for quantitative work.

In CAPILLARY ELECTROPHORESIS the conducting buffer is retained within a capillary tube whose inner diameter is typically 25–75 μm. Samples are injected into one end of the capillary tube. As the sample migrates through the capillary its components separate and elute from the column at different times. The resulting ELECTROPHEROGRAM looks similar to a GC or an HPLC chromatogram, providing both qualitative and quantitative information. Only capillary electrophoretic methods receive further consideration in this section.

12G.1 Theory of Capillary Electrophoresis

In capillary electrophoresis we inject the sample into a buffered solution retained within a capillary tube. When an electric field is applied across the capillary tube, the sample's components migrate as the result of two types of action: electrophoretic mobility and electroosmotic mobility. ELECTROPHORETIC MOBILITY is the solute's response to the applied electrical field. As described earlier, cations move toward the negatively charged cathode, anions move toward the positively charged anode, and neutral species remain stationary. The other contribution to a solute's migration is ELECTROOSMOTIC FLOW, which occurs when the buffer moves through the capillary in response to the applied electrical field. Under normal conditions the buffer moves toward the cathode, sweeping most solutes, including the anions and neutral species, toward the negatively charged cathode.

Electrophoretic Mobility

The velocity with which a solute moves in response to the applied electric field is called its ELECTROPHORETIC VELOCITY, ν_{ep}; it is defined as

$$\nu_{ep} = \mu_{ep} E \qquad 12.34$$

where μ_{ep} is the solute's electrophoretic mobility, and E is the magnitude of the applied electrical field. A solute's electrophoretic mobility is defined as

$$\mu_{ep} = \frac{q}{6\pi\eta r} \qquad 12.35$$

where q is the solute's charge, η is the buffer viscosity, and r is the solute's radius. Using equation 12.34 and equation 12.35 we can make several important conclusions about a solute's electrophoretic velocity. Electrophoretic mobility and, therefore, electrophoretic velocity, increases for more highly charged solutes and for solutes of smaller size. Because q is positive for a cation and negative for an anion, these species migrate in opposite directions. Neutral species, for which q is zero, have an electrophoretic velocity of zero.

Electroosmotic Mobility

When an electrical field is applied to a capillary filled with an aqueous buffer we expect the buffer's ions to migrate in response to their electrophoretic mobility. Because the solvent, H_2O, is neutral we might reasonably expect it to remain stationary. What we observe under normal conditions, however, is that the buffer solution moves towards the cathode. This phenomenon is called the electroosmotic flow.

Electroosmotic flow occurs because the walls of the capillary tubing are electrically charged. The surface of a silica capillary contains large numbers of silanol groups (–SiOH). At pH levels greater than approximately 2 or 3, the silanol groups ionize to form negatively charged silanate ions ($–SiO^-$). Cations from the buffer are attracted to the silanate ions. As shown in Figure 12.56, some of these cations bind tightly to the silanate ions, forming a fixed layer. Because the cations in the fixed layer only partially neutralize the negative charge on the capillary walls, the solution adjacent to the fixed layer—what we call the diffuse layer—contains more cations than anions. Together these two layers are known as the double layer. Cations in the diffuse layer migrate toward the cathode. Because these cations are solvated, the solution is also pulled along, producing the electroosmotic flow.

The anions in the diffuse layer, which also are solvated, try to move toward the anode. Because there are more cations than anions, however, the cations win out and the electroosmotic flow moves in the direction of the cathode.

