Eigth Week

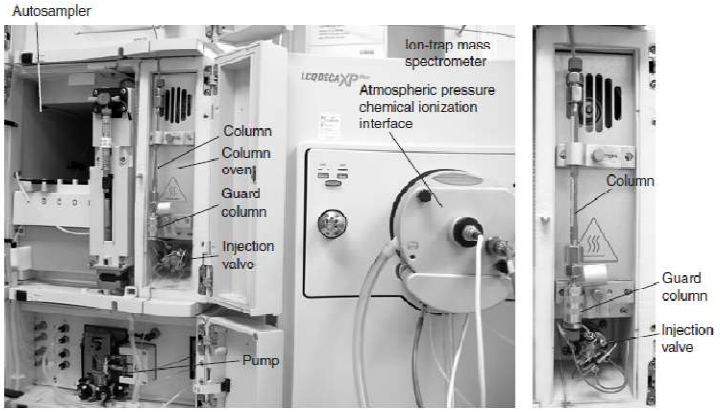


FIGURE 24-1 High-performance liquid chromatography (HPLC) equipment including a mass spectrometer for detection. The column is enlarged at the right. In operation, the door to the column oven would be closed to maintain constant temperature. [Courtesy E. Erickson, Michelson Laboratory, China Lake, CA.]

The pioneer of high-performance liquid chromatography was C. Horváth at Yale University in 1965.

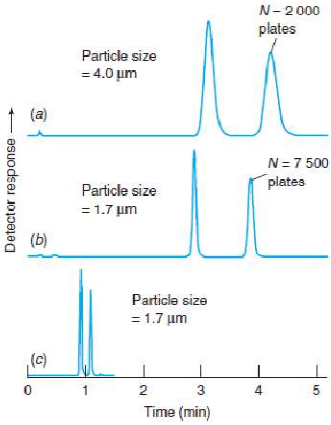


FIGURE 24-2 (*a* and *b*) Chromatograms of the same sample run at the same linear velocity on 5.0-cm-long columns packed with C_{18}-silica. (*c*) A stronger solvent was used to elute solutes more rapidly from the column in panel *b*. [From Y. Yang and C. C. Hodges, "Assay Transfer from HPLC to UPLC for Higher Analysis Throughput," *LCGC Supplement*, May 2005, p. 31.]

High-performance liquid chromatography (HPLC) uses high pressure to force solvent through closed columns containing fine particles that give high-resolution separations.[2–6] The HPLC system in Figure 24-1 consists of an autosampler, a solvent delivery system, a sample injection valve, a high-pressure chromatography column, and a mass spectrometer, which serves as a detector. Not shown in the photograph are solvent reservoirs, a photodiode array absorbance detector, and a computer to control the hardware and display results. The column is housed in an oven whose door is normally closed to keep the column at constant temperature. In this chapter, we discuss liquid-liquid partition and liquid-solid adsorption chromatography. Chapter 25 deals with ion-exchange, molecular exclusion, affinity, and hydrophobic interaction chromatography.

Chromatographers generally choose gas chromatography over liquid chromatography when there is a choice, because gas chromatography is normally less expensive and generates much less waste. Liquid chromatography is important because most compounds are not sufficiently volatile for gas chromatography.

24-1 The Chromatographic Process

Increasing the rate at which solute equilibrates between stationary and mobile phases increases the efficiency of chromatography. For gas chromatography with an open tubular column, rapid equilibration is accomplished by reducing the diameter of the column so that molecules can diffuse quickly between the channel and the stationary phase on the wall. Diffusion in liquids is 100 times slower than diffusion in gases. Therefore, in liquid chromatography, it is not generally feasible to use open tubular columns, because the diameter of the solvent channel is too great to be traversed by a solute molecule in a short time. Liquid chromatography is conducted with packed columns so that a solute molecule does not have to diffuse very far to encounter the stationary phase.

Small Particles Give High Efficiency but Require High Pressure

The efficiency of a packed column increases as the size of the stationary phase particles decreases. Typical particle sizes in HPLC are 1.7 to 5 µm. Figure 24-2a and b illustrate the increased resolution afforded by decreasing particle size. Plate number increased from 2 000 to 7 500 when the particle size decreased, so the peaks are sharper with the smaller particle size. In Figure 24-2c, a stronger solvent was used to elute the peaks in less time. Decreasing particle size permits us to improve resolution or to maintain the same resolution while decreasing run time.

