High Performance Liquid Chromatography (HPLC)
Harris Chapter 25

HPLC – separation of nonvolatile or thermally unstable compounds. If the analyte/sample can be found to be sufficiently soluble in a solvent system, then that system can usually to used as the m.p. in an HPLC separation.

Common method used for analysis of

Biological compounds
Pharmaceuticals
Low- or Non-volatile environmental cpds. e.g. PCB, DDT

LC Origins.

Michael Tswett (1906) separation of plant pigments by organic solvent mobile phase & chalk stationary phase.

Martin and Synge (1941) liquid-liquid partition chromatography, 1952 Nobel Prize in chemistry.

Other variants – Paper chromatography
Thin-layer chromatography (TLC)
Preparative column chromatography
Medium pressure chromatography
Ion-exchange chromatography*
Size-exclusion chromatography*

HPLC components:

Liquid
Mobile => Pump => Injection => Separation => Detector
Phase Valve Column

Also an integrator usually records the detector response.

We discuss each component, but let's first discuss the band broadening aspects of LC. This discussion tells us why high pressures are required for analytical separations.

Band Broadening in LC

Back to the van Deemter Equation,

$$H = A + B/u + Cu$$

Which of the three components is the largest contribution to H? Consider the following:

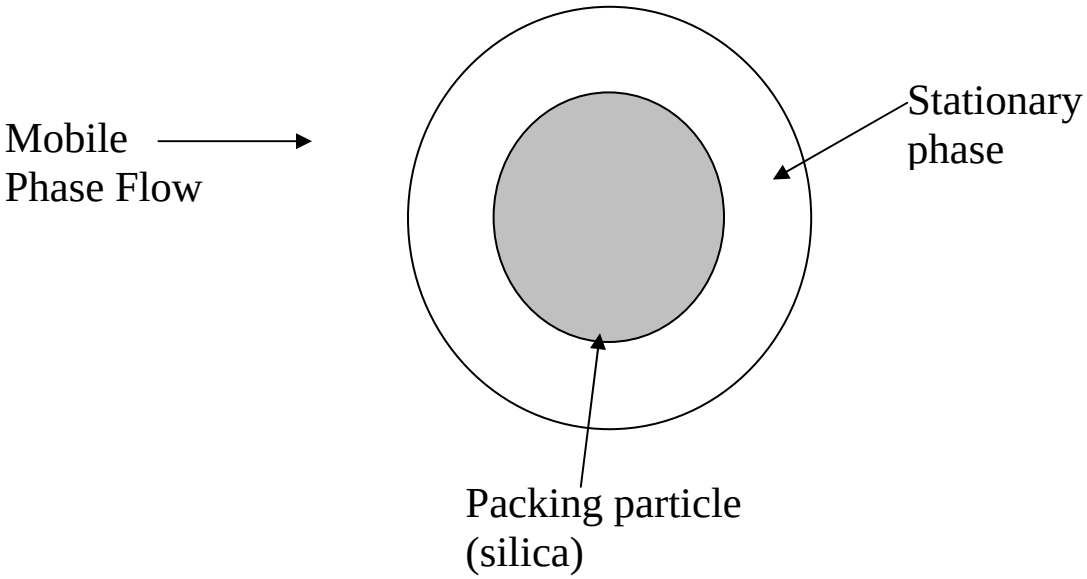
<u>B/u effects</u> – Diffusion is usually 100x less in liquids than in the gas phse.

<u>Cu effects</u> – By process of elimination we will assume that mass transport effects are the largest contribution to H in LC.

Review of MT effects

MT in the m.p. $C_m \ \alpha \ \frac{f(k')d_p^2}{D_m}$

Where d_p is the diameter of the packing particle in LC,



Smaller d_p increases the surface area/volume ratio and thus increases M.T. in the m.p.

Volume = $4/3\ \pi\ r^3$ Surface Area = $4\ \pi\ r^2$

s.a./vol. = $1/3 \times r$

The effect is dramatic in figures 25-2 & 25-3

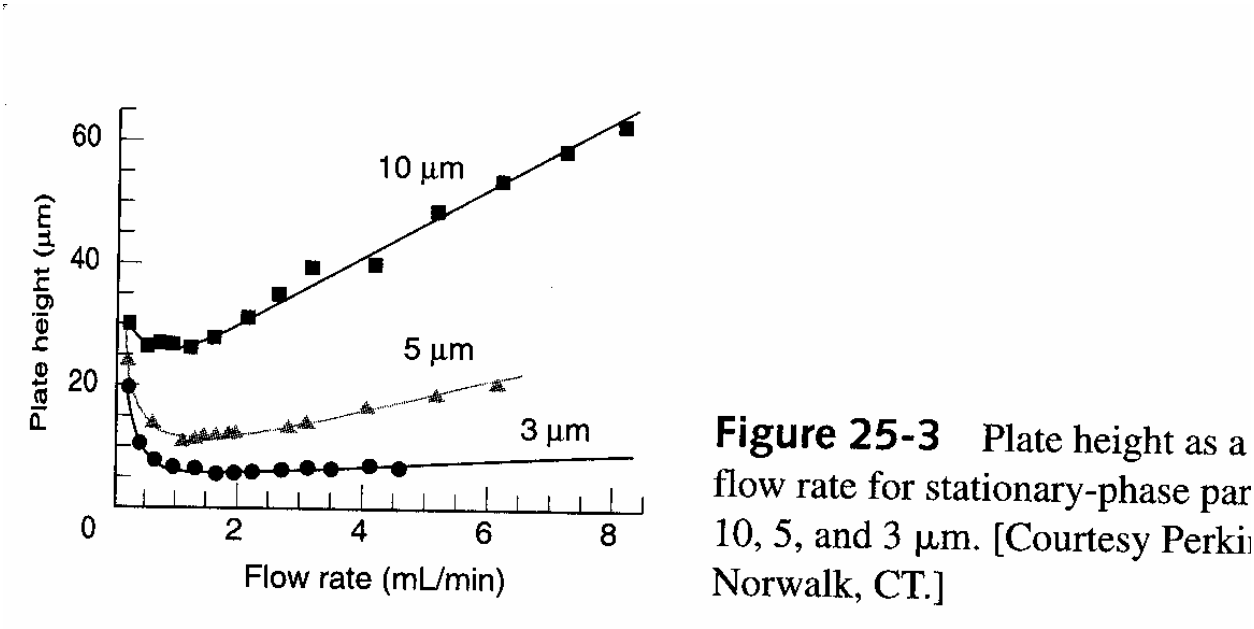


Figure 25-3 Plate height as a flow rate for stationary-phase par 10, 5, and 3 μm. [Courtesy Perki Norwalk, CT.]

