11th Week

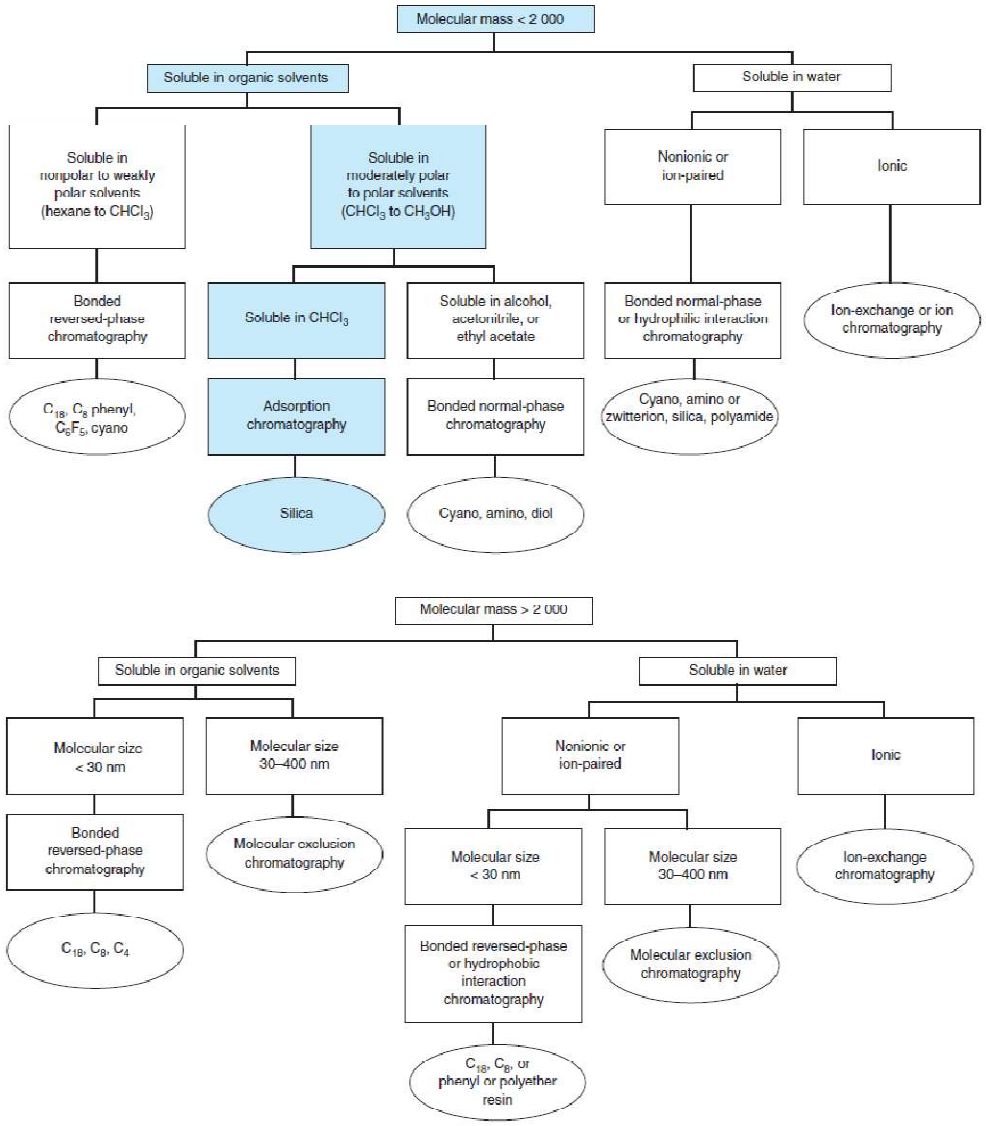

FIGURE 24-15 Guide to HPLC mode selection.

