

## CHAPTER 8

# Separation and Purification Methods

**Read This Chapter to Learn About**

- > Principal Techniques
- > Special Applications for Biomolecules

## PRINCIPAL TECHNIQUES

The separation of mixtures is important for two reasons. First, separatory techniques are required for analyzing any number of complex mixtures—from contaminants in well water to forensic DNA samples to pharmaceutical formulations. Second, it is often necessary to purify compounds for further use—for example, the isolation of morphine from poppy seeds or the purification of intermediates in a multistep organic synthesis.

The most common techniques fall into three broad categories—**extraction, chromatography, distillation**. Each are based on slightly different chemical principles—in some respects overlapping, in others complementary. From a practical standpoint, a significant point of differentiation is scalability. For example, distilling 1 kg of solvent poses no particular technical challenges. However, purifying even 20 g of a reaction mixture by **chromatography** is an expensive and laborious proposition. Extending this to an industrial scale (on the order of 100 kg or more) is no less of a challenge, although it can be done.

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TABLE 8-1 General Methods for Separation and Purification

	Extraction	Chromatography	Distillation
<b>Principle of Separation</b>	Differential solubility between two or more solvent systems (e.g., water and dichloromethane)	Differential affinity between a stationary phase (e.g., silica or cellulose) and a mobile phase	Differential vapor pressures
<b>Common Applications</b>	Initial aqueous workup of reactions; separation of inorganic salts from organic products	Final purification of reaction mixtures (e.g., flash columns); analysis of products (e.g., HPLC and GC)	Purification of solvents and volatile products; concentration of reaction mixtures
<b>Scope and Limitations</b>	Broadly applicable; can be used with any two immiscible solvents	Can be used on a wide variety of compounds and mixtures	Limited to products with appreciable vapor pressure at or below 300°C
<b>Scalability</b>	Excellent	Limited	Very good
<b>Relative Expense</b>	Low	High	Moderate

By the same token, the various methods also differ in their scope and range of application. Clearly, a compound must exhibit some degree of volatility under reasonable conditions to be distilled. However, column chromatography can be used to purify a wide array of compounds, whether solid or liquid, polar or nonpolar, volatile or nonvolatile. It is sometimes advantageous to use combinations of methods—for example, a “quick and dirty” chromatography column to remove baseline impurities from a complex reaction mixture, followed by a careful distillation to obtain an analytically pure sample.

## Extraction

The basis of extractive techniques is the “like dissolves like” rule. Water typically dissolves inorganic salts (such as lithium chloride) and other ionized species, while solvents (ethyl acetate, methylene chloride, diethyl ether, etc.) dissolve neutral organic molecules. However, some compounds (e.g., alcohols) exhibit solubility in both media. Therefore, it is important to remember that this method of separation relies on **partitioning**—that is, the preferential dissolution of a species into one solvent over another. For example, 2-pentanol is somewhat soluble in water (i.e., 17 g/100 mL H<sub>2</sub>O), but infinitely soluble in diethyl ether. Thus 2-pentanol can be preferentially partitioned into ether.

One of the most common uses of **extraction** is during aqueous workup, as a way to remove inorganic materials from the desired organic product. On a practical note, workup is usually carried out using two immiscible solvents—that is, in a biphasic system. If a reaction has been carried out in tetrahydrofuran, dioxane, or methanol, then it is generally desirable to remove those solvents by evaporation before workup because

they have high solubilities in both aqueous and organic phases, and can set up single-phase systems (i.e., nothing to separate) or emulsions. Typical extraction solvents include ethyl acetate, hexane, chloroform, methylene chloride, and diethyl ether. All of these form crisp delineations between phases.

The two layers are commonly referred to as the **aqueous phase** and the **organic phase**. It is important to keep track of the phases, as their positions are solvent dependent. For example, diethyl ether is lighter than water, so the organic phase will rest on top in the separatory funnel, whereas methylene chloride is heavier than water and so will sink to the bottom (see Figure 8-1).

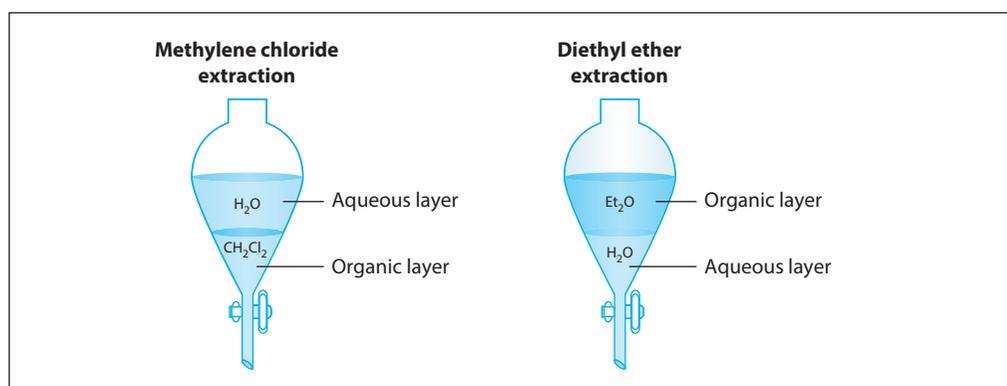


FIGURE 8-1 Two manifestations of an organic/aqueous extraction.

By way of vocabulary, actually two operations are encountered in the separatory funnel. When components are removed from an organic layer by shaking with an aqueous solution, the organic phase is said to be **washed** (e.g., “The combined ether extracts were washed with aqueous sodium bicarbonate solution”). On the other hand, when components are removed from water by treatment with an organic solvent, the aqueous phase is said to be **extracted** (e.g., “The aqueous layer was extracted with three portions of ethyl acetate”). Thus aqueous layers are extracted, and organic layers are washed—although these two terms are sometimes (erroneously) used interchangeably.

Aqueous workup can involve more than just separation. For example, reactions that produce anions (e.g., Grignard reactions) are usually “quenched” with a mildly acidic aqueous solution (e.g., saturated ammonium chloride) at the end of the reaction to neutralize any residual base. The same holds true for very acidic reactions. Thus aromatic nitration reactions ( $\text{HNO}_3/\text{H}_2\text{SO}_4$ ) are usually quenched by being poured onto a large quantity of ice, which dilutes the acidic environment.