EXAMPLE Scaling Relations Between Columns

Commonly, silica particles occupy ~40% of the column volume and solvent occupies ~60% of the column volume, regardless of particle size. The column used in Figure 24-2a has an inside diameter of 4.6 mm and was run at a volume flow rate (u_v) of 3.0 mL/min with a

sample size of 20 μL. The column used in Figure 24-2b has a diameter of d_c = 2.1 mm. What flow rate should be used in trace *b* to achieve the same linear velocity (u_x) as in trace *a*? What sample volume should be injected?

Solution Column volume is proportional to the square of column diameter. Changing the diameter from 4.6 to 2.1 mm, reduces volume by a factor of $(2.1/4.6)^2 = 0.208$. Therefore, u_v should be reduced by a factor of 0.208 to maintain the same linear velocity.

$$u_v(\text{small column}) = 0.208 \times u_v(\text{large column}) = (0.208)(3.0\ \text{mL/min}) = 0.62\ \text{mL/min}$$

To maintain the same ratio of injected sample to column volume,

$$\text{Injection volume in small column} = 0.208 \times (\text{injection volume in large column})$$
$$= (0.208)(20\ \mu\text{L}) = 4.2\ \mu\text{L}$$

Test Yourself What should be the volume flow rate and injected volume for a 1.5-mm-diameter column? (*Answer:* 0.32 mL/min, 2.1 μL)

van Deemter plots of plate height versus linear flow rate in Figure 24-3 show that small particles reduce plate height and that plate height is not very sensitive to increased flow rate when the particles are small. At the optimum flow rate for each column (minimum plate height in Figure 24-3), the number of theoretical plates in a column of length L (cm) is *approximately*[7]

$$N \approx \frac{3\,000\,L\ (\text{cm})}{d_p\ (\mu\text{m})} \qquad (24\text{-}1)$$

where d_p is the particle diameter in μm. The 5.0-cm-long column in Figure 24-2a with 4.0-μm-diameter particles is predicted to provide ~(3 000)(5.0)/4.0 = 3 800 plates. The observed plate number for the second peak is 2 000. Perhaps the column was not run at optimum flow rate. When the stationary phase particle diameter is reduced to 1.7 μm, the optimum plate number is expected to be ~(3 000)(5.0)/1.7 = 8 800. The observed value is 7 500.

One reason why small particles give better resolution is that they provide more uniform flow through the column, thereby reducing the multiple path term, A, in the van Deemter equation (22-33). A second reason is that the distance through which solute must diffuse in the mobile and stationary phases is on the order of the particle size. The smaller the particles, the less distance solute must diffuse. This effect decreases the C term in the van Deemter equation for finite equilibration time. Optimum flow rate for small particles is faster than for large particles because solutes diffuse through smaller distances.

Increasing efficiency is equivalent to decreasing plate height, H, in the van Deemter equation (22-33):

$$H \approx A + \frac{B}{u_x} + Cu_x$$

u_x = linear flow rate

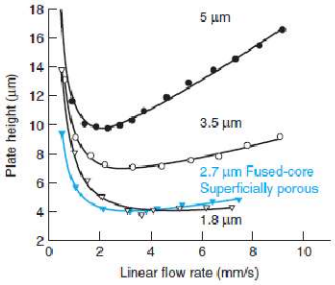


FIGURE 24-3 van Deemter curves: Plate height as a function of *linear flow rate* (mm/s) for microporous (Figure 24-5) stationary phase particle diameters of 5.0, 3.5, and 1.8 μm, as well as *superficially porous* particles (Figure 24-9) with a diameter of 2.7 μm (0.5 μm porous layer thickness). Measurements for naphthalene eluted from C_{18}-silica (50 mm long × 4.6 mm diameter) with 60 vol% acetonitrile/40 vol% H_2O at 24°C. [Courtesy MAC-MOD Analytical, Chadds Ford, PA.]

Smaller particle size leads to
- higher plate number
- higher pressure
- shorter optimum run time
- lower detection limit

An added benefit of small particle size, coupled with a narrow column and higher flow is that analyte is not diluted so much as it travels through the column. The limit of quantitation for conditions in Figure 24-2c (50 μg/L) is four times lower than the limit of quantitation in Figure 24-2a (200 μg/L).

The penalty for small particle size is resistance to solvent flow. The pressure required to drive solvent through a column is

Column pressure:
$$P = f\frac{u_x \eta L}{\pi r^2 d_p^2} \qquad (24\text{-}2)$$

Viscosity measures resistance of a fluid to flow. The more viscous a liquid, the slower it flows at a given pressure.

where u_x is linear flow rate, η is the viscosity of the solvent, L is the length of the column, r is column radius, and d_p is the particle diameter. The factor f depends on particle shape and particle packing. The physical significance of Equation 24-2 is that pressure in HPLC is proportional to flow rate and column length and inversely proportional to the square of column radius (or diameter) and the square of particle size. The difference between traces *a* and *b* in Figure 24-2 is that particle size was decreased from 4.0 μm to 1.7 μm and column diameter was decreased from 4.6 mm to 2.1 mm. Therefore, the required pressure increases by a factor of $(4.6\text{ mm}/2.1\text{ mm})^2(4.0\ \mu\text{m}/1.7\ \mu\text{m})^2 = 27$. That is, 27 *times more pressure* is required to operate the column in Figure 24-2b.