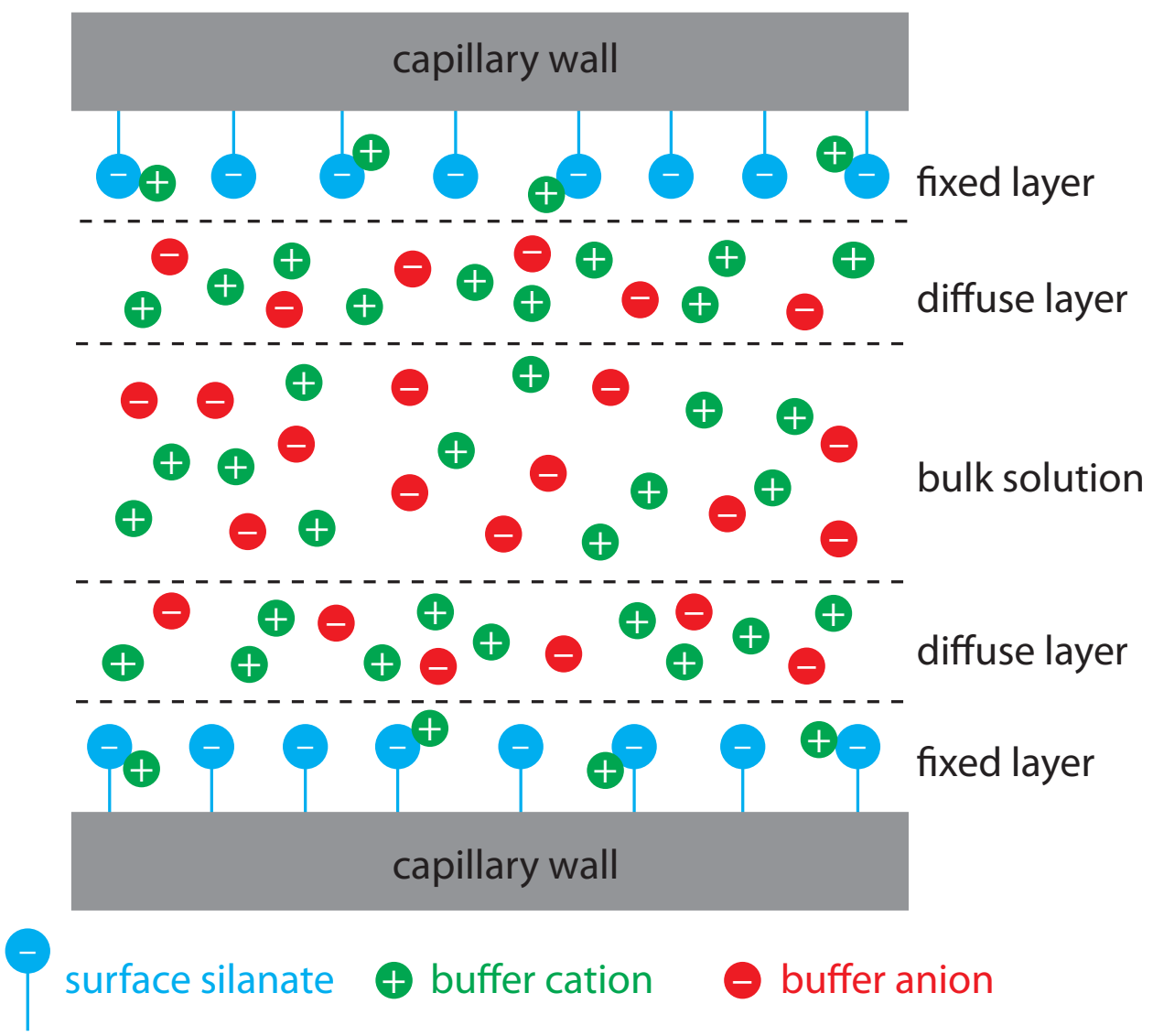


Figure 12.56 Schematic diagram showing the origin of the double layer within a capillary tube. Although the net charge within the capillary is zero, the distribution of charge is not. The walls of the capillary have an excess of negative charge, which decreases across the fixed layer and the diffuse layer, reaching a value of zero in bulk solution.

The rate at which the buffer moves through the capillary, what we call its ELECTROOSMOTIC FLOW VELOCITY, ν_{eof}, is a function of the applied electric field, E, and the buffer's electroosmotic mobility, μ_{eof}.

$$\nu_{eof} = \mu_{eof} E \qquad 12.36$$

Electroosmotic mobility is defined as

$$\mu_{eof} = \frac{\varepsilon\zeta}{4\pi\eta} \qquad 12.37$$

where ε is the buffer dielectric constant, ζ is the zeta potential, and η is the buffer viscosity.