While extractions are usually carried out with a neutral aqueous phase, sometimes pH modulation can be used to advantage. For example, a mixture of naphthalenesulfonic acid and naphthalene can be separated by washing with bicarbonate, in which case the sulfonic acid is deprotonated and partitioned into the aqueous phase. Similarly, a mixture of naphthalene and quinoline can be separated by an acid wash

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(see Figure 8-2), taking advantage of the basic nature of the heterocyclic nitrogen ( $pK_a$  9.5). If it is necessary to isolate the quinoline, it is neutralized with bicarbonate and extracted back into an organic solvent.

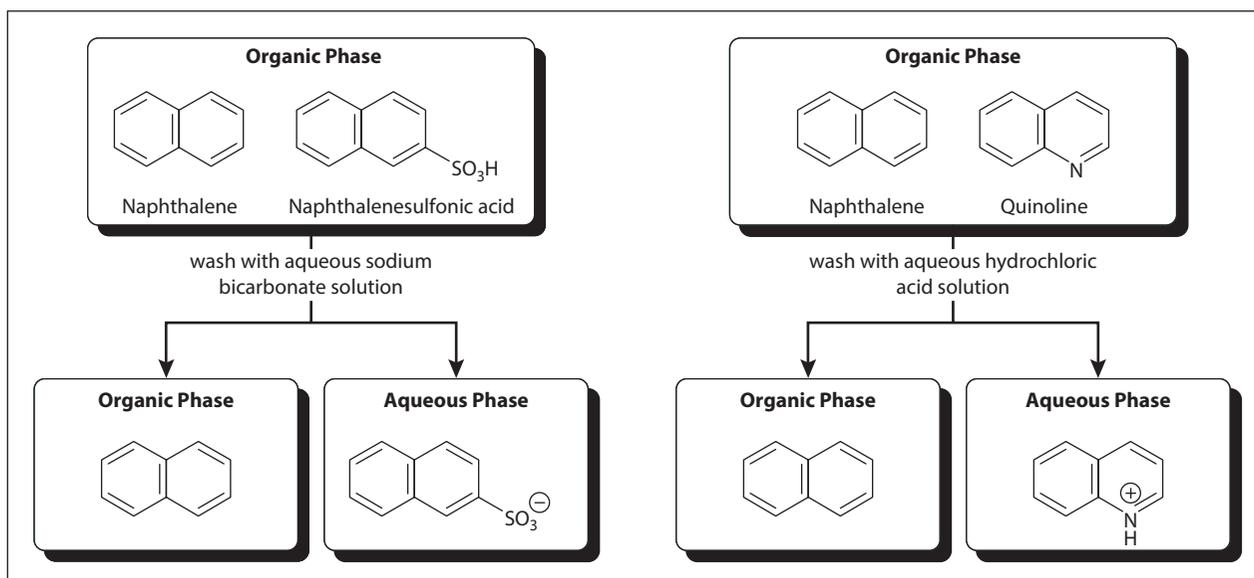


FIGURE 8-2 Two pH-controlled extractions.

Organic solvents are used for workup because they are easily removed by evaporation, leaving behind the organic compound of interest. A common problem encountered at this point is residual water from the aqueous washes. Ethyl acetate and diethyl ether both dissolve large quantities of water (3.3% and 1.2%, respectively). Therefore, it is advantageous to wash these organic layers with brine (saturated NaCl solution) at the end of the extraction sequence—the brine draws out the dissolved water through an osmotic-like effect. For methylene chloride and chloroform, a brine wash is unnecessary, since the solubility of water in these solvents is quite low. Once freed from the bulk of residual water, the organic layer is dried over a desiccant, such as sodium sulfate, calcium chloride, or magnesium sulfate, and then decanted or filtered before evaporation.

## Distillation

If chromatography is the most versatile separation method in the laboratory, it might be argued that **distillation** is the most common. This technique is used very frequently for purifying solvents and reagents before use.

### SIMPLE AND FRACTIONAL DISTILLATION

When volatile components are being removed from nonvolatile impurities, the method of **simple distillation** is employed (see Figure 8-3). In this familiar protocol, a liquid is heated to the boil, forcing vapor into a water-cooled condenser, where it is converted

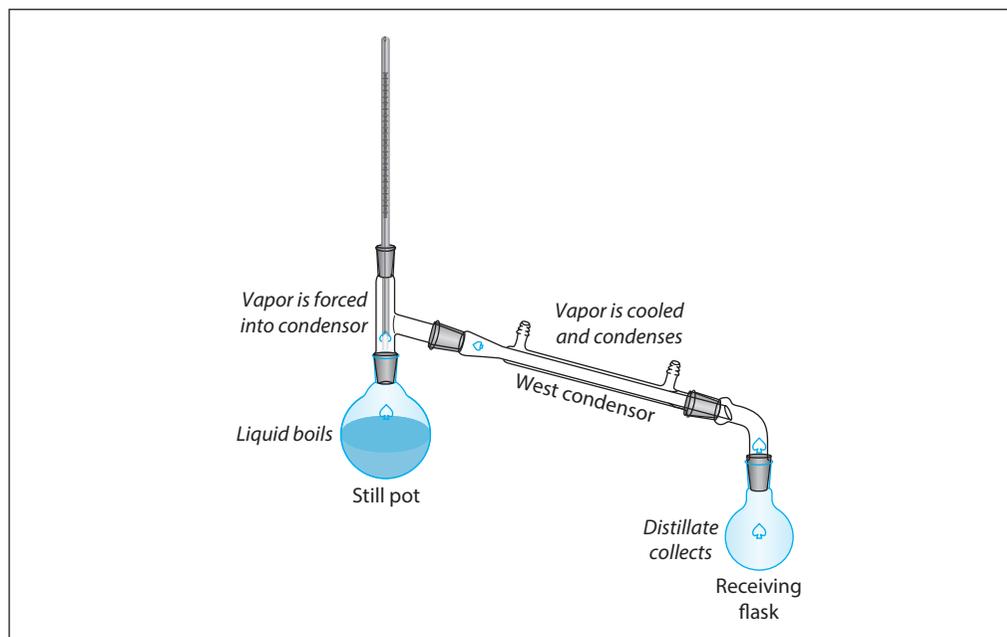


FIGURE 8-3 Simple distillation.