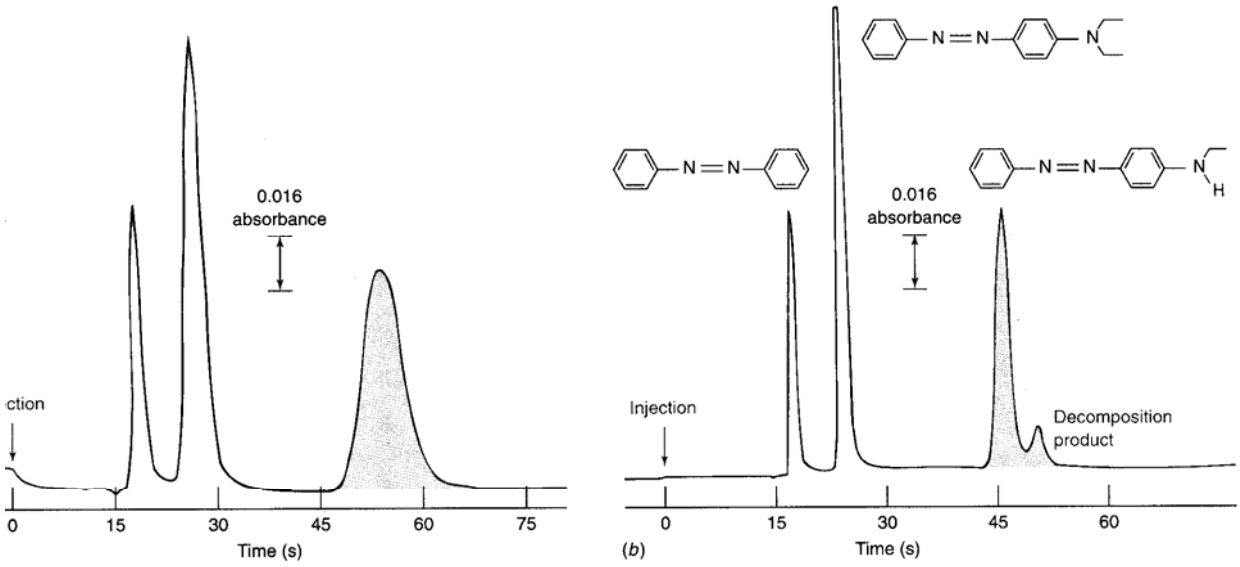


Figure 25-2 Chromatograms of the same sample run on columns packed with (*a*) 10-μm and (*b*) 5-μm particle diameter silica. [From R. E. Majors, *J. Chromatogr. Sci.* **1973**, *11*, 88.]

The cost of small packing particles is that the pressure required to force liquid through the column follows as:

$$\Delta P \alpha 1/d_p^3$$

The typical particle sizes in HPLC is 3-10 μm. In order to achieve flow rates of 0.5 to 5 mL/min, for a 10-30 cm column, pressures of 70 to 400 atm (1000 to 6000 psi) are required.

HPLC pumps

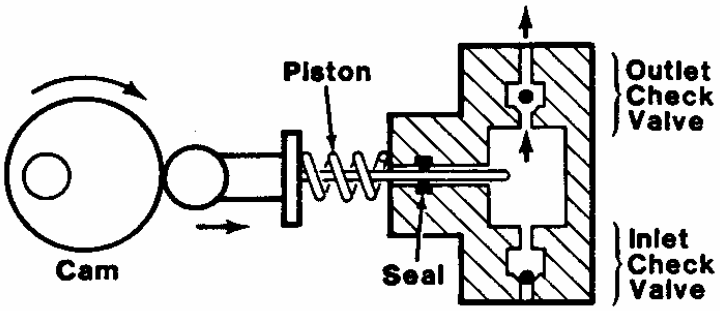
Requirements for HPLC

- pressures to 6000 psi
- pulse free, prevents remixing of solutes
- control flow rate from 0.1 to 10 mL/min

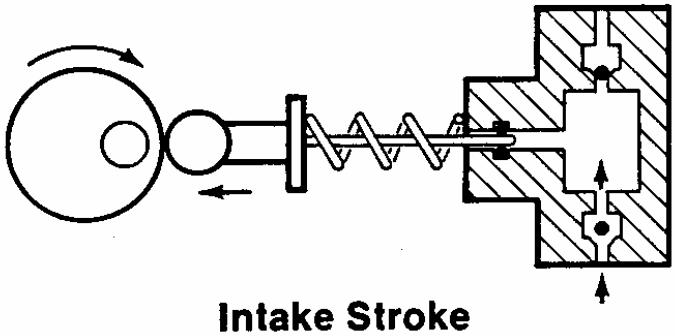
Types of HPLC pumps

1. Reciprocating pumps

most commercial systems are based on this design.



Compression Stroke



Intake Stroke

Disadvantages – pulses from single piston. See dual piston design in figure 25-14 of your text.

2. Syringe pumps

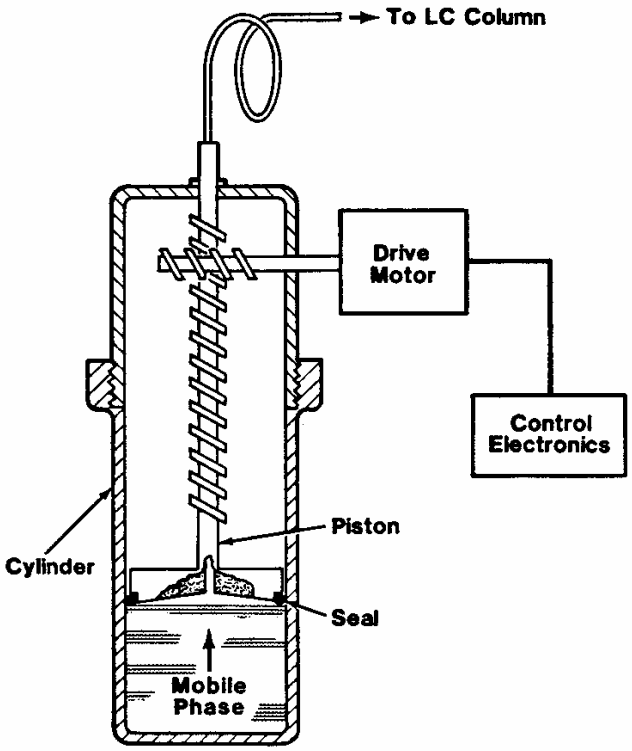


Fig. 26.6 Cross section of a syringe pump. The piston drive motor forces the piston down into the reservoir cylinder by means of a screw drive. The mobile phase is forced out through a channel up the center of the screw gear.

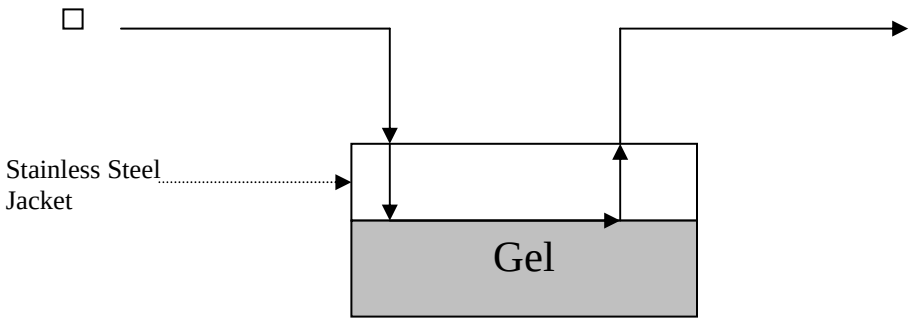
Pulse-free output, limited mobile phase capacity.

Pulse Dampers. Evens out flow from reciprocating pumps.

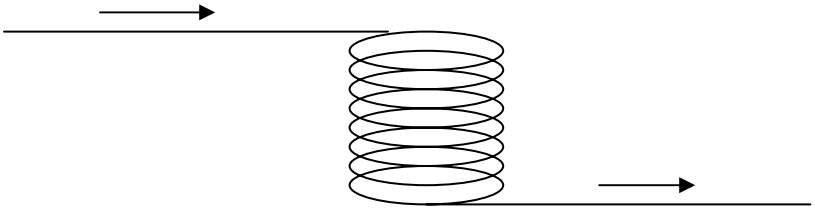
Mobile => Reciprocating => *Pulse* => Injection => Column….
Phase Pump *Damper* Valve

Two common types of dampers – coil & diaphragm.

Diaphragm:



Coil – 3 to 20 meters in length:



Injection (Sampling) Valves

Introduces sample to the column.

Mobile => Pump => *Injection* => Column
Phase *Valve*

Valve consists of a rotor and stator (stationary back-plane). See schematics below and figure 25-15 of your text.