The ZETA POTENTIAL—the potential of the diffuse layer at a finite distance from the capillary wall—plays an important role in determining the electroosmotic flow velocity. Two factors determine the zeta potential's value. First, the zeta potential is directly proportional to the charge on the capillary walls, with a greater density of silanate ions corresponding to a larger zeta potential. Below a pH of 2 there are few silanate ions, and the zeta potential and electroosmotic flow velocity are zero. As the pH increases, both the zeta potential and the electroosmotic flow velocity increase. Second, the zeta potential is directly proportional to the thickness of the double layer. Increasing the buffer's ionic strength provides a higher concentration of cations, decreasing the thickness of the double layer and decreasing the electroosmotic flow.

The definition of zeta potential given here is admittedly a bit fuzzy. For a much more technical explanation see Delgado, A. V.; González-Caballero, F.; Hunter, R. J.; Koopal, L. K.; Lyklema, J. "Measurement and Interpretation of Electrokinetic Phenomena," *Pure. Appl. Chem.* **2005**, *77*, 1753–1805. Although this a very technical report, Sections 1.3–1.5 provide a good introduction to the difficulty of defining the zeta potential and measuring its value.

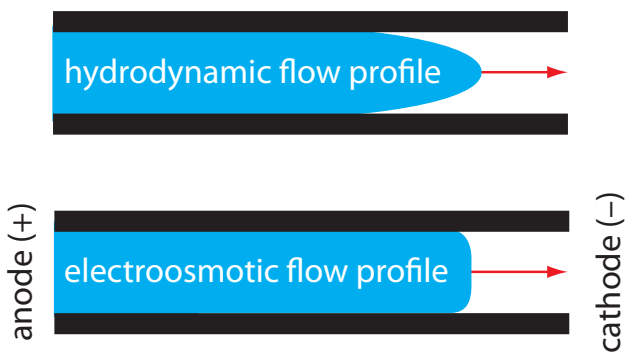


Figure 12.57 Comparison of hydrodynamic flow and electroosmotic flow. The nearly uniform electroosmotic flow profile means that the electroosmotic flow velocity is nearly constant across the capillary.

The electroosmotic flow profile is very different from that of a fluid moving under forced pressure. Figure 12.57 compares the electroosmotic flow profile with that the hydrodynamic flow profile in gas chromatography and liquid chromatography. The uniform, flat profile for electroosmosis helps minimize band broadening in capillary electrophoresis, improving separation efficiency.

Total Mobility

A solute's total velocity, ν_{tot}, as it moves through the capillary is the sum of its electrophoretic velocity and the electroosmotic flow velocity.

$$\nu_{tot} = \nu_{ep} + \nu_{eof}$$

As shown in Figure 12.58, under normal conditions the following general relationships hold true.