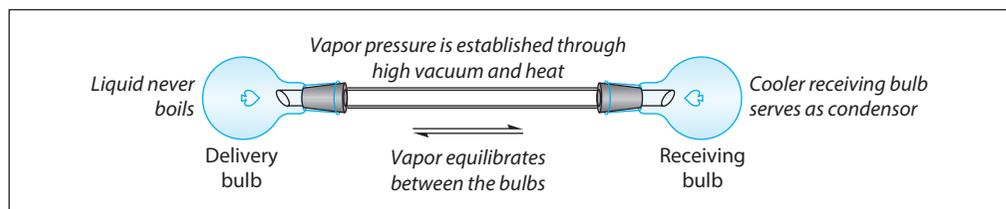
back to liquid and is conveyed by gravity to a receiving flask. When volumes are small (1–10 mL) a special apparatus known as a short-path distillation head is used. This piece of equipment is designed to minimize dead volume in the assembly and maximize recovery of the distillate. Otherwise, the principle is the same.

When separating two liquids with similar boiling points—or substances that tend to form azeotropes, **fractional distillation** is used. In this method, a connector with large surface area (such as a Vigreux column) is inserted between the still pot and the distillation head. The purpose of this intervening portion is to provide greater surface area upon which the vapor can condense and revolatilize, leading to greater efficiency of separation. In a more sophisticated apparatus, the Vigreux column and condenser are separated by an automated valve that opens intermittently, thus precisely controlling the rate of distillation.

## DISTILLING HIGH-BOILING COMPOUNDS

For compounds with limited volatility, the technique of **bulb-to-bulb distillation** (see Figure 8-4) is sometimes successful. In this distillation, the liquid never truly boils—that is to say, the vapor pressure of the compound does not reach the local pressure of the environment. Instead, the sample is placed in a flask (or bulb) and subjected to high vacuum and heat, which sets up a vapor pressure adequate to equilibrate through a passage to another bulb, which is cooled with air, water, or dry ice. The temperature differential drives the vapor equilibrium into the cooler bulb, where the compound condenses. This is the same principle behind the Kugelrohr (from German, “bulb and tube”) distillation.

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UNIT II:  
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Biological Systems**FIGURE 8-4** Bulb-to-bulb distillation.

The effect of temperature on the volatility of compounds is well-known, but the impact of reduced pressure is much less appreciated. However, distillation at reduced pressure, or **vacuum distillation**, brings many advantages. For example, consider a liquid that has a boiling point of  $180^{\circ}\text{C}$  at atmospheric pressure (760 Torr). If you were to attempt that distillation, you would not be surprised to observe charring of the compound at such elevated temperature. Fortunately, reducing the pressure using a water aspirator (approximately 20 Torr total pressure) results in a significant lowering of the boiling point. Figure 8-5 shows this effect using a pressure-temperature nomograph. First, the normal boiling point at atmospheric pressure is located on the center scale; then a straightedge is used to span that point and the distillation pressure on the rightmost scale; tracing the straightedge back to the leftmost scale will provide an estimate of the boiling point at that pressure. Thus a compound with a boiling point of  $180^{\circ}\text{C}$  at 760 Torr will boil at about  $80^{\circ}\text{C}$  under a vacuum of 20 Torr. At 1 Torr (easily attainable with a vacuum pump), the boiling point is well below room temperature! For the purposes of purification, however, you are better off with the aspirator, because at 1 Torr you could not recover the compound using a water-cooled (approximately  $20^{\circ}\text{C}$ ) condenser.

Another way to distill sparingly volatile compounds is by **steam distillation**. In a true steam distillation, live steam is introduced into the still pot by means of a metal tube, or dipleg. The steam heats up the pot and carries any vaporized material from the head space to the condenser, where it collects and drains into the receiving flask with the condensed water. In a common modification, water is simply added to the still pot and the mixture is heated in the same fashion as a simple distillation—the steam is thus produced *in situ*. The underlying principle of steam distillation is that an azeotrope forms with water, which has a lower boiling point than the pure compound itself. For example, naphthalene has a boiling point of  $218^{\circ}\text{C}$ , but in the presence of steam an azeotrope is formed, which contains 16% naphthalene and boils at  $99^{\circ}\text{C}$ . In addition, the continual physical displacement of the head space by water vapor also aids in the collection of slightly volatile components.

## ROTARY EVAPORATION

There are many occasions when you simply need to remove solvent (e.g., after working up a reaction). In such cases, the volatile compound is the undesired component, so

recovering it quantitatively is not of great concern. For this application, the technique of **rotary evaporation** has been developed (see Figure 8-6). A solution of the desired product in some common solvent (ether, methylene chloride, etc.) is placed in a round-bottom flask and placed under an aspirator vacuum while being mechanically rotated. The rotation maintains a fresh film of solution on the flask walls, which maximizes the solvent vapor pressure in a manner reminiscent of a Kugelrohr distillation. The vapor rises to a water-cooled glass coil, where it condenses and collects into a receiving flask. The delivery flask is kept in an ambient temperature bath to counteract the evaporative cooling effect. In this way, 50 mL of methylene chloride can be removed in about 5 minutes. However, if the desired product is somewhat volatile ( $bp_{760} < 250^{\circ}\text{C}$ ), care must be taken to prevent inadvertent loss on the rotary evaporator.