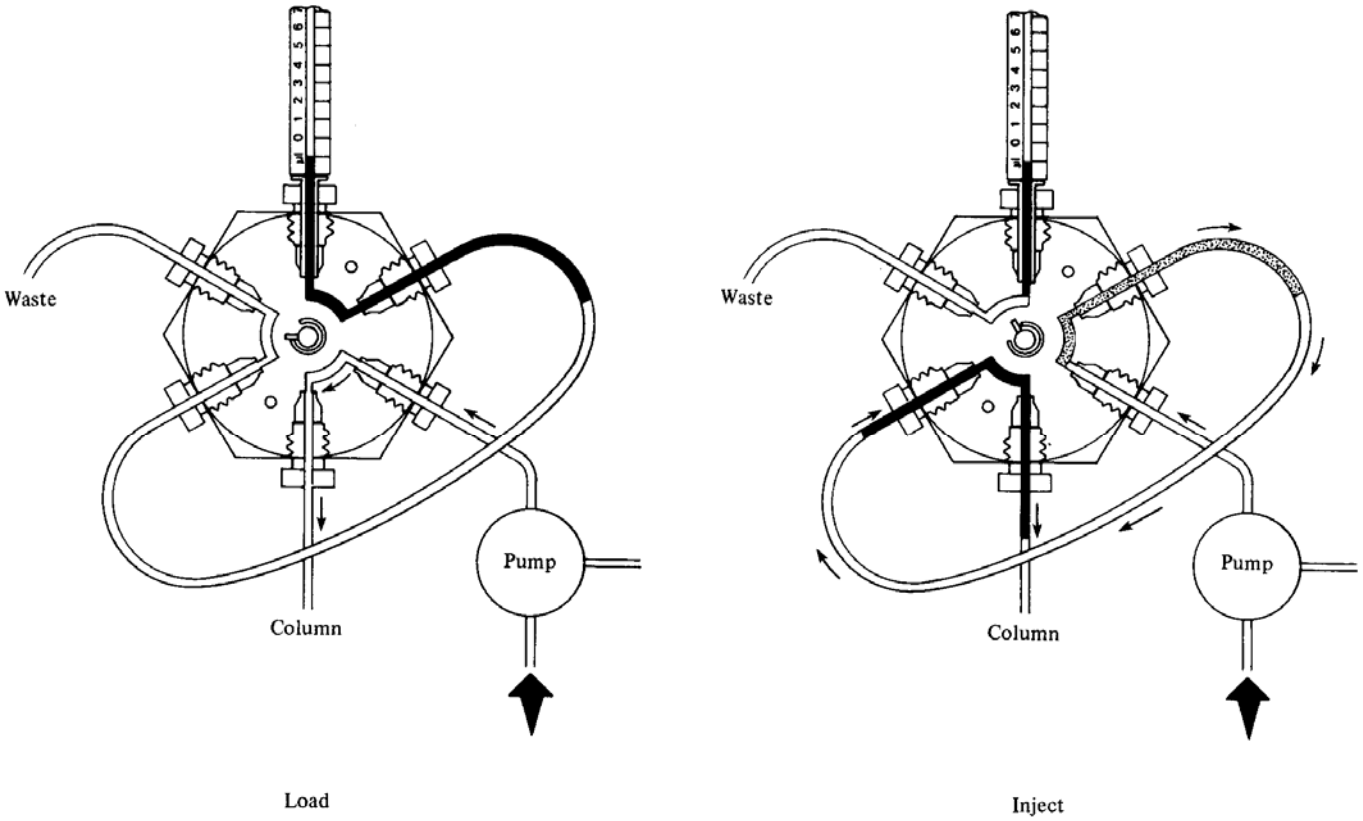


FIGURE 21.18. *Six-port rotary sample-injection valve for HPLC. The arrows indicate the path of solvent flow. Courtesy of Varian Associates.*

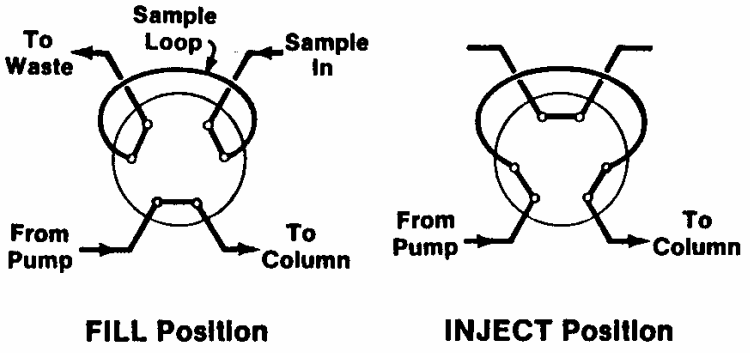


Fig. 26.10 A sample injection valve. The six-port valve shown allows three pairs of ports to be switched synchronously. In the FILL position the mobile phase flows directly to the column through one pair of ports, while the other ports allow new sample to flush out the sample loop. Rotation of the valve to the INJECT position directs the mobile-phase flow into the sample loop, leading to injection of sample onto the column.

Precolumn filters

- 2 types porous stainless frit 0.5 to 2 μm or a little piece of sacrificial column.

Injection => Precolumn => Column => Detector
Valve

Prevents the contamination of the expensive analytical columns with fine particles that can eventually clog the mobile phase flow.

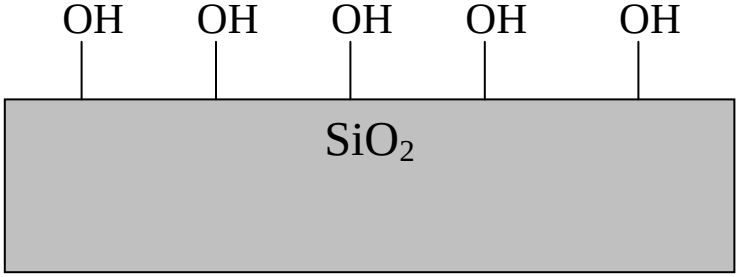
Analytical Column

Common configuration to the right.

Generally stainless steel and teflon components.

The stationary phase packings are microporous silica 2-10 μm in diameter.

Unmodified silica is very polar.



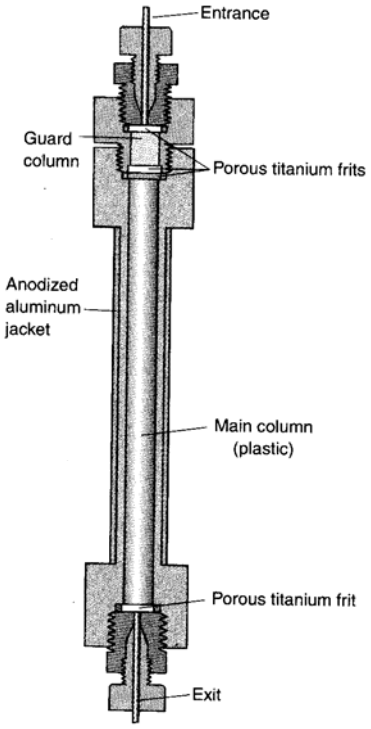
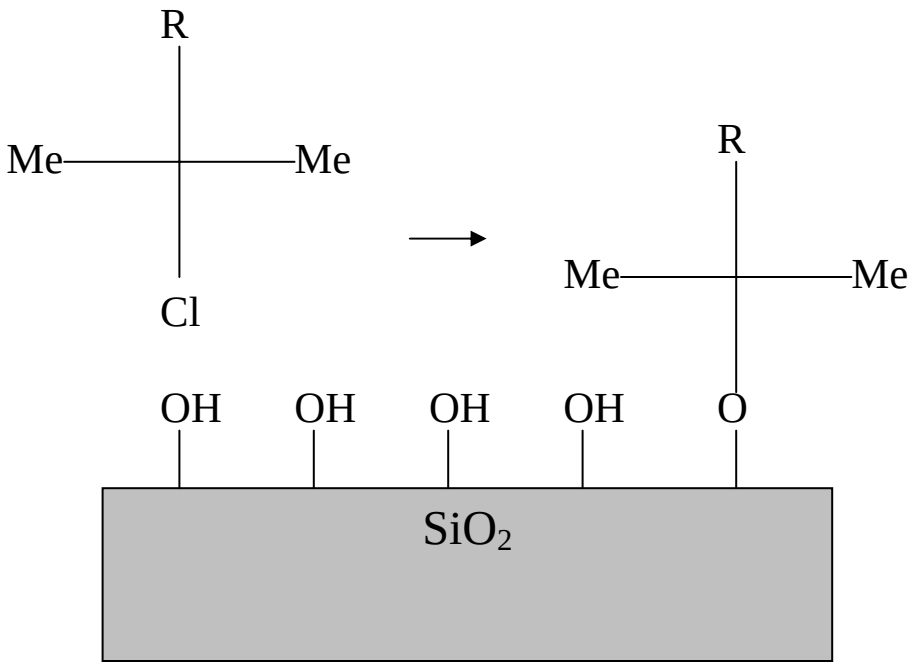