$$(\nu_{tot})_{cations} > \nu_{eof}$$

$$(\nu_{tot})_{neutrals} = \nu_{eof}$$

$$(\nu_{tot})_{anions} < \nu_{eof}$$

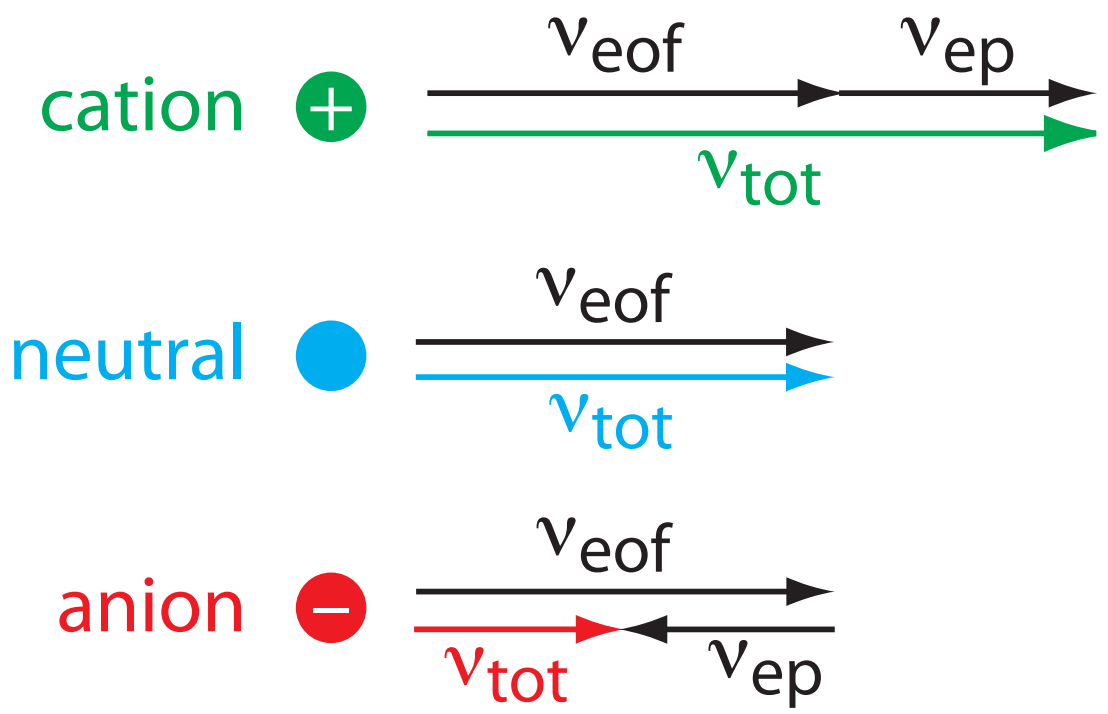


Figure 12.58 Visual explanation for the general elution order in capillary electrophoresis. Each species has the same electroosmotic flow, ν_{eof}. Cations elute first because they have a positive electrophoretic velocity, ν_{ep}. Anions elute last because their negative electrophoretic velocity partially offsets the electroosmotic flow velocity. Neutrals elute with a velocity equal to the electroosmotic flow.

Cations elute first in an order corresponding to their electrophoretic mobilities, with small, highly charged cations eluting before larger cations of lower charge. Neutral species elute as a single band with an elution rate equal to the electroosmotic flow velocity. Finally, anions are the last components to elute, with smaller, highly charged anions having the longest elution time.

Migration Time

Another way to express a solute's velocity is to divide the distance it travels by the elapsed time

$$\nu_{tot} = \frac{l}{t_m} \qquad 12.38$$

where l is the distance between the point of injection and the detector, and t_m is the solute's migration time. To understand the experimental variables affecting migration time, we begin by noting that

$$\nu_{tot} = \mu_{tot} E = (\mu_{ep} + \mu_{eof}) E \qquad 12.39$$

Combining equation 12.38 and equation 12.39 and solving for t_m leaves us with

$$t_m = \frac{l}{(\mu_{ep} + \mu_{eof}) E} \qquad 12.40$$

Finally, the magnitude of the electrical field is

$$E = \frac{V}{L} \qquad 12.41$$

where V is the applied potential and L is the length of the capillary tube. Finally, substituting equation 12.41 into equation 12.40 leaves us with the following equation for a solute's migration time.

$$t_m = \frac{lL}{(\mu_{ep} + \mu_{eof})V} \qquad 12.42$$

To decrease a solute's migration time—and shorten the analysis time—we can apply a higher voltage or use a shorter capillary tube. We can also shorten the migration time by increasing the electroosmotic flow, although this decreases resolution.

From equation 12.10 and equation 12.11, we know that the number of theoretical plates for a solute is

$$N = \frac{l^2}{\sigma^2}$$

where l is the distance the solute travels and σ is the standard deviation for the solute's band broadening. For capillary electrophoresis band broadening is due to longitudinal diffusion and is equivalent to $2Dt_m$, where t_m is the migration time.

It is possible to design an electrophoretic experiment so that anions elute before cations—more about this later—in which smaller, more highly charged anions elute with greater efficiencies.