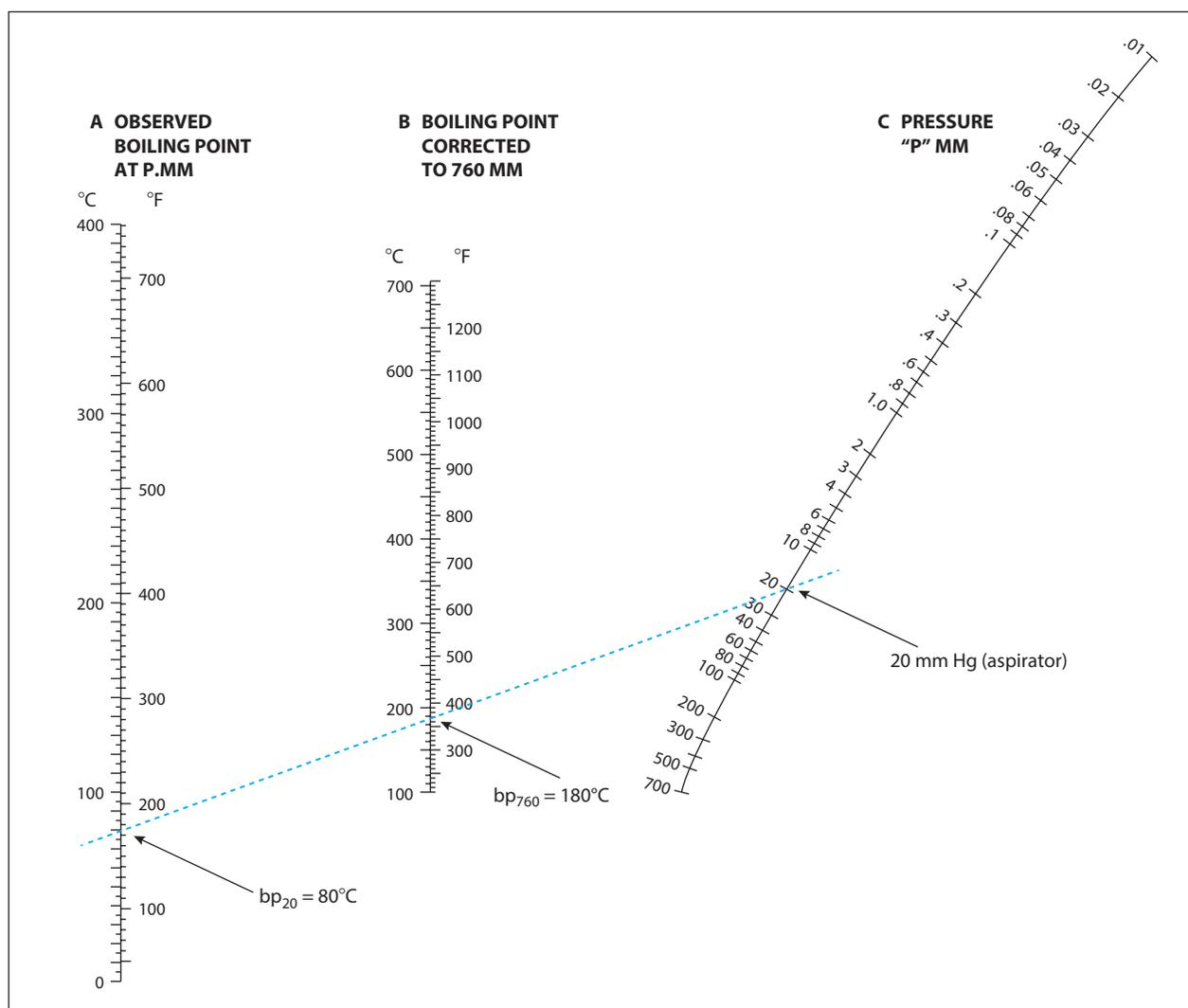


FIGURE 8-5 A pressure-temperature nomograph.

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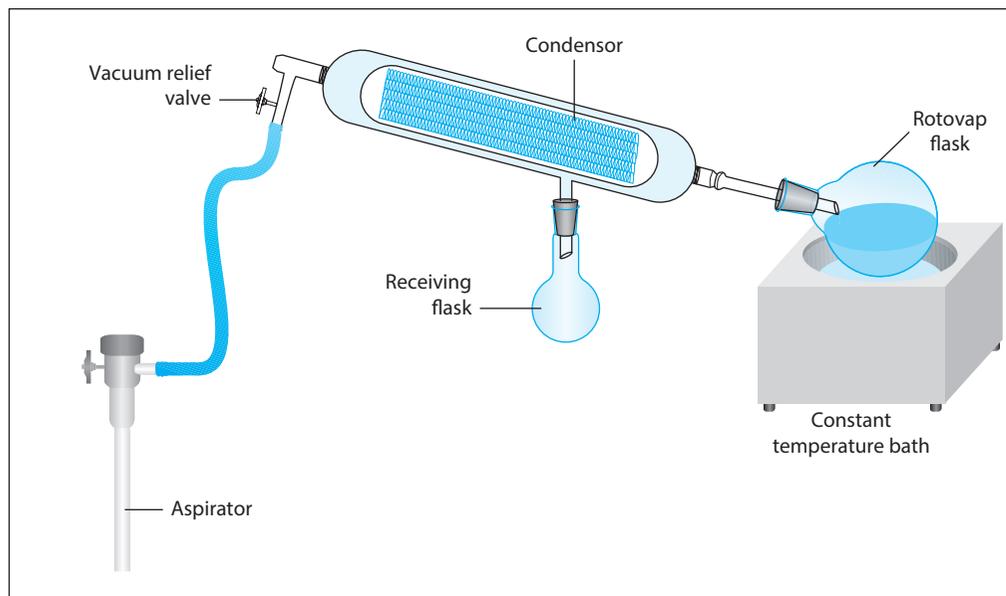
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FIGURE 8-6 Components of a rotary evaporator.

## Chromatography

Chromatography represents the most versatile separation technique readily available to the organic chemist. Even though it suffers from limitations of cost and scalability, it has found broad application in the purification and analysis of complex chemical systems.

### BACKGROUND

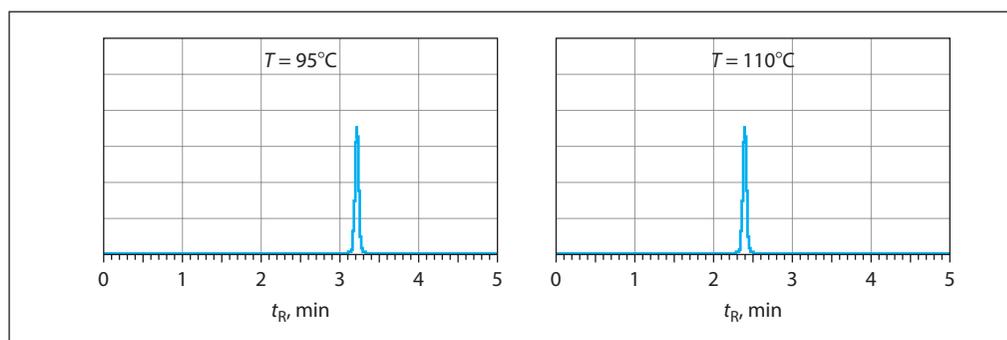
Conceptually, the technique of chromatography is very simple—there are only two components: a **stationary phase** (usually silica or cellulose) and a **mobile phase** (usually a solvent system). Any two compounds usually have different partitioning characteristics between the stationary and mobile phases. Since the mobile phase is moving (thus the name), then the more time a compound spends in that phase, the farther it will travel.

