**Isocratic elution:** one solvent

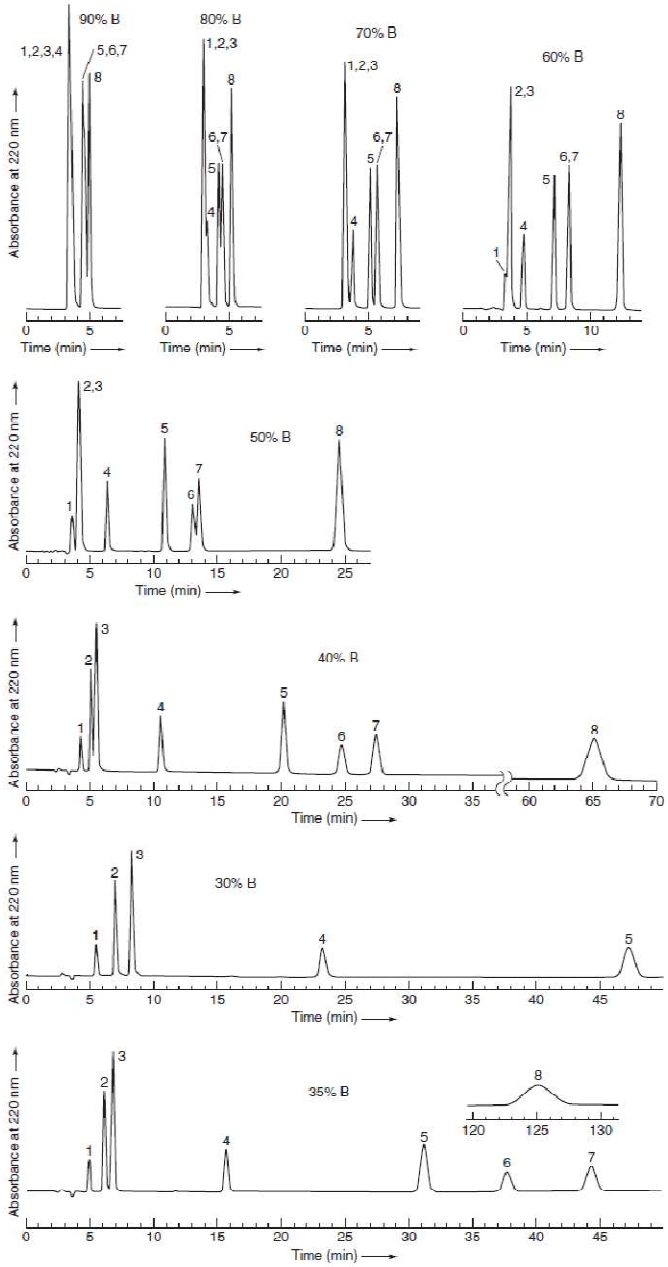
**Gradient elution:** continuous change of solvent composition to increase eluent strength. Gradient elution in HPLC is analogous to temperature programming in gas chromatography. Increased eluent strength is required to elute more strongly retained solutes.

stationary phase interface in reversed-phase chromatography.

## Isocratic and Gradient Elution

**Isocratic elution** is performed with a single solvent (or constant solvent mixture). If one solvent does not provide sufficiently rapid elution of all components, then **gradient elution** can be used. In this case, increasing amounts of solvent B are added to solvent A to create a continuous gradient.

Figure 24-12 shows the effect of increasing eluent strength in the isocratic elution of eight compounds from a reversed-phase column. In a reversed-phase separation, eluent strength *decreases* as the solvent becomes *more* polar. The first chromatogram (upper left) was obtained with a solvent consisting of 90 vol% acetonitrile and 10 vol% aqueous buffer. Acetonitrile has a high eluent strength, and all compounds are eluted rapidly. Only




- Aqueous buffer for HPLC is prepared and the pH adjusted *prior* to mixing with organic solvent.[15]
- Ultrapure water for HPLC should be freshly prepared by a purification train or by distillation. Water extracts impurities from polyethylene or glass after storage for a few hours.
- To prepare 70% B, for example, mix 70 mL of B with 30 mL of A. *The result is different* from placing 70 mL of B in a volumetric flask and diluting to 100 mL with A because there is a volume change when A and B are mixed.

**General elution problem:** For a complex mixture, isocratic conditions can often be found to produce adequate separation of early-eluting peaks or late-eluting peaks, but not both. This problem drives us to use gradient elution.

**FIGURE 24-12** Isocratic HPLC separation of a mixture of aromatic compounds at 1.0 mL/min on a 0.46 × 25 cm Hypersil ODS column ($C_{18}$ on 5 μm silica) at ambient temperature (~22°C): (1) benzyl alcohol; (2) phenol; (3) 3′,4′-dimethoxyacetophenone; (4) benzoin; (5) ethyl benzoate; (6) toluene; (7) 2,6-dimethoxytoluene; (8) *o*-methoxybiphenyl. Eluent consisted of aqueous buffer (designated A) and acetonitrile (designated B). The notation "90% B" in the first chromatogram means 10 vol% A and 90 vol% B. The buffer contained 25 mM $KH_2PO_4$ plus 0.1 g/L sodium azide adjusted to pH 3.5 with HCl.

three peaks are observed because of overlap. It is customary to call the aqueous solvent A and the organic solvent B. The first chromatogram was obtained with 90% B. When eluent strength is reduced by changing the solvent to 80% B, there is slightly more separation and five peaks are observed. At 60% B, we begin to see a sixth peak. At 40% B, there are eight clear peaks, but compounds 2 and 3 are not fully resolved. At 30% B, all peaks would be resolved, but the separation takes too long. Backing up to 35% B (the bottom trace) separates all peaks in a little over 2 h (which is still too long for many purposes).