Efficiency

As we learned in Section 12B.4, the efficiency of a separation is given by the number of theoretical plates, N. In capillary electrophoresis the number of theoretic plates is

$$N = \frac{l^2}{2Dt_m} = \frac{(\mu_{ep} + \mu_{eof})Vl}{2DL} \qquad 12.43$$

where D is the solute's diffusion coefficient. From equation 12.43, the efficiency of a capillary electrophoretic separation increases with higher voltages. Increasing the electroosmotic flow velocity improves efficiency, but at the expense of resolution. Two additional observations deserve comment. First, solutes with larger electrophoretic mobilities—in the same direction as the electroosmotic flow—have greater efficiencies; thus, smaller, more highly charged cations are not only the first solutes to elute, but do so with greater efficiency. Second, efficiency in capillary electrophoresis is independent of the capillary's length. Theoretical plate counts of approximately 100,000–200,000 are not unusual.

Selectivity

In chromatography we defined the selectivity between two solutes as the ratio of their retention factors (see equation 12.9). In capillary electrophoresis the analogous expression for selectivity is

$$\alpha = \frac{\mu_{ep,1}}{\mu_{ep,2}}$$

where $\mu_{ep,1}$ and $\mu_{ep,2}$ are the electrophoretic mobilities for the two solutes, chosen such that $\alpha \geq 1$. We can often improve selectivity by adjusting the pH of the buffer solution. For example, NH_4^+ is a weak acid with a pK_a of 9.75. At a pH of 9.75 the concentrations of NH_4^+ and NH_3 are equal. De-

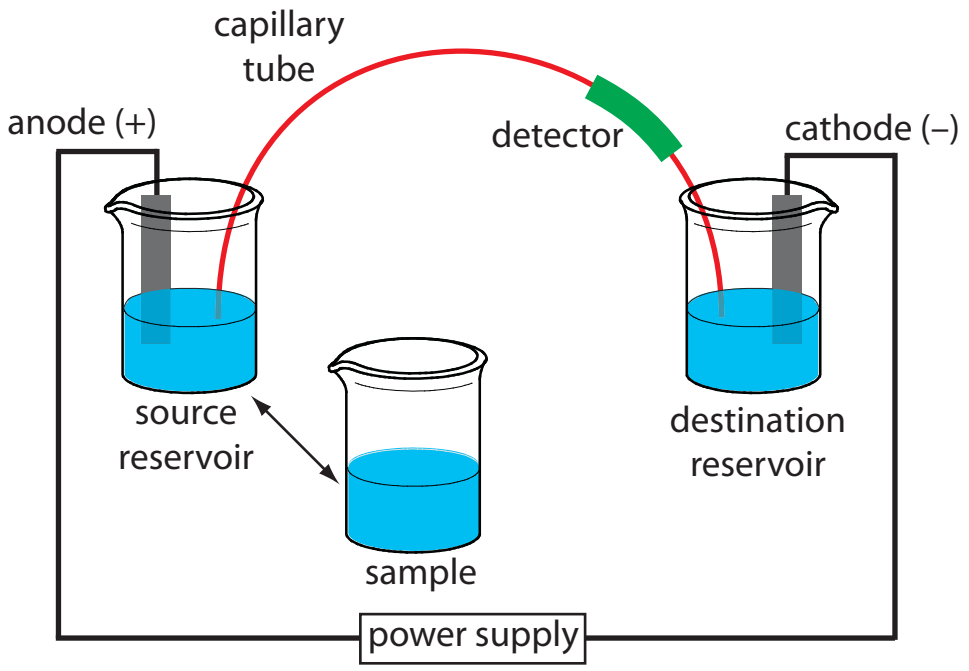


Figure 12.59 Schematic diagram of the basic instrumentation for capillary electrophoresis. The sample and the source reservoir are switched when making injections.

creasing the pH below 9.75 increases its electrophoretic mobility because a greater fraction of the solute is present as the cation NH_4^+. On the other hand, raising the pH above 9.75 increases the proportion of the neutral NH_3, decreasing its electrophoretic mobility.