Chromatographic techniques fall into one of two categories: analytical and preparative. **Analytical techniques** are used to follow the course of reactions and determine purity of products. These methods include gas chromatography (GC), high-performance liquid chromatography (HPLC), and thin-layer chromatography (TLC). Sample sizes for these procedures are usually quite small, from microgram to milligram quantities. In some cases, the chromatograph is coupled to another analytical instrument, such as a mass spectrometer or nuclear magnetic resonance (NMR) spectrometer, so the components that elute can be easily identified.

**Preparative techniques** are used to purify and isolate compounds for characterization or further use. The most common techniques in this category are preparative HPLC, preparative TLC, and column chromatography. One popular modification of column chromatography is the flash column, which operates at medium pressures (about 10 psi) and provides very rapid separation (approximately 5 minutes elution times). Column chromatography is suitable for sample sizes ranging from a few milligrams to several grams.

## PARAMETERS

For gas chromatography (GC), the mobile phase is an inert carrier gas, such as helium or argon. Therefore, for the components to move, they must be volatile under the analytical conditions. Toward this end, GC columns are usually heated during analysis. The retention time ( $t_R$ ) can be controlled by the oven temperature—the higher the temperature, the more quickly the sample elutes (see Figure 8-7).



**FIGURE 8-7** Effect of oven temperature on GC retention time.

The other methods fall under the category of LC (liquid chromatography), where the mobile phase is a solvent system, which can be used instead of temperature to leverage retention. Occasionally, this is a single solvent, but more often it is a binary mixture of solvents with different polarities. The advantage of the latter is that the bulk polarity can be modulated by varying the ratio of the two solvents.

For example, consider a typical TLC plate (see Figure 8-8) developed in a 1:1 mixture of ethyl acetate and hexane, which exhibits two well-separated components. The spots can be characterized by their  **$R_f$  value**, which is defined as the distance traveled from the origin divided by the distance traveled by the mobile phase. Generally speaking, the slower moving component ( $R_f$  0.29) is either larger, more polar, or both. If you wanted a larger  $R_f$  value, you could boost the solvent polarity by increasing the proportion of ethyl acetate in the mobile phase. Conversely, more hexane would result in lower-running spots.

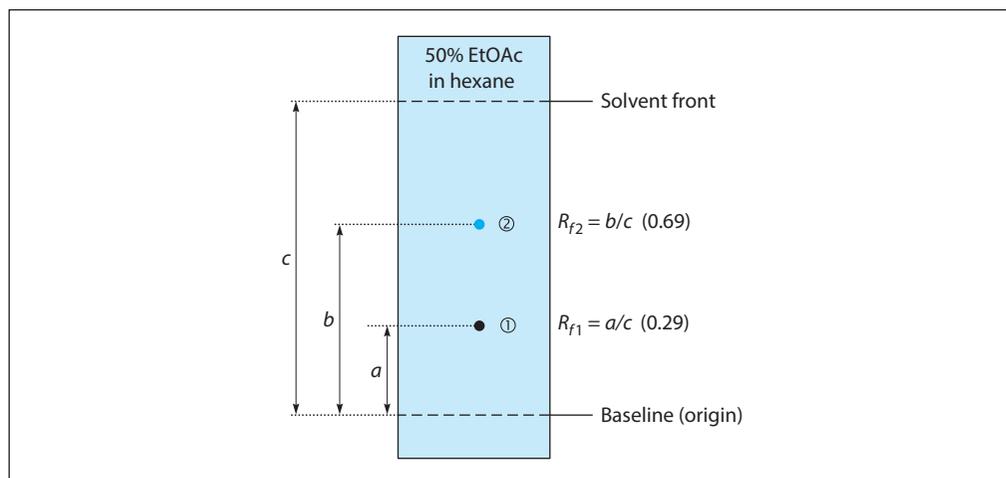


FIGURE 8-8 A typical TLC plate.