From the isocratic elutions in Figure 24-12, the gradient in Figure 24-13 was selected to resolve all peaks in 38 min. First, 30% B (B = acetonitrile) was run for 8 min to separate components 1, 2, and 3. Then eluent strength was increased over 5 min to 45% B and held there for 15 min to elute peaks 4 and 5. Finally, the solvent was changed to 80% B over 2 min and held there to elute the last peaks.

Box 24-3 describes gradient elution in *supercritical fluid chromatography*.

**Hydrophilic:** "water loving"—soluble in water, surface is wetted by water

**Hydrophobic:** "water hating"—insoluble in water, surface is not wetted by water

## Hydrophilic Interaction Chromatography (HILIC)

**Hydrophilic substances** are soluble in water or attract water to their surfaces. Polar organic molecules have hydrophilic regions. **Hydrophilic interaction chromatography** is most useful for small molecules that are too polar to be retained by reversed-phase columns. Stationary phases for hydrophilic interaction chromatography, such as those in Figure 24-14, are strongly polar. The mobile phase typically contains (25–97 vol%) $CH_3CN$ or other polar organic solvent mixed with aqueous buffer. Solutes equilibrate between the mobile phase and a layer of aqueous phase on the surface of the stationary phase. In biochemistry, HILIC is useful for separating peptides and saccharides (sugars).

In HILIC, eluent strength is increased by *increasing* the fraction of water in the mobile phase. Gradient elution goes from low aqueous content to high aqueous content. In normal-phase chromatography, the solvent is nonaqueous. To increase eluent strength, we increase the polarity of the nonaqueous solvent. In reversed-phase chromatography, the solvent is usually aqueous, and eluent strength is increased by *decreasing* the fraction of water in the mobile phase to increase the solubility of solutes in the mobile phase.

## Selecting the Separation Mode

There can be many ways to separate components of a given mixture. Figure 24-15 is a decision tree for choosing a starting point. If the molecular mass of analyte is below 2 000, use the upper part of the figure; if the molecular mass is greater than 2 000, use the lower part. In

There are no firm rules in Figure 24-15. Methods in either part of the diagram might work for molecules whose size belongs to the other part.

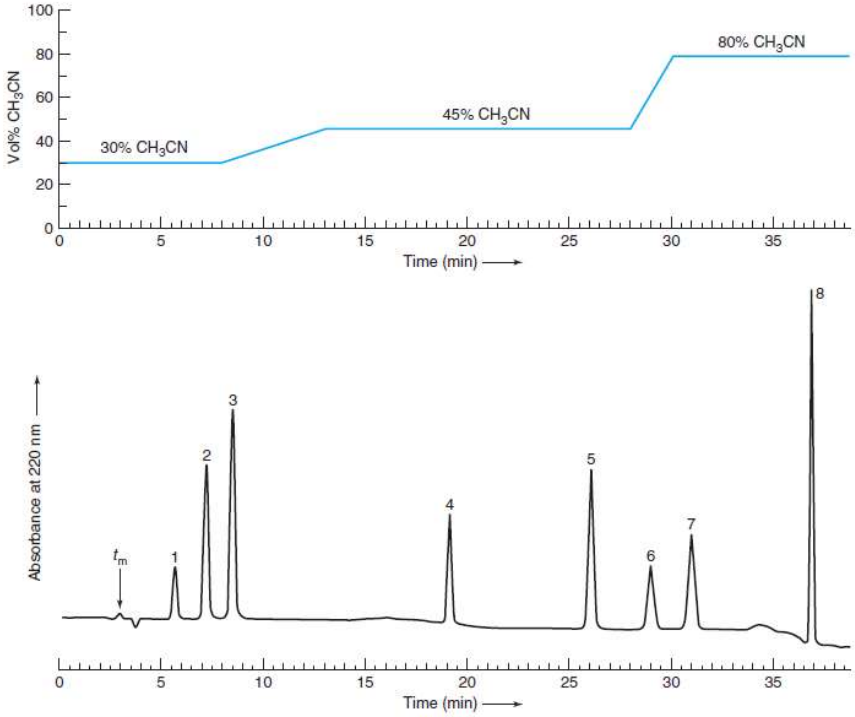



**FIGURE 24-13** Gradient elution of the same mixture of aromatic compounds in Figure 24-12 with the same column, flow rate, and solvents. The upper trace is the *segmented gradient* profile, so named because it is divided into several different segments.

either part, the first question is whether the solutes dissolve in water or in organic solvents. Suppose we have a mixture of small molecules (molecular mass <2 000) soluble in dichloromethane. Table 24-2 is essentially a ranking of solvent polarity, with the most polar solvents at the bottom. The eluent strength of dichloromethane (0.30) is closer to that of chloroform (0.26) than it is to those of alcohols, acetonitrile, or ethyl acetate (≥0.48). Therefore, Figure 24-15 suggests that we try adsorption chromatography on silica. The decision path is highlighted in color.

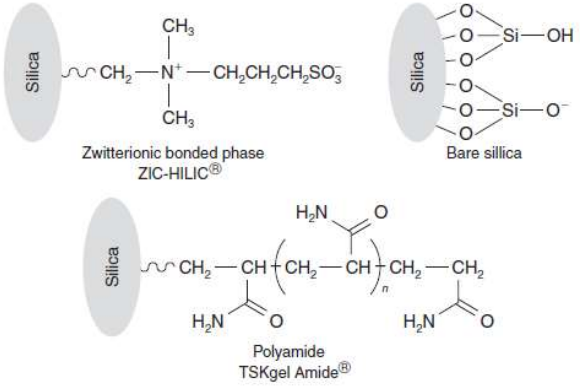



**FIGURE 24-14** Stationary phases for hydrophilic interaction chromatography (HILIC).