If solutes dissolve only in nonpolar or weakly polar solvents, the decision tree suggests that we try reversed-phase chromatography. Our choices include bonded phases containing octadecyl (C_{18}), octyl, phenyl, pentafluorophenyl, and cyano groups.

If molecular masses of solutes are >2 000 and if they are soluble in organic solvents and their molecular diameter is >30 nm, Figure 24-15 tells us to try molecular exclusion chromatography, described in Section 25-3. If molecular masses of solutes are >2 000, and they are soluble in water, but not ionic, and have diameters <30 nm, the decision tree says to use reversed-phase chromatography, or *hydrophobic interaction chromatography*, which is

Solvents

Pure HPLC-grade (expensive) solvents are required to prevent degradation of costly columns with impurities and to minimize detector background signals from contaminants. Before use, solvents are purged with He or evacuated to remove dissolved air. Air bubbles create difficulties for pumps, columns, and detectors. Dissolved O_2 absorbs ultraviolet radiation in the 250- to 200-nm wavelength range,[18] interfering with ultraviolet detection. A filter is used on the intake tubing in the solvent reservoir to exclude >0.5-μm particles.

Purging with gas is called *sparging*.

Sample and solvent are passed through a short, expendable *guard column* (Figures 24-1 and 24-4) that has the same stationary phase as the analytical column and retains strongly adsorbed species. At the end of a reversed-phase separation, the column should be washed with 10–20 mobile phase volumes (V_m) of strong eluent to remove strongly retained solutes.[19] To clean the column after a series of runs, first replace aqueous buffer by water. For example, if the last eluent was acetonitrile-aqueous buffer (40:60 vol/vol), wash the column with 5–10 volumes (V_m) of acetonitrile-water (40:60 vol/vol). Then wash the column with 10–20 mobile phase volumes of strong solvent such as acetonitrile-water (95:5 vol/vol) and store the column with this solvent. This procedure is suitable for alkyl, aryl, cyano, and embedded polar-phase columns. A different procedure is recommended for normal-phase columns.[20]

V_m = volume of solvent in column between stationary phase particles and inside pores of particles.

If time for mobile phase or unretained solute to transit the column is t_m, and flow rate is F, then $V_m = t_m F$.

If you don't know t_m, you can estimate $V_m(\text{mL}) \approx \frac{1}{2} d_c^2 L$, where d_c = column inner diameter (cm) and L = column length (cm).

Normal-phase separations are sensitive to water in the solvent. To speed the equilibration of stationary phase with changing eluents, organic solvents for normal-phase chromatography should be 50% saturated with water. Saturation can be achieved by adding a few milliliters of water to dry solvent and stirring. Then the wet solvent is separated from the excess water and mixed with an equal volume of dry solvent.

For gradient elution in reversed-phase separations, 10–20 volumes (V_m) of initial solvent should be passed through the column after a run to equilibrate the stationary phase with solvent for the next run. Equilibration can take as long as the separation. By adding 3 vol% 1-propanol to each solvent (so that there is always 3% 1-propanol at any point in the gradient), you can reduce the re-equilibration volume down to $3V_m$.[21] Propanol is thought to coat the stationary phase with a monolayer of alcohol that does not change much throughout the gradient.

Reduce Waste Solvent Without Sacrificing Resolution

- Use shorter columns with smaller diameter particles.
- Switch from 4.6-mm-diameter column to 2.1 mm.
- For isocratic separations, use an electronic recycler that recycles eluate when no peak is being eluted.

Maintaining Symmetric Bandshape

HPLC columns should provide narrow, symmetric peaks. If a new column does not reproduce the quality of the separation of a standard mixture whose chromatogram comes with the column, and if you have satisfied yourself that the problem is not in the rest of your system, return the column.

A standard mixture should be injected each day to evaluate the HPLC system. Changes in peak shapes or retention times alert you to a problem.