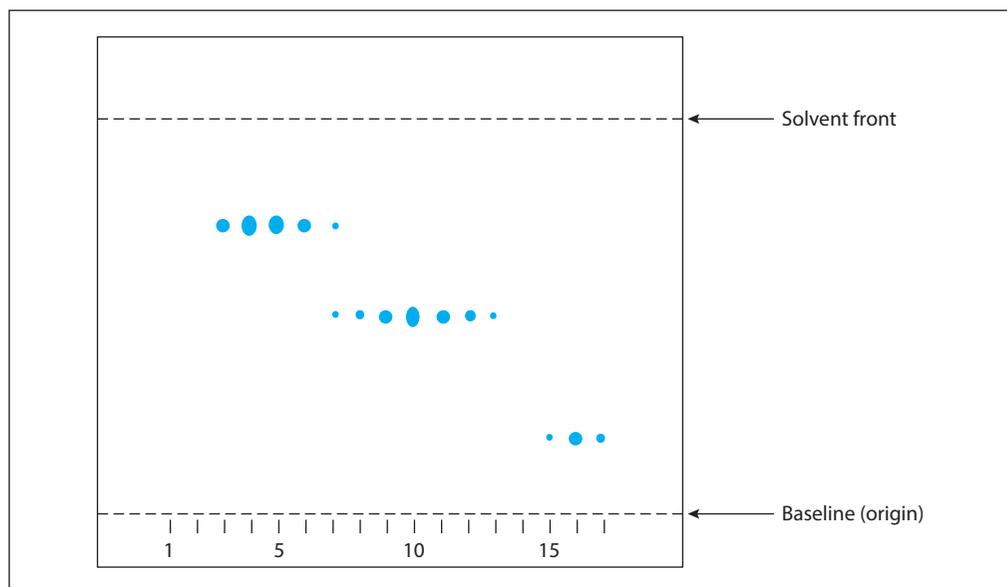
## NORMAL AND REVERSE-PHASE SYSTEMS

Sometimes one or more of the components interact so strongly with the silica gel that departure from the origin comes only with great difficulty. One answer to this problem is to derivatize the silica with nonpolar substituents, such as long-chain aliphatic residues. This creates a situation known as a **reverse phase system**. In normal phase chromatography, the stationary phase (i.e., silica) is *more polar* than the mobile phase; in reverse phase, the stationary phase is *less polar* than the mobile phase. The derivatized silica is not only much less polar itself, but it also allows for the use of very polar solvents, such as water and methanol. The reversed phase results in some counterintuitive outcomes. For example, more polar components actually elute *faster*, and a more polar solvent system results in an *increased* retention time (i.e., lower  $R_f$  value). These parameters are summarized in the following table.

TABLE 8-2 Comparison of Normal and Reverse-Phase Chromatography

Parameter	Normal Phase	Reverse Phase
typical stationary phase	underivatized silica gel	C <sub>8</sub> -hydrocarbon derivatized silica
representative mobile phase	ethyl acetate/hexane mixture	acetonitrile/water mixture
more polar components	have lower $R_f$ values	have higher $R_f$ values
increasing solvent polarity	increases $R_f$ values	decreases $R_f$ values

Generally speaking, most analytical methods employ reverse phase systems, whereas the majority of preparative techniques are based on normal silica gel (an outcome largely driven by cost, as the derivatized silica is quite expensive). Among



**FIGURE 8-9** A typical flash column separation.

preparative techniques, flash columns are the most prevalent. Typical columns range from 0.5–5.0 cm in diameter, with a silica column height of about 15 cm. A sample is loaded to the top of the column and then rapidly eluted by forcing solvent through under medium pressure. A series of fractions are collected, the volume of which is determined by the size of the column—for a 2-cm column, one usually collects 10 mL fractions.

Once collected, the fractions are spotted on a long TLC plate and developed in the same solvent system used for eluting the flash column. Figure 8-9 shows an ideal scenario, in which three components have been successfully isolated in separate fractions. The next step is to combine like fractions into “cuts”—for example, the first cut might contain fractions 3–6; the second cut, fractions 8–13; and the third cut, fractions 15–17. The solvent is then removed by evaporation.

## SPECIAL APPLICATIONS FOR BIOMOLECULES

In addition to the standard techniques discussed above, there are two special methods of particular interest to the purification and analysis of molecules of biological interest.

### Electrophoresis

One important methodology for the separation and characterization of amino acids and oligopeptides is **electrophoresis**. In this analytical technique, a mixture of amino acids (or oligopeptides) is spotted onto the center of a conductive gel, across which

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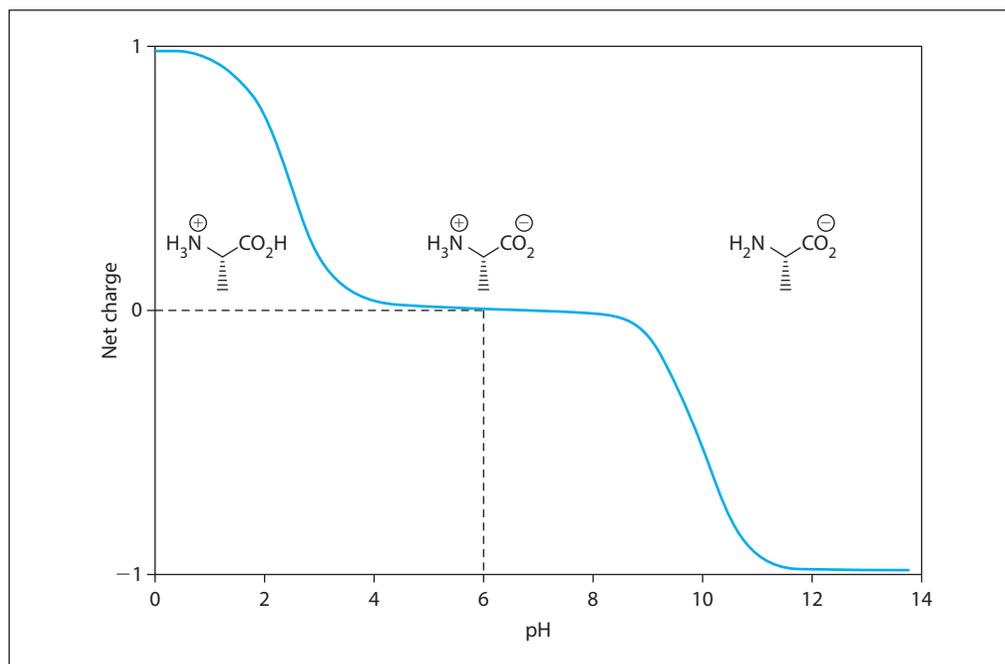


FIGURE 8-10 Net charge of alanine as a function of pH.

is applied an electrical potential. The amino acids then migrate toward the anode or cathode, depending upon the net charge on each molecule (positively-charged species move to the cathode and negatively-charged species move to the anode). The mobility of each ion depends upon its net charge and its mass.