Resolution

The resolution between two solutes is

$$R = \frac{0.177(\mu_{ep,1} - \mu_{ep,2})\sqrt{V}}{\sqrt{D(\mu_{avg} - \mu_{eof})}} \qquad 12.44$$

where μ_{avg} is the average electrophoretic mobility for the two solutes. Increasing the applied voltage and decreasing the electroosmotic flow velocity improves resolution. The latter effect is particularly important. Although increasing electroosmotic flow improves analysis time and efficiency, it decreases resolution.

12G.2 Instrumentation

The basic instrumentation for capillary electrophoresis is shown in Figure 12.59 and includes a power supply for applying the electric field, anode and cathode compartments containing reservoirs of the buffer solution, a sample vial containing the sample, the capillary tube, and a detector. Each part of the instrument receives further consideration in this section.

Capillary Tubes

Figure 12.60 shows a cross-section of a typical capillary tube. Most capillary tubes are made from fused silica coated with a 15–35 μm layer of polyimide to give it mechanical strength. The inner diameter is typically 25–75 μm—smaller than the internal diameter of a capillary GC column—with an outer diameter of 200–375 μm.

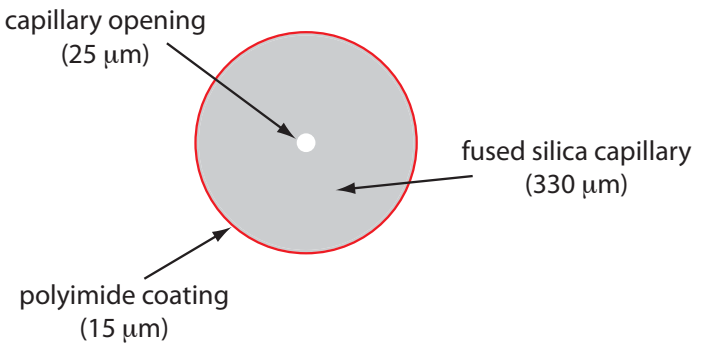


Figure 12.60 Cross section of a capillary column for capillary electrophoresis. The dimensions shown here are typical and are scaled proportionally.

The capillary column's narrow opening and the thickness of its walls are important. When an electric field is applied to the buffer solution within the capillary, current flows through the capillary. This current leads to the release of heat—what we call JOULE HEATING. The amount of heat released is proportional to the capillary's radius and the magnitude of the electrical field. Joule heating is a problem because it changes the buffer solution's viscosity, with the solution at the center of the capillary being less viscous than that near the capillary walls. Because a solute's electrophoretic mobility depends on viscosity (see equation 12.35), solute species in the center of the capillary migrate at a faster rate than those near the capillary walls. The result is an additional source of band broadening that degrades the separation. Capillaries with smaller inner diameters generate less Joule heating, and capillaries with larger outer diameters are more effective at dissipating the heat. Placing the capillary tube inside a thermostated jacket is another method for minimizing the effect of Joule heating; in this case a smaller outer diameter allows for a more rapid dissipation of thermal energy.

Injecting the Sample

There are two commonly used method for injecting a sample into a capillary electrophoresis column: hydrodynamic injection and electrokinetic injection. In both methods the capillary tube is filled with the buffer solution. One end of the capillary tube is placed in the destination reservoir and the other end is placed in the sample vial.

HYDRODYNAMIC INJECTION uses pressure to force a small portion of sample into the capillary tubing. A difference in pressure is applied across the capillary by either pressurizing the sample vial or by applying a vacuum to the destination reservoir. The volume of sample injected, in liters, is given by the following equation

For a hydrodynamic injection we move the capillary from the source reservoir to the sample. The anode remains in the source reservoir.

A hydrodynamic injection is also possible by raising the sample vial above the destination reservoir and briefly inserting the filled capillary.

$$V_{inj} = \frac{Pd^4\pi t}{128\eta L} \times 10^3 \qquad 12.45$$

where ΔP is the difference in pressure across the capillary in pascals, d is the capillary's inner diameter in meters, t is the amount of time that the pressure is applied in seconds, η is the buffer's viscosity in kg m^{-1} s^{-1}, and L is the length of the capillary tubing in meters. The factor of 10^3 changes the units from cubic meters to liters.