Figure 25-4 HPLC column with replaceable guard column to collect irreversibly adsorbed impurities. Titanium frits distribute the liquid evenly over the diameter of the column. [Courtesy Upchurch Scientific, Oak Harbor, WA.]

Most analytical stationary phases are modified.



Where R can vary, typically…. C_{18}, C_8, , -CH_2-C_6H_5

-{CH_2}$_3$-NH_2, -{CH_2}$_5$-CN

“Reversed” & “Normal” Phase Separations.

Normal Phase – Polar s.p. & Nonpolar m.p.

Early HPLC work was conducted on unmodified silica (highly polar) this required the use of nonpolar mobile phases in order to get adequate separations.

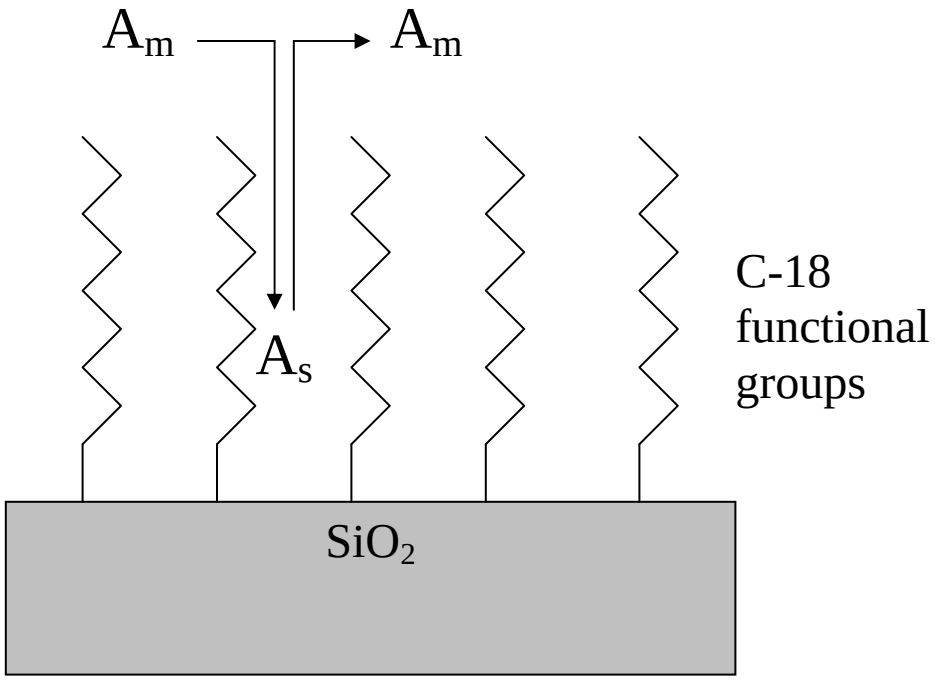
Reversed Phase – Nonpolar s.p. & Polar m.p.

Later HPLC research led to silica C_{18} modified surfaces which required the use of polar mobile phases.

Partition vs. Adsorption Chromatography

Adsorption Chromatography – Based on the unmodified silica surface, which is very polar. Solute analyte species is *adsorbed* to this surface.

Partition Chromatography – Based on modified silica surfaces.



The C-18 bonded phases dissolve rather than adsorb the analyte solute species, thus partitioning of the solute between two essentially liquid phases.

When should we use Partition (nonpolar s.p.) vs. Adsorption (polar s.p.) phases in chormatographic separations?