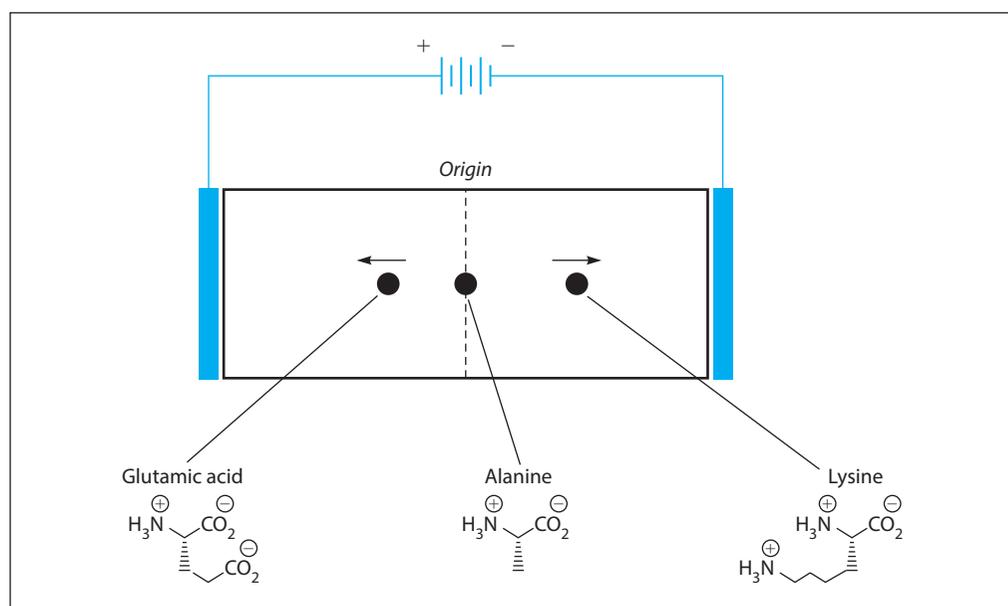
Since amino acids are polyfunctional ionizable molecules, their net charge is a function of pH. In the case of alanine, for example, at very low pH the net charge is +1 (both the carboxylic acid and amine functionalities are protonated)—thus at pH = 2, alanine would move toward the cathode (see Figure 8-10). Conversely, at very high pH the net charge is -1 (both the carboxylic acid and amine functionalities are deprotonated)—thus at pH = 10, alanine would move toward the anode. At pH = 6, there is no net charge on the alanine molecule (positive and negative charges exactly cancel each other)—therefore, alanine would not migrate on an electrophoresis gel at pH = 6. This is known as the **isoelectric point** (pI).

The isoelectric point varies with the identity of the amino acid (or oligopeptide), as shown in the following table. For example, glutamic acid has two carboxylic acid groups and one amine group, which means its isoelectric point is in the acidic range. This makes sense if you consider that for a net charge of zero, each of the carboxylic acid moieties must be half-protonated, which requires a lower pH. As a complementary example, lysine has two amine groups and one carboxylic acid group, which results in an isoelectric point in the basic range. In this case, half of the amines must be deprotonated to achieve a net charge of zero for the molecule.

**TABLE 8-3** Isoelectric Points of the Amino Acids

Amino Acid	pI	Amino Acid	pI
Alanine	6.00	Arginine	10.76
Asparagine	5.41	Aspartic acid	2.77
Cysteine	5.07	Glutamic acid	3.22
Glutamine	5.65	Glycine	5.97
Histidine	7.59	Isoleucine	6.02
Leucine	5.98	Lysine	9.74
Methionine	5.74	Phenylalanine	5.48
Proline	6.30	Serine	5.68
Threonine	5.60	Tryptophan	5.89
Tyrosine	5.66	Valine	5.96

This background provides a theoretical framework for understanding the electrophoresis gel shown in Figure 8-11, in which glutamic acid, alanine, and lysine are separated at pH = 6. Because alanine is *at* its isoelectric point, it does not migrate. Glutamic acid, however, is *above* its isoelectric point (3.22), so it bears a net negative charge and will move toward the anode. Conversely, lysine is *below* its isoelectric point (9.74), so it bears a net positive charge and will move toward the cathode.

**FIGURE 8-11** Gel electrophoresis of three amino acids at pH 6.0.

A common contemporary method for electrophoretic analysis is known as **capillary electrophoresis** or CE (see Figure 8-12). The separatory medium in this technique is not a gel, but rather a capillary with either end immersed in a buffer solution. Application of an electrical potential across the capillary induces a flow of buffer

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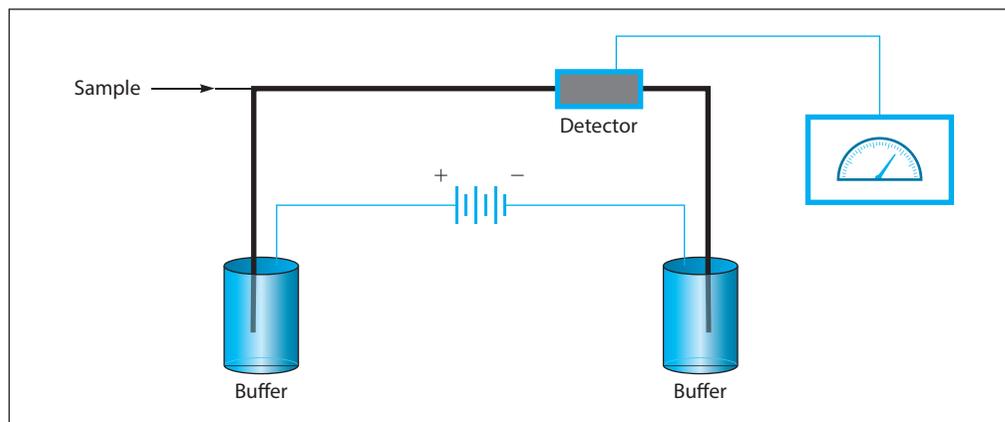
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FIGURE 8-12 Schematic of a capillary electrophoresis apparatus.