If you want to verify the units in equation 12.45, recall from Table 2.2 that 1 Pa is equivalent to 1 kg m^{-1} s^{-2}.

Example 12.9

In a hydrodynamic injection we apply a pressure difference of 2.5×10^3 Pa (a $\Delta P \approx 0.02$ atm) for 2 s to a 75-cm long capillary tube with an internal diameter of 50 μm. Assuming that the buffer's viscosity is 10^{-3} kg m^{-1} s^{-1}, what volume and length of sample did we inject?

SOLUTION

Making appropriate substitutions into equation 12.45 gives the sample's volume as

$$V_{inj} = \frac{(2.5\times10^{3}\ \text{kg m}^{-1}\ \text{s}^{-2})(50\times10^{-6}\ \text{m})^{4}(3.14)(2\ \text{s})}{(128)(0.001\ \text{kg m}^{-1}\ \text{s}^{-1})(0.75\ \text{m})}\times10^{3}\ \text{L/m}^{3}$$

$$V_{inj} = 1\times10^{-9}\ \text{L} = 1\ \text{nL}$$

Because the interior of the capillary is cylindrical, the length of the sample, l, is easy to calculate using the equation for the volume of a cylinder; thus

$$l = \frac{V_{inj}}{\pi r^{2}} = \frac{(1.0\times10^{-9}\ \text{L})(10^{-3}\ \text{m}^{3}/\text{L})}{(3.14)(25\times10^{-6}\ \text{m})^{2}} = 5\times10^{-4}\ \text{m} = 0.5\ \text{mm}$$

Practice Exercise 12.9

Suppose that you need to limit your injection to less than 0.20% of the capillary's length. Using the information from Example 12.9, what is the maximum injection time for a hydrodynamic injection?

Click here to review your answer to this exercise.

In an ELECTROKINETIC INJECTION we place both the capillary and the anode into the sample and briefly apply an potential. The volume of injected sample is the product of the capillary's cross sectional area and the length of the capillary occupied by the sample. In turn, this length is the product of the solute's velocity (see equation 12.39) and time; thus

$$V_{inj} = \pi r^{2}\times L = \pi r^{2}\times(\mu_{ep} + \mu_{eof})E't \qquad 12.46$$

where r is the capillary's radius, L is the length of the capillary, and E' is effective electric field in the sample. An important consequence of equation 12.46 is that an electrokinetic injection is inherently biased toward solutes with larger electrophoretic mobilities. If two solutes have equal concentrations in a sample, we inject a larger volume—and thus more moles—of the solute with the larger μ_{ep}.

When a analyte's concentration is too small to detect reliably, it may be possible to inject it in a manner that increases its concentration in the capillary tube. This method of injection is called STACKING. Stacking is accomplished by placing the sample in a solution whose ionic strength is significantly less than that of the buffer in the capillary tube. Because the sample plug has a lower concentration of buffer ions, the effective field strength across the sample plug, E' is larger than that in the rest of the capillary.

We know from equation 12.34 that electrophoretic velocity is directly proportional to the electrical field. As a result, the cations in the sample

The electric field in the sample is different that the electric field in the rest of the capillary because the sample and the buffer have different ionic compositions. In general, the sample's ionic strength is smaller, which makes its conductivity smaller. The effective electric field is

$$E' = E\times\frac{\kappa_{buf}}{\kappa_{sam}}$$

where κ_{buf} and κ_{sam} are the conductivities of the buffer and the sample, respectively.