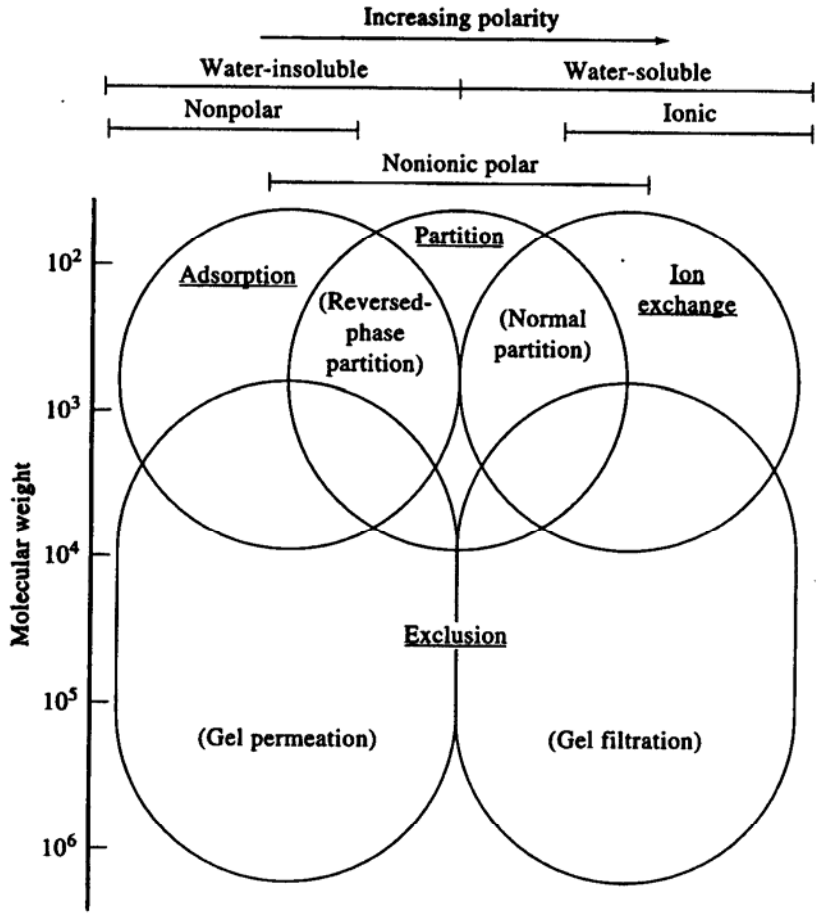
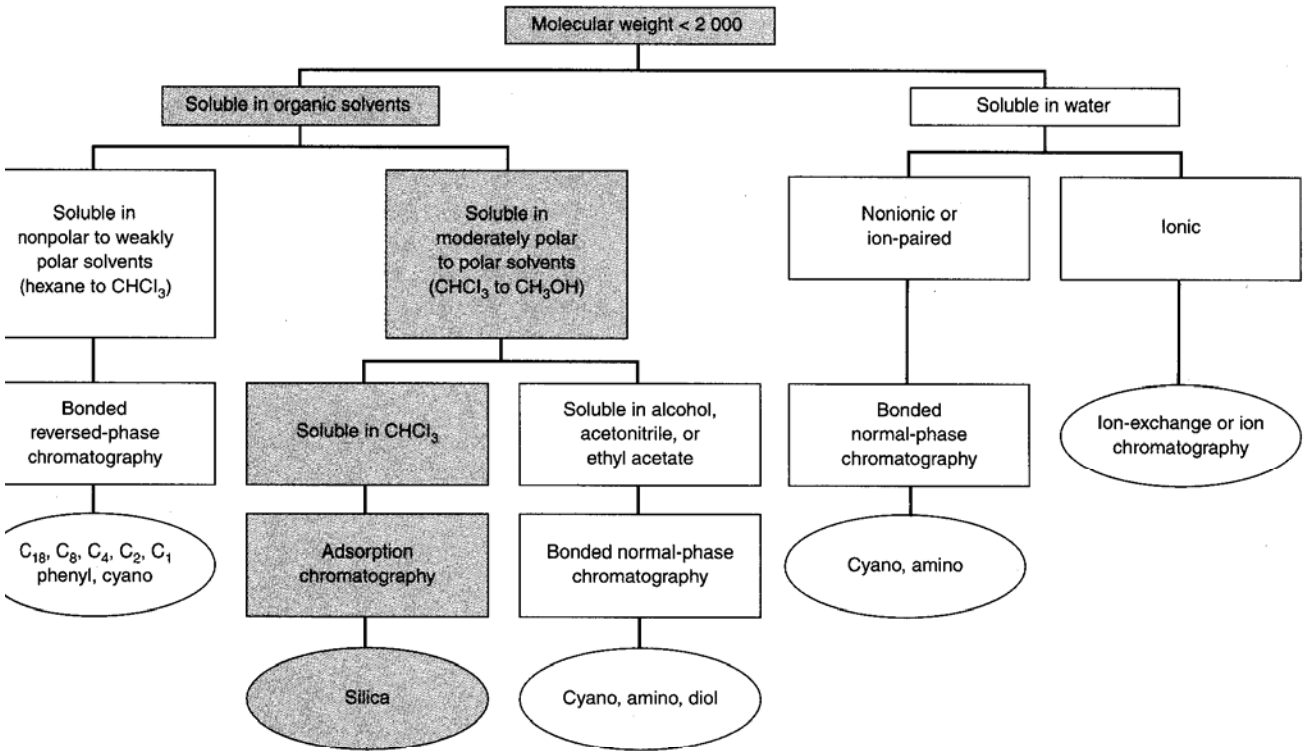
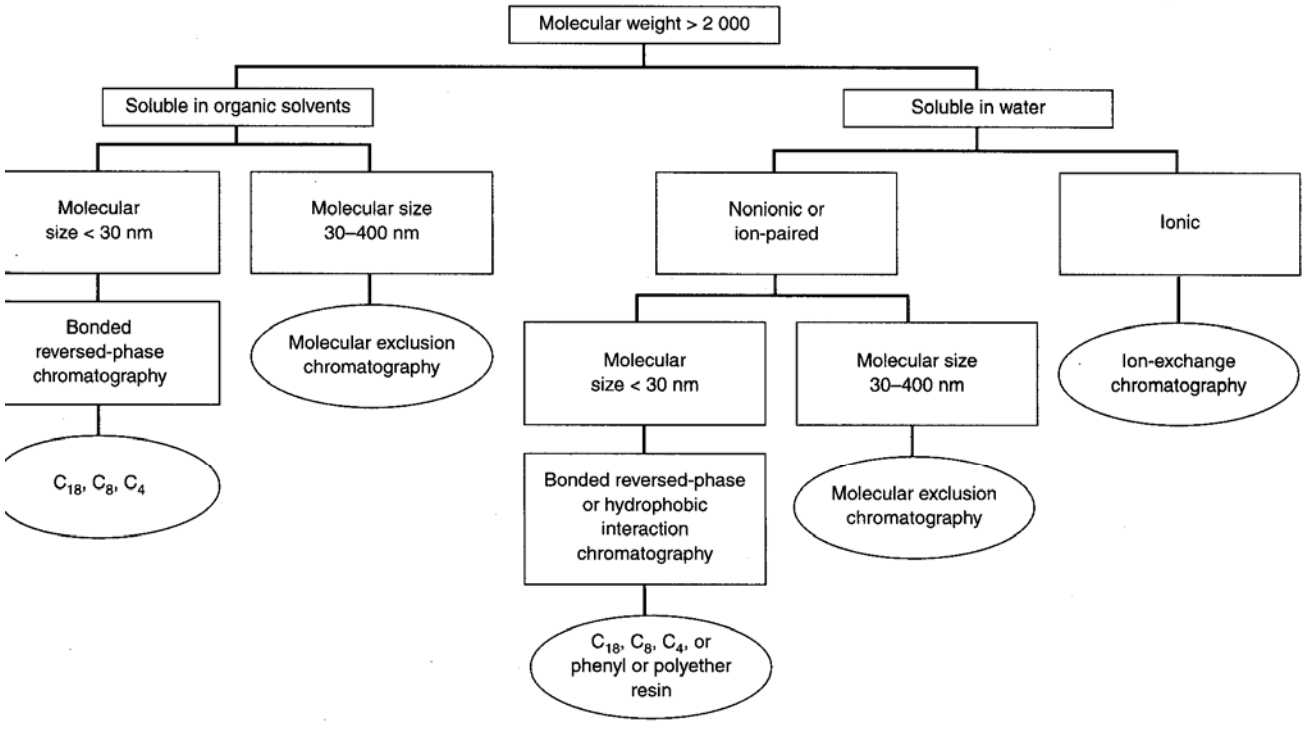


Figure 28-1 Applications of liquid chromatography. *(From D. L. Saunders, in* Chromatography, *3rd ed., E. Heftmann, Ed., p. 81. New York: Van Nostrand Reinhold, 1975. With permission.)*


Molecular weight < 2 000
Soluble in organic solvents
Soluble in water
Soluble in nonpolar to weakly polar solvents (hexane to $CHCl_3$)
Soluble in moderately polar to polar solvents ($CHCl_3$ to CH_3OH)
Nonionic or ion-paired
Ionic
Bonded reversed-phase chromatography
Soluble in $CHCl_3$
Soluble in alcohol, acetonitrile, or ethyl acetate
Bonded normal-phase chromatography
Ion-exchange or ion chromatography
C_{18}, C_8, C_4, C_2, C_1 phenyl, cyano
Adsorption chromatography
Bonded normal-phase chromatography
Cyano, amino
Silica
Cyano, amino, diol


Molecular weight > 2 000
Soluble in organic solvents
Soluble in water
Molecular size < 30 nm
Molecular size 30–400 nm
Nonionic or ion-paired
Ionic
Bonded reversed-phase chromatography
Molecular exclusion chromatography
Molecular size < 30 nm
Molecular size 30–400 nm
Ion-exchange chromatography
C_{18}, C_8, C_4
Bonded reversed-phase or hydrophobic interaction chromatography
Molecular exclusion chromatography
C_{18}, C_8, C_4, or phenyl or polyether resin

HPLC solvents.

Operator experience plays a large role in the design and selection of an HPLC solvent system.