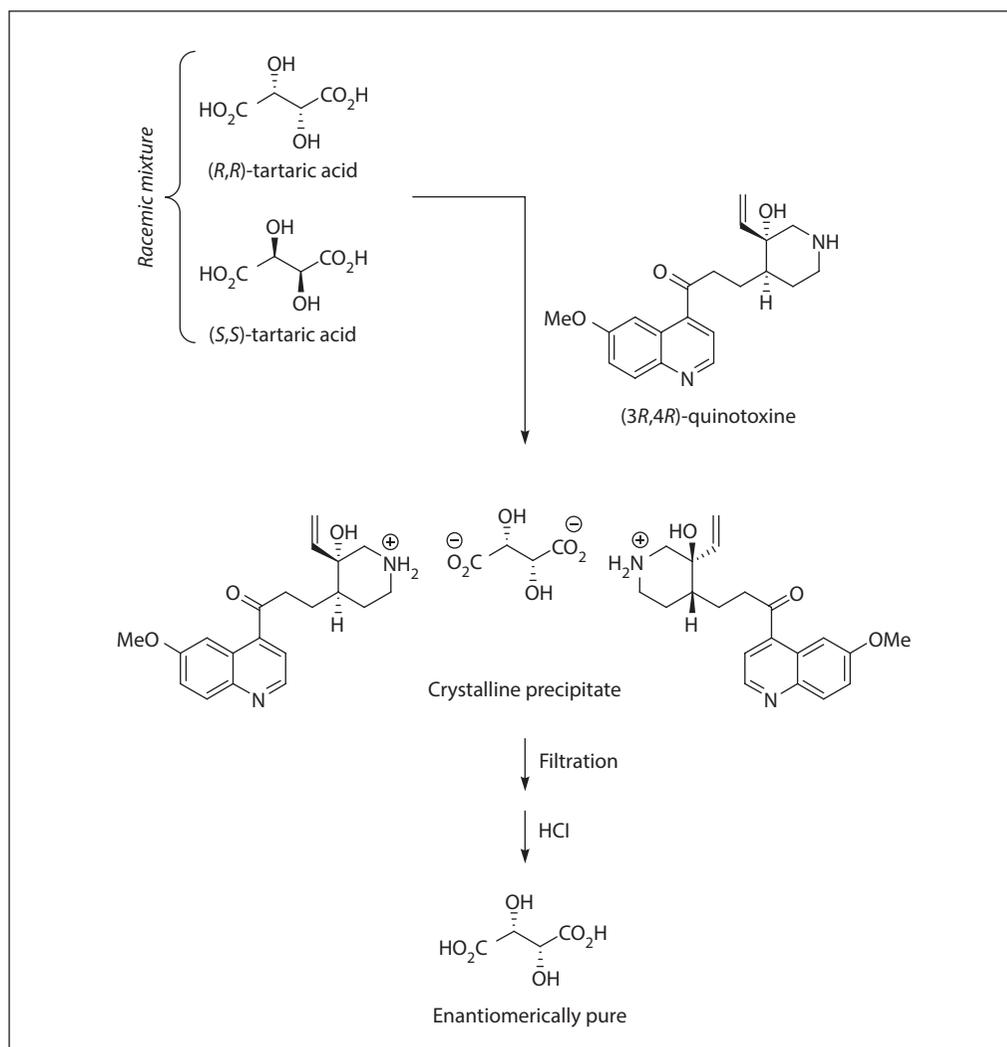
toward the cathode, a phenomenon known as **electroosmotic flow** (EOF). The sample is introduced either by temporarily replacing the anode-side buffer with a solution of the sample or via a more sophisticated in-line injection system. While the finer details regarding the mode of separation are different for CE versus gel electrophoresis, the general principle of separation based on charge still holds.

## Separation of Enantiomers

It is often desirable to prepare compounds as a single enantiomer. There are two general approaches to this problem: (1) by designing a synthesis that results only in a single enantiomer (chiral synthesis), or (2) by synthesizing the product in racemic form and then separating the enantiomers from one another (chiral resolution). The first approach is beyond the scope of this text. The second approach can be accomplished using one of two methods: (1) by preferential crystallization and (2) by chiral HPLC.

In **preferential crystallization**, a racemic mixture is treated with an optically pure compound that can coordinate very tightly with the racemate. Very often this is done with salt formation. For example, consider the chiral resolution of racemic tartaric acid shown in Figure 8-13. When the racemic acid is treated with enantiomerically pure (3*R*, 4*R*)-quinotoxine, a crystalline salt is formed with the (*R*, *R*)-tartrate only. The (*S*, *S*)-enantiomer remains in solution. The crystalline salt can be isolated by filtration and then acidified to reform the carboxylic acid as the enantiomerically pure (*R*, *R*)-enantiomer.

Two fundamental concepts are important to recognize with this example. First, there is no particular reason the (*R*, *R*)-base preferentially formed a precipitate with the (*R*, *R*)-acid; it was merely coincidence that these two isomers formed the insoluble salt. Second, the acid exists as a mixture of enantiomers. Recall that enantiomers have

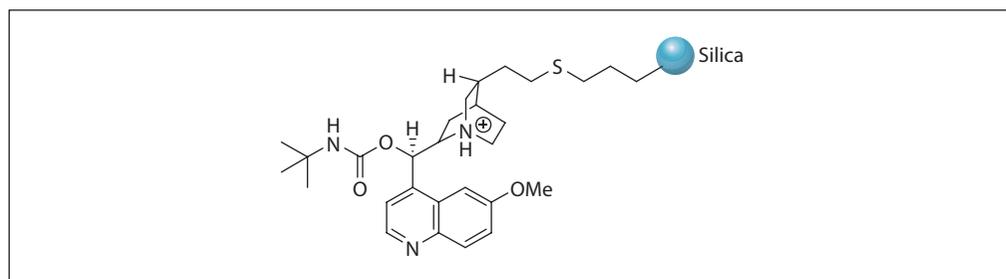


**FIGURE 8-13** Chiral resolution of racemic tartaric acid using quinotoxine.

identical physical properties (solubility, melting point, etc.), so it would be impossible to separate enantiomers based on solubility differences. However, with the addition of a chiral amine (quinotoxine), a set of *diastereomeric* salts are formed (*R, R*-acid + *R, R*-base, and *S, S*-acid + *R, R*-base). Unlike enantiomers, diastereomers can have radically different physical properties. In this case, the (*R, R*-acid + *R, R*-base) diastereomer is insoluble, but the (*S, S*-acid + *R, R*-base) diastereomer remains in solution.

The second general approach to chiral resolution is through HPLC with the use of a **chiral stationary phase**. In this technique, silica gel is derivatized with an enantiomerically pure chiral species (such as the quinine-based stationary phase shown in Figure 8-14). When a mixture of enantiomers travels through such a column, one enantiomer tends to interact more strongly with the chiral stationary phase (another diastereomeric relationship), thereby slowing down its progress through the column.

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**FIGURE 8-14** Example of a chiral HPLC stationary phase.

As with the standard techniques discussed earlier, chromatographic separation is a more expensive approach to chiral separation, but generally a more versatile option.