Analogy of fluid flow with electric current:

Electric power (W) = current × voltage

Chromatographic heat generation:

Power (W) = volume flow rate (m^3/s) × pressure drop (Pa = $kg/(m \cdot s^2)$)

Until recently, HPLC operated at pressures of ~7–40 MPa (70–400 bar, 1 000–6 000 pounds/inch^2) to attain flow rates of ~0.5–5 mL/min. In 2004, commercial equipment became available to employ 1.5- to 2-μm-diameter particles at pressures up to 100 MPa (1 000 bar, 15 000 pounds/inch^2). These instruments provide substantially increased resolution or decreased run time. Table 24-1 shows theoretical performance for different particle sizes; such performance was realized in research with ultrahigh-pressure equipment. Chromatography with 1.5- to 2-μm-diameter particles at high pressure is commonly called UPLC (Ultra Performance Liquid Chromatography), which is a trademark of Waters Corporation. Peaks eluted from a UPLC column could be so narrow that there is not enough time for mass spectral detection.

Another penalty of small particle size is increased frictional heating as solvent is forced through the particle bed.[8] The center of a column is warmer than the outer wall, and the outlet is warmer than the inlet. A 100-mm-long × 2.1-mm-diameter column containing 1.7-μm particles eluted with acetonitrile generates a temperature difference of ~10°C from the inlet to the outlet at a flow rate of 1.0 mL/min. The centerline of the column can be ~2°C warmer than the wall. To avoid undue band broadening from temperature differences, column diameter should be ≤2.1 mm for 1.7-μm particles.

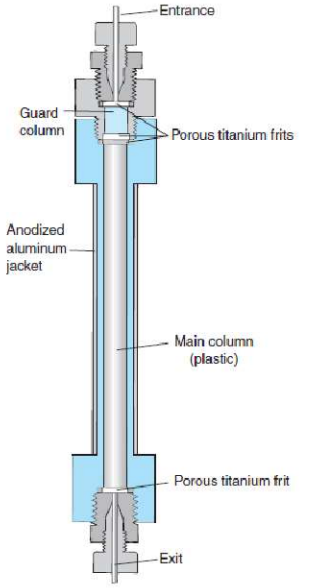


FIGURE 24-4 HPLC column with replaceable guard column to collect irreversibly adsorbed impurities. Titanium frits contain the stationary phase and distribute liquid evenly over the diameter of the column. [Courtesy Upchurch Scientific, Oak Harbor, WA.] In Figure 24-1, flow direction is bottom to top, which is opposite the flow direction in this figure.

The Column

Columns are expensive and easily degraded by dust or particles in the sample or solvent and by irreversible adsorption of impurities from the sample or solvent. To avoid introducing particulate matter into the column, samples should be centrifuged and/or filtered through a 0.5-μm filter before they are loaded into vials for an autosampler or taken into a syringe for manual injection. An in-line 0.5-μm filter should be installed immediately downstream of the autosampler.

The HPLC equipment in Figure 24-1 uses steel or plastic columns that are 5–30 cm in length, with an inner diameter of 1–5 mm (Figure 24-4). The entrance to the main column is protected by a short **guard column** containing the same stationary phase as the main column.

TABLE 24-1 Performance as a function of particle diameter

Particle size d_p (μm)	Retention time (min)	Plate number (N)	Required pressure (bar)
5.0	30	25 000	19
3.0	18	42 000	87
1.5	9	83 000	700
1.0	6	125 000	2 300

NOTE: Theoretical performance of 33-μm-diameter × 25-cm-long capillary for minimum plate height for solute with retention factor $k = 2$ and diffusion coefficient $= 6.7 \times 10^{-10}$ m^2/s in water-acetonitrile eluent.

SOURCE: J. E. MacNair, K. D. Patel, and J. W. Jorgenson, "Ultrahigh-Pressure Reversed-Phase Capillary Liquid Chromatography with 1.0-μm Particles," Anal. Chem. 1999, 71, 700.

Fine particles and strongly adsorbed solutes are retained in the guard column, which is periodically replaced when column pressure increases or after a set number of injections or time in service. While guard columns make sense with 10- to 30-cm-long chromatography columns, many people do not consider a guard column to be cost effective for a 5-cm column.