Generally we want a significant difference between the polarities of the s.p. and the m.p., the reason being is that separation is based on solubility differences between the m.p. and s.p. (partitioning)

$$K = C_s/C_m$$

Table 25-1

Eluotropic series and ultraviolet cutoff wavelengths of solvents for adsorption chromatography on silica

Solvent	Eluent strength (ϵ°)	Ultraviolet cutoff (nm)
Pentane	0.00	190
Hexane	0.01	195
Heptane	0.01	200
Trichlorotrifluoroethane	0.02	231
Toluene	0.22	284
Chloroform	0.26	245
Dichloromethane	0.30	233
Diethyl ether	0.43	215
Ethyl acetate	0.48	256
Methyl *t*-butyl ether	0.48	210
Dioxane	0.51	215
Acetonitrile	0.52	190
Acetone	0.53	330
Tetrahydrofuran	0.53	212
2-Propanol	0.60	205
Methanol	0.70	205

Increasing Polarity

The ultraviolet cutoff for water is 190 nm.

SOURCES: L. R. Snyder in *High-Performance Liquid Chromatography* (C. Horváth, ed.), Vol. 3 (New York: Academic Press, 1983); *Burdick & Jackson Solvent Guide*, 3rd ed. (Muskegon, MI: Burdick & Jackson Laboratories, 1990).

UV cutoff is important to keep in mind when we get to detectors

Assume that water is the most polar of all possible solvents

Practical notes

– Separation of most organic compounds can be handled by C-18 stationary phases.

– Most mobile compositions can be handled by either

  – CH_3CN/H_2O or

  – CH_3OH/H_2O

- Solvents must be miscible e.g. water/ethanol. An immiscible solvent system such as water/toluene would create a mess in the column!

Mobile Phase Compositions

Isocratic Elutions – Constant solvent composition, mobile phase polarity stays constant throughout elution process. This is equivalent to isothermal separations in GC.

Gradient Elutions – Mobile phase composition (and thus polarity) varies throughout elution. This is equivalent to temperature programming in GC.

Consider the series of isocratic elutions on the next page.

We can see that an efficient separation is never achieved.

$A = H_2O$ $B = CH_3CN$

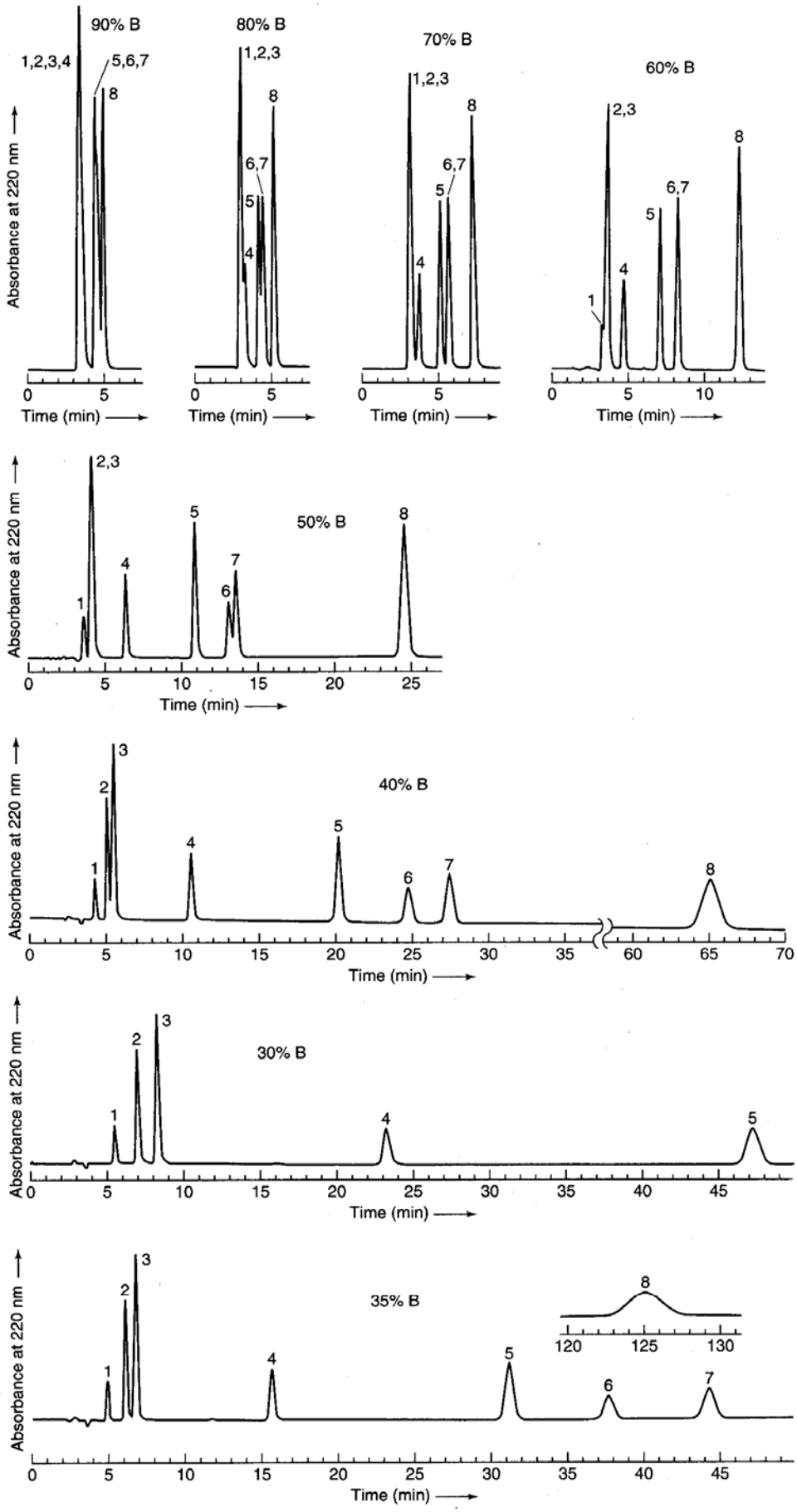


Figure 25-10 Isocratic HPLC separation of a mixture of aromatic compounds at 1.0 mL/min on a 0.46 × 25 cm Hypersil ODS column (C_{18} on 5-μm silica) at ambient temperature (~22° C): (1) benzyl alcohol; (2) phenol; (3) 3′,4′-dimethoxyacetophenone; (4) benzoin; (5) ethyl benzoate; (6) toluene; (7) 2,6-dimethoxytoluene; (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.

Now using gradient elution:

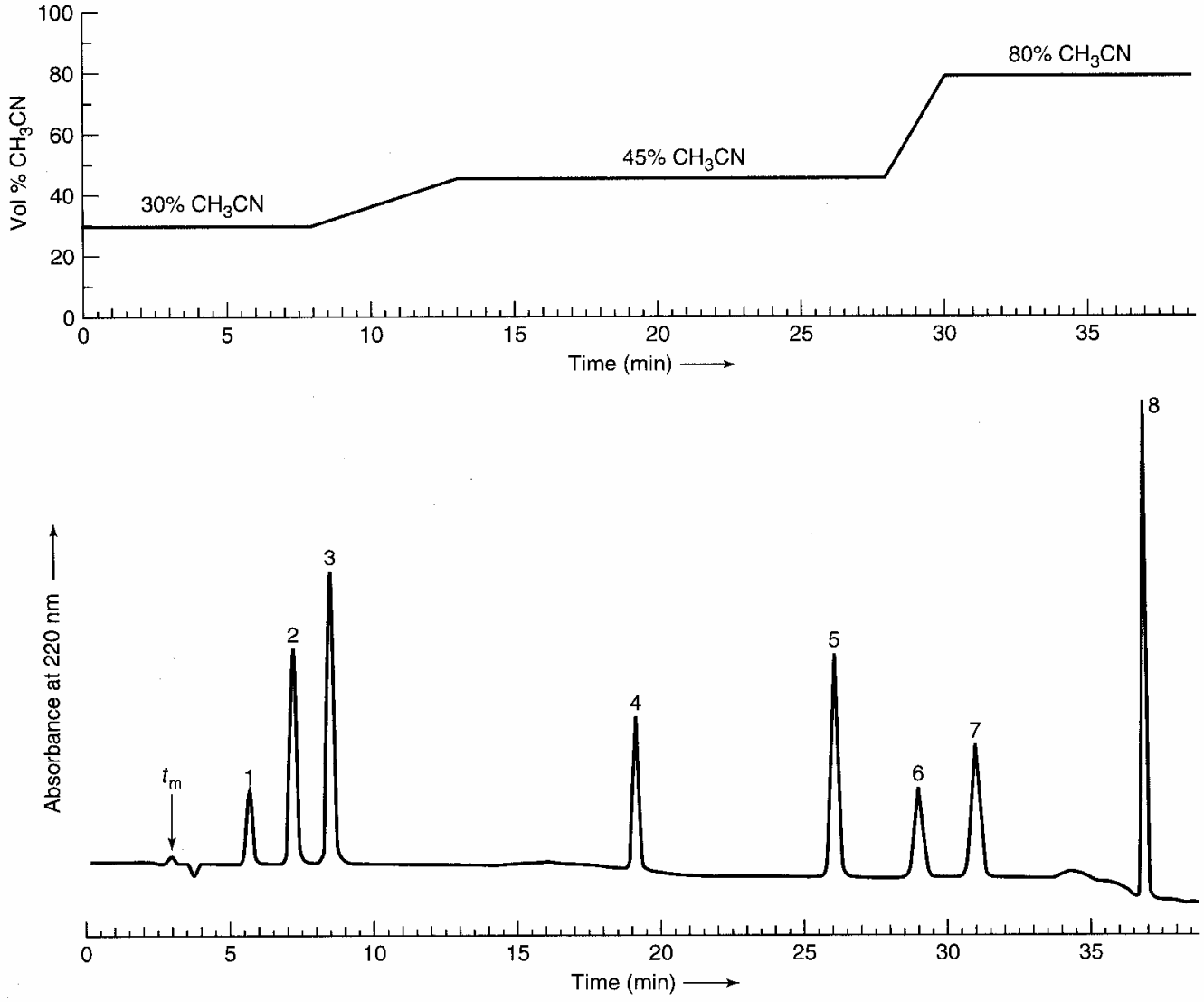


Figure 25-11 Gradient elution of the same mixture of aromatic compounds from Figure 25-10 with the same column, flow rate, and solvents. The upper trace is the *segmented gradient* profile, so named because it is divided into several different segments.

– The pump system for gradient elution is quite a bit more expensive than for isocratic systems. The metering valves require electronic control:

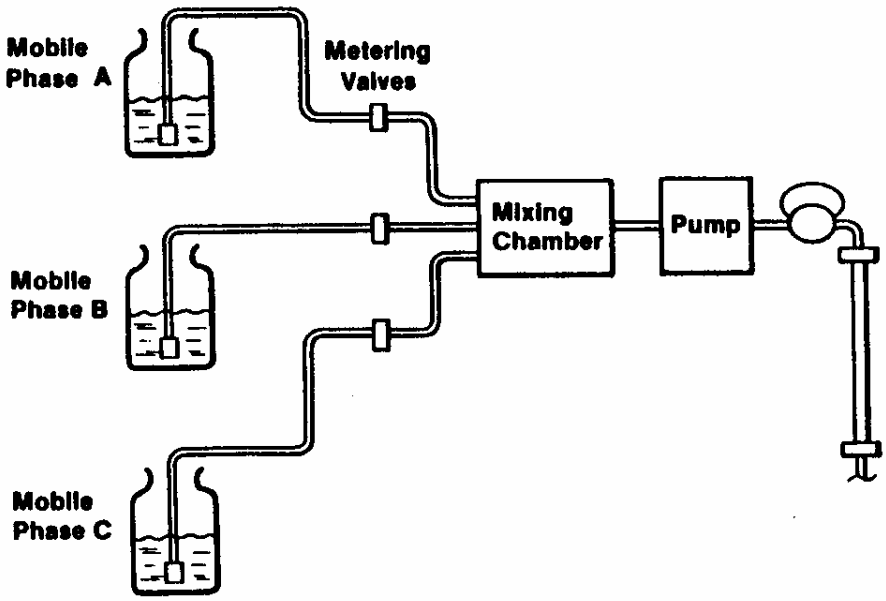


Fig. 26.8 A system for low-pressure gradient generation for a ternary solvent system. The mobile phase composition is controlled by metering valves located in the lines feeding the mixing chamber. The mixed solvent enters the high-pressure pump to be pumped through the column. Changes in mobile-phase composition are accomplished by changing the settings for the metering valves.

– Solute elution times under gradient programs are not as reproducible as isocratic elutions.

Column Heaters in HPLC

Heating the column in HPLC will improve MT, decreases the C_u term in the van Deemter equation.

Consider the following example:

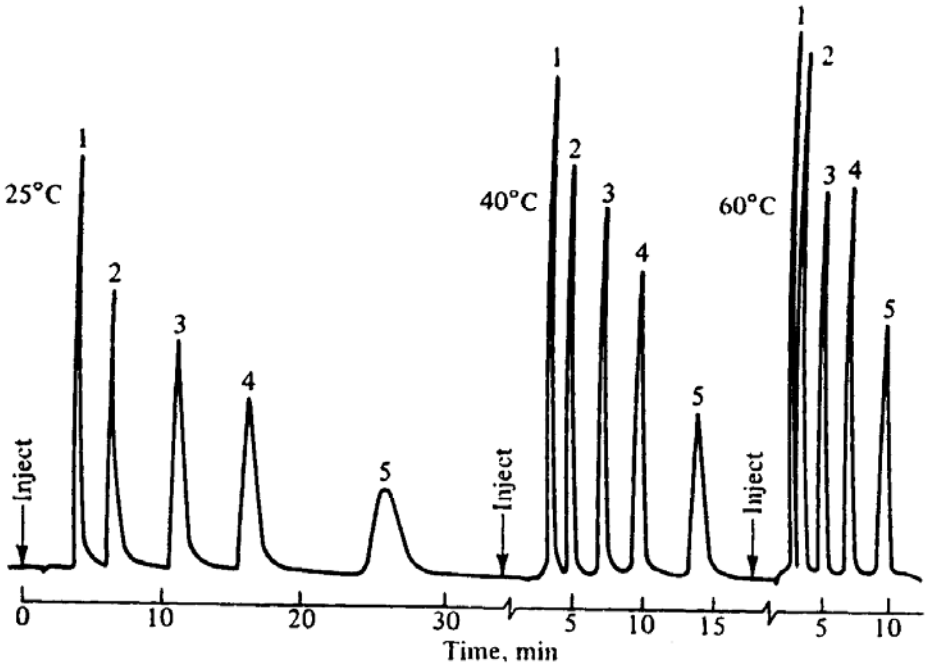


FIGURE 19.2
Influence of column temperature in liquid column chromatography. Mobile phase is 45% methanol/55% water. Peaks: (1) 9,10-anthraquinone; (2) 2-methyl-9,10-anthraquinone; (3) 2-ethyl-9,10-anthraquinone; (4) 1,4-dimethyl-9,10-anthraquinone; and (5) 2-*t*-butyl-9,10-anthraquinone.

Notice that the t_r for each solute changes with temperature, this is because of the solubility changes we should expect with T.

Detectors in HPLC

Ideal Characteristics

- Universal
- Small volume, prevents remixing & band braoadening
- Fast response to flowing system

<u>Refractive Index (RI) detector</u>

- Nearly universal but poor detection limit
- Passes visible light through 2 compartments, sample & reference.