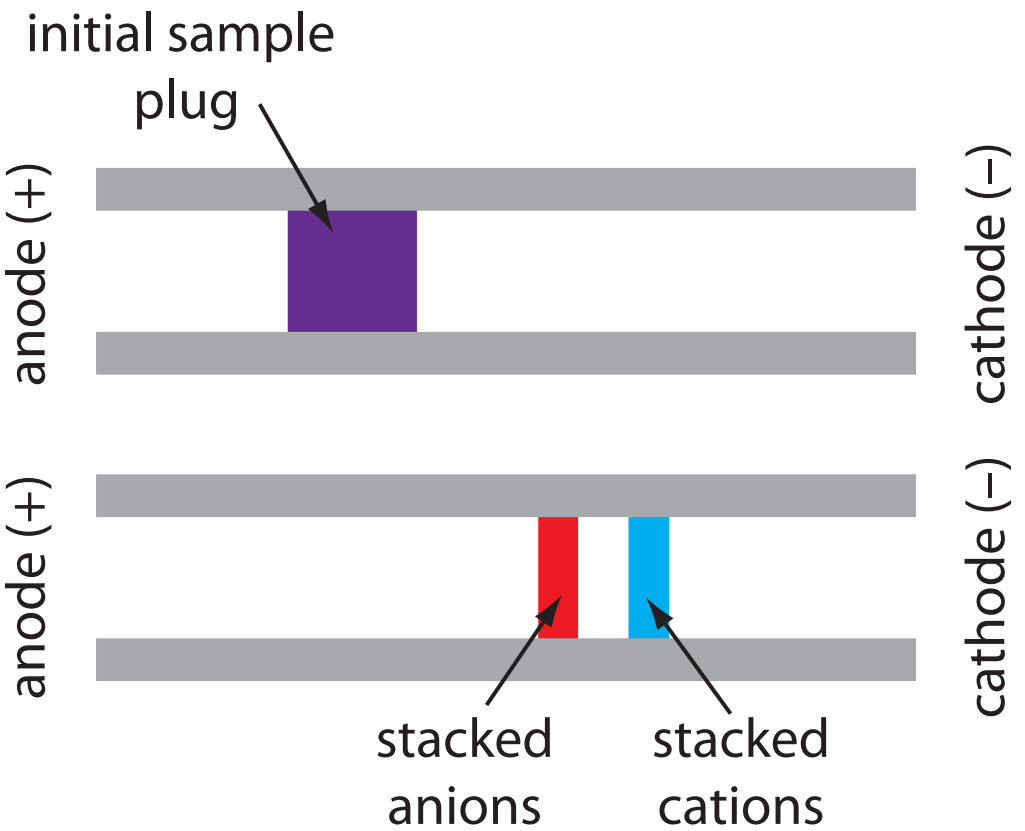


Figure 12.61 The stacking of cations and anions. The top diagram shows the initial sample plug and the bottom diagram shows how the cations and anions become concentrated at opposite sides of the sample plug.

plug migrate toward the cathode with a greater velocity, and the anions migrate more slowly—neutral species are unaffected and move with the electroosmotic flow. When the ions reach their respective boundaries between the sample plug and the buffering solution, the electrical field decreases and the electrophoretic velocity of cations decreases and that for anions increases. As shown in Figure 12.61, the result is a stacking of cations and anions into separate, smaller sampling zones. Over time, the buffer within the capillary becomes more homogeneous and the separation proceeds without additional stacking.

Applying the Electrical Field

Migration in electrophoresis occurs in response to an applied electrical field. The ability to apply a large electrical field is important because higher voltages lead to shorter analysis times (see equation 12.42), more efficient separations (equation 12.43), and better resolution (equation 12.44). Because narrow bored capillary tubes dissipate Joule heating so efficiently, voltages of up to 40 kV are possible.

Because of the high voltages, be sure to follow your instrument's safety guidelines.

Detectors

Most of the detectors used in HPLC also find use in capillary electrophoresis. Among the more common detectors are those based on the absorption of UV/Vis radiation, fluorescence, conductivity, amperometry, and mass spectrometry. Whenever possible, detection is done "on-column" before the solutes elute from the capillary tube and additional band broadening occurs.

UV/Vis detectors are among the most popular. Because absorbance is directly proportional to path length, the capillary tubing's small diameter leads to signals that are smaller than those obtained in HPLC. Several approaches have been used to increase the pathlength, including a Z-shaped