The asymmetry factor *A*/*B* in Figure 22-14 should rarely be outside the range 0.9–1.5. Tailing of amines (Figure 24-7) might be eliminated by adding 30 mM triethylamine to the mobile phase. The additive binds to sites on silica that would otherwise strongly bind analyte. Tailing of acidic compounds might be eliminated by adding 30 mM ammonium acetate. For unknown mixtures, 30 mM triethylammonium acetate is useful. If tailing persists, 10 mM dimethyloctylamine or dimethyloctylammonium acetate might be effective. A problem with additives is that they increase the equilibration time required when changing solvents. Improved grades of silica (types B and C) have reduced tailing and, therefore, reduced the need for additives.

Tailing or splitting of peaks can occur if the frit at the beginning of the column becomes clogged with particles.[22] You can try to unclog the frit by disconnecting and reversing the column and flushing it with 20–30 mL. The column should not be connected to the detector during reverse flushing. If peaks remain distorted, replace the column.

Doubled peaks or altered retention times (Figure 24-16) sometimes occur if the solvent in which sample is dissolved has much greater eluent strength than the mobile phase. Try to dissolve the sample in a solvent of lower eluent strength or in the mobile phase itself.

Overloading causes the distorted shape shown in Figure 22-21.[23] To see if overloading is occurring, reduce the sample mass by a factor of 10 and see if retention times increase or peaks become narrower. If either change occurs, reduce the mass again until the injection size does not affect retention time and peak shape. In general, reversed-phase columns can handle 1–10 μg of sample per gram of silica. A 4.6-mm-diameter column contains 1 g of silica in a length of 10 cm. To prevent peak broadening from too large an injection volume, the injected volume should be less than 15% of the peak volume measured at the baseline. For example, if a peak eluted at 1 mL/min has a width of 0.2 min, the peak volume is 0.2 mL. The injection volume should not exceed 15% of 0.2 mL, or 30 μL.

The volume of a chromatography system outside of the column from the point of injection to the point of detection is called the **dead volume,** or the *extra-column volume*. Excessive dead volume allows bands to broaden by diffusion or mixing. Use short, narrow tubing whenever possible, and be sure that connections are made with matched fittings to minimize dead volume and thereby minimize extra-column band spreading.

HPLC columns have a typical lifetime of 500 to 2 000 injections.[7] You can monitor the health of a column by keeping a record of pressure, resolution, and peak shape. The pressure required to maintain a given flow rate increases as a column ages. System wear becomes serious when pressure exceeds 17 MPa (2 500 pounds/inch^2). It is desirable to develop methods in which pressure does not exceed 14 MPa (2 000 pounds/inch^2). When the pressure reaches 17 MPa, the in-line 0.5-μm frit between the autosampler and the column should be replaced. If this does not help, it is probably time for a new column. If you use a column for repetitive analyses, replace the column when the required resolution is lost or when tailing becomes significant. Resolution and tailing criteria should be established during method development.

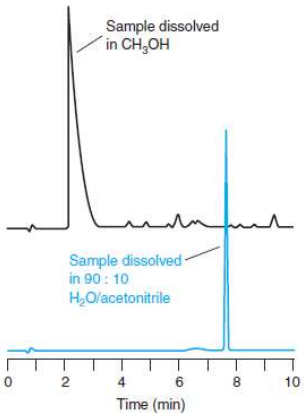

FIGURE 24-16 Effect of sample solvent on retention time and peak shape of *n*-butylaniline. Eluent (1 mL/min) is 90:10 (vol/vol) H_2O/acetonitrile with 0.1 wt% trifluoroacetic acid. Lower sample was dissolve in eluent. Upper sample was dissolved in methanol, which is a much stronger solvent than eluent. Column: 15 cm × 4.6 mm, 5-μm-diameter C_{18}-silica, 30°C. Injection: 10 μL containing 0.5 μg analyte. Ultraviolet detection at 254 nm. [Courtesy Supelco, Bellefonte, PA.]