Heating a chromatography column[9] usually decreases the viscosity of the solvent, thereby reducing the required pressure or permitting faster flow. Increased temperature decreases retention times (Figure 22-19) and improves resolution by hastening diffusion of solutes. However, increased temperature can degrade the stationary phase and decrease column lifetime. When column temperature is not controlled, it fluctuates with the ambient temperature. Using a column heater set 10°C above room temperature improves the reproducibility of retention times and the precision of quantitative analysis. Some chromatographers routinely conduct all separations at 50° or 60°C. For a heated column, the mobile phase should be passed through a preheating metal coil between the injector and the column so that the solvent and column are at the same temperature. If their temperatures are different, peaks become distorted and retention times change.

In the recent past, the most common HPLC column diameter was 4.6 mm. Now, 2.1 mm is becoming most common. The narrow column is more compatible with mass spectrometry, which requires low solvent flow. The narrow column requires less sample and produces less waste. Instruments that used the 4.6-mm column also operate with the 2.1-mm column. Columns narrower than 2.1 mm require specially designed instruments to reduce band broadening outside the column. Capillary columns as narrow as 25 μm can be used.

The Stationary Phase

The most common support is highly pure, spherical, **microporous particles** of silica (Figure 24-5) that are permeable to solvent and have a surface area of several hundred square meters per gram. Most silica cannot be used above pH 8, because it dissolves in base. Figure 24-6 shows the structure of ordinary silica and silica with ethylene bridges, which resist hydrolysis up to pH 12.[10] For separation of basic compounds at pH 8–12, ethylene-bridged silica or polymeric supports such as polystyrene (Figure 25-1) can be used. Stationary phase is covalently attached to the polymer.

A silica surface (Figure 24-6) has up to 8 μmol of silanol groups (Si—OH) per square meter. Silanol groups are protonated at pH ~2–3. They dissociate to negative Si—O^- over a

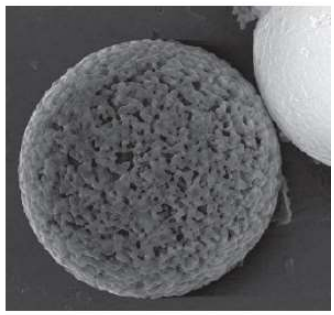


FIGURE 24-5 Scanning electron micrograph of 4.4-μm-diameter microporous silica chromatography particle from an experimental batch made by K. Wyndham at Waters Corporation. [Photo kindly provided by J. Jorgensen, University of North Carolina.]

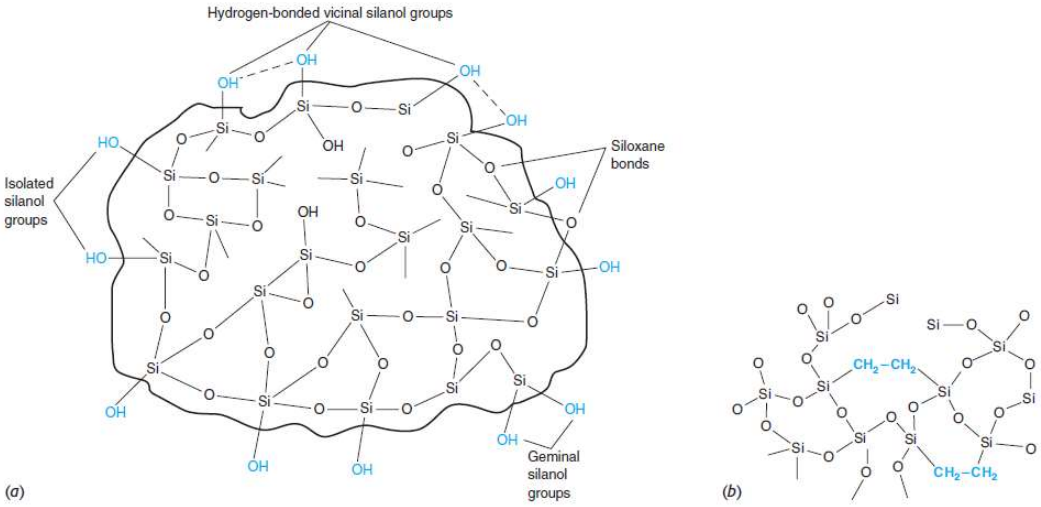


FIGURE 24-6 (*a*) Schematic structure of silica particle. [From R. E. Majors, *LCGC*, May 1997, p. 58.] (*b*) Base-hydrolysis-resistant silica incorporates ethylene bridges in place of oxide bridges between some silicon atoms. Ethylene-bridged structure is more rigid and well suited for particles that are <2 μm in diameter, which must withstand high pressure.