24-2 Injection and Detection in HPLC

We now consider the hardware required to inject sample and solvent onto the column and to detect compounds as they leave the column. Mass spectrometric detection, which is extremely powerful and important, was discussed in Section 21-4.

Pumps and Injection Valves

The quality of a pump for HPLC is measured by how steady and reproducible a flow it can produce. A fluctuating flow rate can create detector noise that obscures weak signals. Figure 24-17 shows a pump with two sapphire pistons that produce a programmable, constant flow rate up to 10 mL/min at pressures up to 40 MPa (400 bar, 6 000 pounds/inch^2). Gradients made from up to four solvents are constructed by proportioning the liquids through a four-way valve at low pressure and then pumping the mixture at high pressure into the column. The gradient is electronically controlled and programmable in 0.1 vol% increments.

The *injection valve* in Figure 24-18 has interchangeable sample loops, each of which holds a fixed volume. Loops of different sizes hold volumes that range from 2 to 1 000 μL. In the load position, a syringe is used to wash and load the loop with fresh sample at atmospheric pressure. High-pressure flow from the pump to the column passes through the lower left segment of the valve. When the valve is rotated 60° counterclockwise, the content of the sample loop is injected into the column at high pressure.

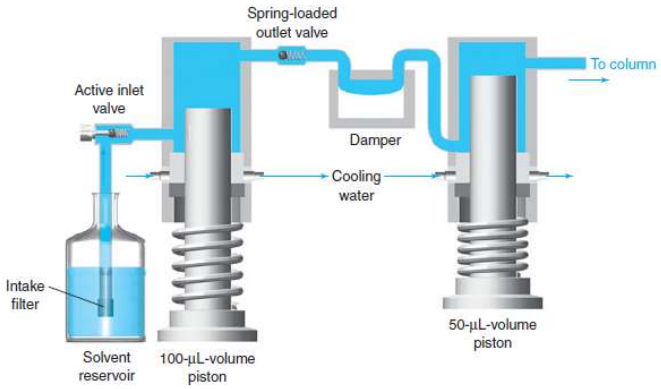

FIGURE 24-17 High-pressure piston pump for HPLC. Solvent at the left passes through an electronic inlet valve synchronized with the large piston and designed to minimize the formation of solvent vapor bubbles during the intake stroke. The spring-loaded outlet valve maintains a constant outlet pressure, and the damper further reduces pressure surges. Pressure surges from the first piston are decreased in the damper that "breathes" against a constant outside pressure. Pressure surges are typically <1% of the operating pressure. As the large piston draws in liquid, the small piston propels liquid to the column. During the return stroke of the small piston, the large piston delivers solvent into the expanding chamber of the small piston. Part of the solvent fills the chamber while the remainder flows to the column. Delivery rate is controlled by the stroke volumes. [Adapted from Hewlett-Packard Co., Palo Alto, CA.]

- Pass samples through a 0.5-μm filter to remove particles prior to injection.
- The HPLC syringe needle is *blunt*, not pointed, to prevent damage to the injection port.

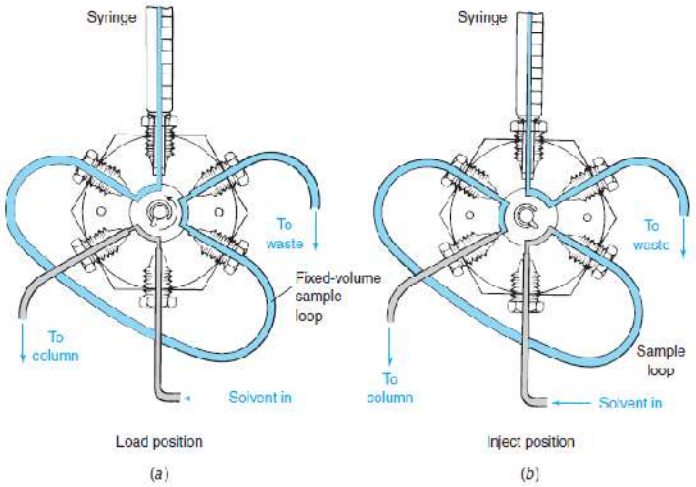

FIGURE 24-18 Injection valve for HPLC.

Linear range: analyte concentration range over which detector response is proportional to concentration

Dynamic range: range over which detector responds in any manner (not necessarily linearly) to changes in analyte concentration (see page 89)

Detection limit: concentration of analyte that gives a specified signal-to-noise ratio

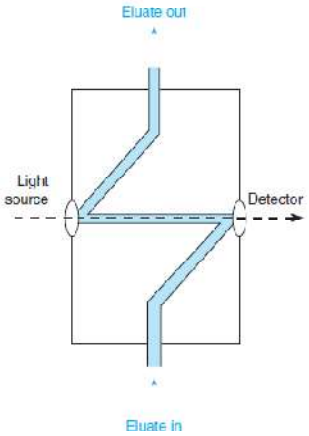

FIGURE 24-19 Light path in a spectrophotometric micro flow cell. One common cell with a pathlength of 1 cm has a volume of 8 μL. Another cell with a 0.5-cm pathlength contains only 2.5 μL. In the future, it should be possible to lower detection limits by 1–2 orders of magnitude by placing mirrors on both ends of the cavity. Light transits the cell multiple times, with ~1% of the power leaking through the mirror to the detector on each pass.[24]

Spectrophotometric Detectors

An ideal detector of any type (Table 24-3) is sensitive to low concentrations of every analyte, provides linear response, and does not broaden the eluted peaks. It is also insensitive to changes in temperature and solvent composition. To prevent peak broadening, the detector volume should be less than 20% of the volume of the chromatographic band. Gas bubbles in the detector create noise, so back pressure may be applied to the detector to prevent bubble formation during depressurization of eluate.

An **ultraviolet detector** using a flow cell such as that in Figure 24-19 is the most common HPLC detector, because many solutes absorb ultraviolet light. Simple systems employ the intense 254-nm emission of a mercury vapor lamp or other discrete ultraviolet wavelengths from zinc or cadmium vapor lamps. More versatile instruments have broadband deuterium, xenon, or tungsten lamps and a monochromator, so you can choose an optimum wavelength for your analytes. The system in Figure 24-20 uses a *photodiode array* to record the spectrum of each solute as it is eluted. High-quality detectors provide full-scale absorbance ranges from 0.000 5 to 3 absorbance units, with a noise level near 1% of full scale. The linear range extends over five orders of magnitude of solute concentration (which is another way of saying that Beer's law is obeyed over this range). Ultraviolet detectors are

TABLE 24-3 Comparison of commercial HPLC detectors

Detector	Approximate limit of detection[a] (ng)	Useful with gradient?
Ultraviolet	0.1–1	Yes
Refractive index	100–1 000	No
Evaporative light-scattering	0.1–1	Yes
Charged aerosol	1	Yes
Electrochemical	0.01–1	No
Fluorescence	0.001–0.01	Yes
Nitrogen ($N \xrightarrow{\text{combustion}} NO \xrightarrow{O_3} NO_2^* \rightarrow h\nu$)	0.3	Yes
Conductivity	0.5–1	No
Mass spectrometry	0.1–1	Yes
Fourier transform infrared	1 000	Yes

*a. Most detection limits from E. W. Yeung and R. E. Synovec, "Detectors for Liquid Chromatography," Anal. Chem. **1986**, 58, 1237A. Detection limit for charged aerosol detector from T. Górecki, F. Lynen, P. Szucs, and P. Sandra, "Universal Response in Liquid Chromatography Using Charged Aerosol Detection," Anal. Chem. **2006**, 78, 3186.*

Optimization with Two or Three Organic Solvents

Figures 24-24 and 24-25 illustrate a systematic process to develop a separation with combinations of solvents. Method development is finished as soon as the separation meets your criteria. There is a good chance of attaining adequate separation without going through all the steps.