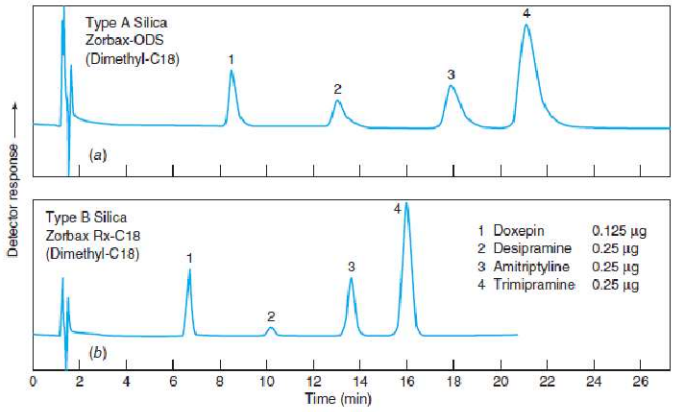
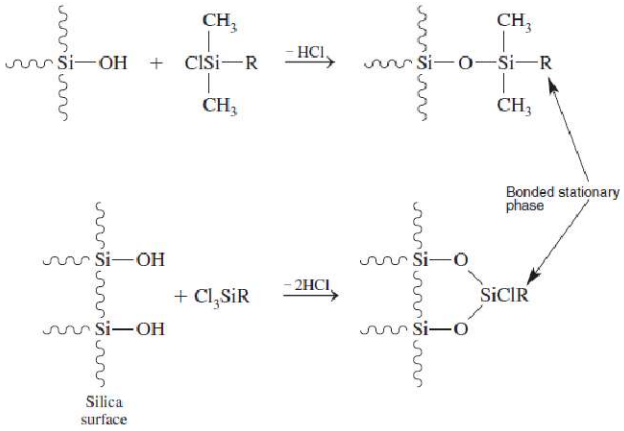


FIGURE 24-7 Tailing of amine bases on silica: (*a*) Type A silica support gives distorted peaks. (*b*) Less acidic Type B silica with fewer Si—OH groups and less metallic impurity gives symmetric peaks with shorter retention time. In both cases, chromatography was performed with a 0.46 × 15 cm column eluted at 1.0 mL/min at 40°C with 30 vol% acetonitrile/70 vol% sodium phosphate buffer (pH 2.5) containing 0.2 wt% triethylamine and 0.2 wt% trifluoroacetic acid. The detector measured ultraviolet absorbance at 254 nm. Additives such as triethylamine and trifluoroacetic acid are often used to mask strong adsorption sites and thereby reduce tailing. [From J. J. Kirkland, *Am. Lab.*, June 1994, p. 28K.]

broad pH range above 3. In the old Type A silica, exposed Si—O^- groups strongly retain protonated bases (for example, RNH_3^+) and lead to tailing (Figure 24-7). Metallic impurities in Type A silica also cause tailing. Type B silica in Figure 24-7, which has fewer exposed silanol groups and fewer metallic impurities, is the most common form used today. Type C silica causes even less tailing because 90% of the Si—OH groups are replaced by Si—H bonds, which do not retain solutes by hydrogen bonding.

Bare silica can be used as the stationary phase for adsorption chromatography. Most commonly, liquid-liquid partition chromatography is conducted with a **bonded stationary phase** covalently attached to the silica surface by reactions such as

Residual silanol groups on the silica surface are capped with trimethylsilyl groups by reaction with $ClSi(CH_3)_3$ to eliminate polar adsorption sites that cause tailing.



Common polar phases		Common nonpolar phases	
R = $(CH_2)_3NH_2$	amino	R = $(CH_2)_{17}CH_3$	octadecyl
R = $(CH_2)_3C{\equiv}N$	cyano	R = $(CH_2)_7CH_3$	octyl
R = $(CH_2)_2OCH_2CH(OH)CH_2OH$	diol	R = $(CH_2)_3C_6H_5$	phenyl
		R = $(CH_2)_3C_6F_5$	F_5-phenyl

There are ~4 µmol of R groups per square meter of support surface area, with little bleeding of the stationary phase from the column during chromatography.

The octadecyl (C_{18}) stationary phase (often abbreviated ODS) is by far the most common in HPLC. Retention factors for a given solute on columns with the same nominal stationary phase from different manufacturers are quite variable. Different surface areas of different columns account for much of the variation.[11] The nonpolar perfluorophenyl (F_5-phenyl)