Step 1 Optimize the separation with acetonitrile/buffer to generate chromatogram A in Figure 24-25.
Step 2 Optimize the separation with methanol/buffer to generate chromatogram B.
Step 3 Optimize the separation with tetrahydrofuran/buffer to generate chromatogram C.
Step 4 Mix the solvents used in A, B, and C, one pair at a time, in 1:1 proportion, to generate chromatograms D, E, and F.
Step 5 Construct a 1:1:1 mixture of the solvents for A, B, and C to generate chromatogram G.
Step 6 If some of the results A through G are almost good enough, select the two best points and mix the solvents to obtain points between those two.

If the separation does not look promising after step 5, try a different stationary phase or a different form of chromatography.

Let's examine Figure 24-25 to see how the systematic procedure works. Step 1 is to generate chromatogram A by varying the proportions of acetonitrile and aqueous buffer (as in Figure 24-12) to obtain the best separation within the constraint that $0.5 \leq k \leq 20$. At the best composition, 30 vol% acetonitrile/70 vol% buffer, peaks 4 and 5 are not resolved adequately for quantitative analysis.

In HPLC, lowering the flow rate usually improves resolution. With the flow rate of 1.0 mL/min in Figure 24-25, we chose to keep $k < 10$ (instead of <20) to obtain a run time below ~25 min. In chromatogram A, $k = 1.1$ for peak 1 and $k = 8.1$ for peak 7. If the flow rate had been 2.0 mL/min, then $k < 20$ would give a run time below ~25 min.

Step 2 seeks a methanol/buffer solvent to obtain the best separation at point B. It is not necessary to start all over again with 90% methanol. Figure 24-26 allows us to select a methanol/water mixture that has approximately the same eluent strength as a particular acetonitrile/water mixture. A vertical line drawn at 30% acetonitrile (the composition used in chromatogram A) intersects the methanol line near 40%. Therefore, 40% methanol has about the same eluent strength as 30% acetonitrile. The first experiment carried out to establish point B in Figure 24-25 utilized 40% methanol. A little trial-and-error (with 45% methanol and 35% methanol) demonstrated that 40% methanol gave the best separation, but the separation is poor. In chromatogram B in Figure 24-25, the seven components give only five peaks. When we changed from acetonitrile to methanol, the order of elution of some compounds changed.

Step 3 generates chromatogram C in Figure 24-25, using tetrahydrofuran. Figure 24-26 tells us that 22% tetrahydrofuran has the same eluent strength as 30% acetonitrile. When 22% tetrahydrofuran was tried, elution times were too long. Trial-and-error demonstrated that 32% tetrahydrofuran was best. All seven compounds are cleanly separated in chromatogram C in an acceptable time. However, a baseline dip associated with the solvent front between peaks 3 and 1 interferes with quantitative analysis of compound 1. The order of elution with tetrahydrofuran is quite different from the order with acetonitrile. In general, changing solvent is a powerful way to change relative retention of different compounds.

Step 4 generates chromatograms D, E, and F. The composition at D is a 1:1 mixture of the solvents used in A and B. Because we used 30% acetonitrile at A and 40% methanol at B,

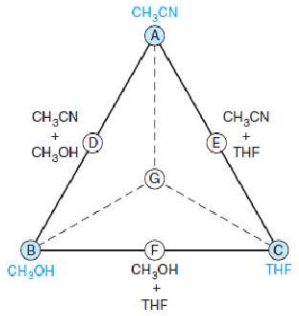

FIGURE 24-24 HPLC method development triangle. THF stands for tetrahydrofuran. Figure 24-25 shows how the procedure is applied to a real chromatographic separation.

The way we know which peak is which is to record the entire ultraviolet spectrum of each peak as it is eluted, using a photodiode array spectrometer.