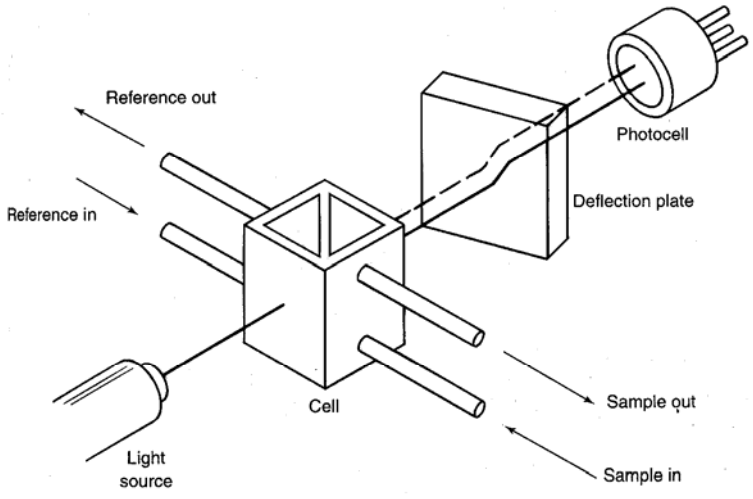


Figure 25-18 Deflection-type refractive index detector.

- When the solvent composition are the same the light passed through the compartments the light beam that passes through is recorded as zero.

- When a solute is in the sample compartment, refractive index changes will shift the light beam from the detector.

- Limit of detection (LOD) 10 ng of solute

UV-vis absorbance detector

- Based on electronic transitions within molecules. (Chapter 19)

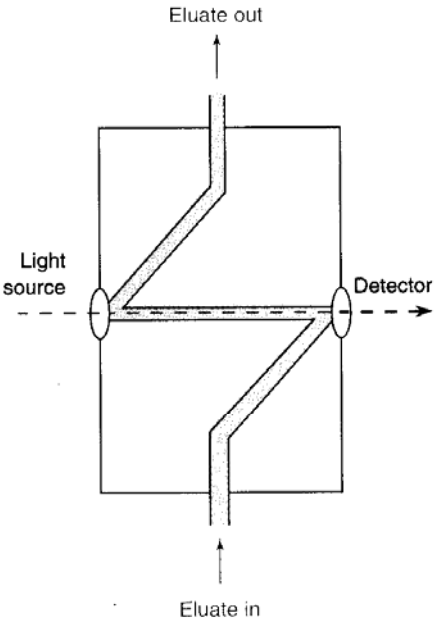


Figure 25-16 Light path in a micro flow cell of a spectrophotometric detector. Cells that have a 0.5-cm pathlength and contain only 8 μL of liquid are common.

- Most common type of detector for LC

- Fixed wavelength, Hg lamp 254 nm ($\pi \Rightarrow \pi^*$)

- Tunable wavelength, selectable for specific wavelengths, monochromators or filters. Still limited to single wavelegths.

- 1 pg LOD

Solvent limitations with UV-vis abs. Detectors

Table 25-1

Eluotropic series and ultraviolet cutoff wavelengths of solvents for adsorption chromatography on silica

Solvent	Eluent strength (ϵ°)	Ultraviolet cutoff (nm)
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Heptane	0.01	200
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Methanol	0.70	205

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SOURCES: L. R. Snyder in *High-Performance Liquid Chromatography* (C. Horváth, ed.), Vol. 3 (New York: Academic Press, 1983); *Burdick & Jackson Solvent Guide*, 3rd ed. (Muskegon, MI: Burdick & Jackson Laboratories, 1990).

Diode array detector

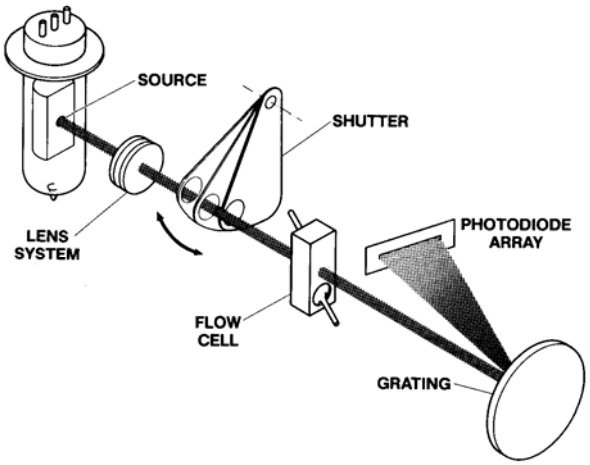

FIGURE 21.20. *Optical system of a photodiode-array LC detector. Courtesy of the Hewlett-Packard Company.*

See lecture notes on the diode array spectrometer, Chapter 21.

Allows for the recording of the entire spectrum of each solute as it passed through the diode array detector

Typical output:

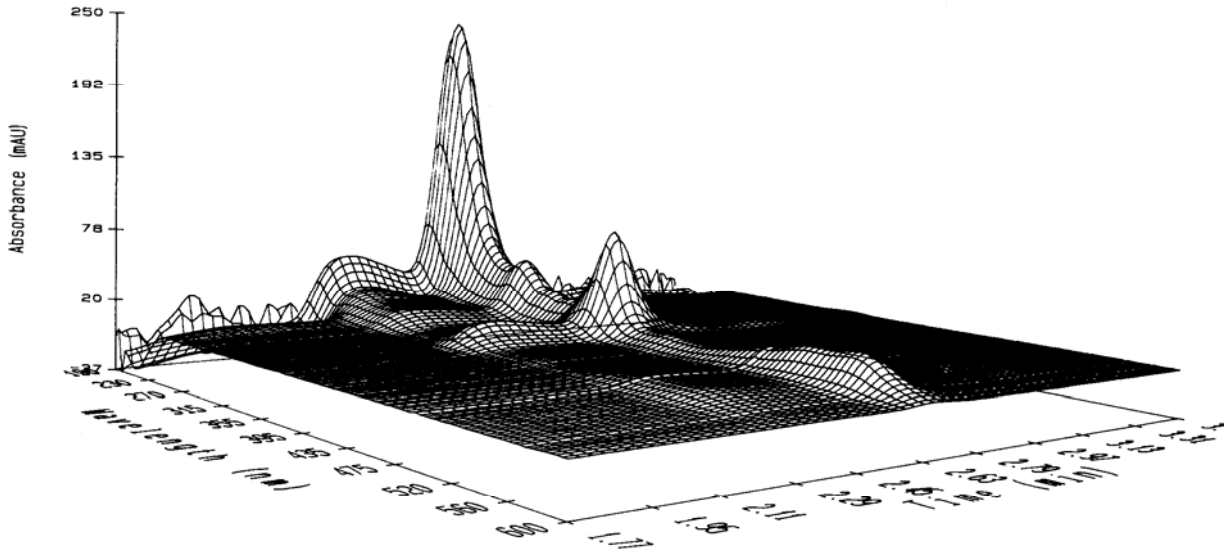

FIGURE 21.21. *Typical three-dimensional plot (absorbance versus wavelength verses time) for a liquid chromatograph. Separation of hydroxylcobalamine (peak 1, at about 2.5 min) and cyanocobalamine (peak 2, at about 3.0 min). Courtesy of the Hewlett-Packard Company.*

Fluorescence Detectors

-Review based on emission of excited state molecules.

-Detector 90^0 from excitation axis.

-LOD 10 fg

IR Detectors

-FT-IR allows for spectrum records of flowing systems analgous to the diode array system.

-Water/alcohols can be major interferences to solute detection

-LOD 100 ng

Evaporative Light Scattering Detector

-Responds to any analyte that is significantly less volatile than the mobile phase.

-Eluate is mixed with $N_2(g)$ and forms a fine mist.

-Solvent (m.p.) evaporates leaving fine particles of analyte. The particles themselves are detected by light scattering.

-Response is proportional to analyte mass.

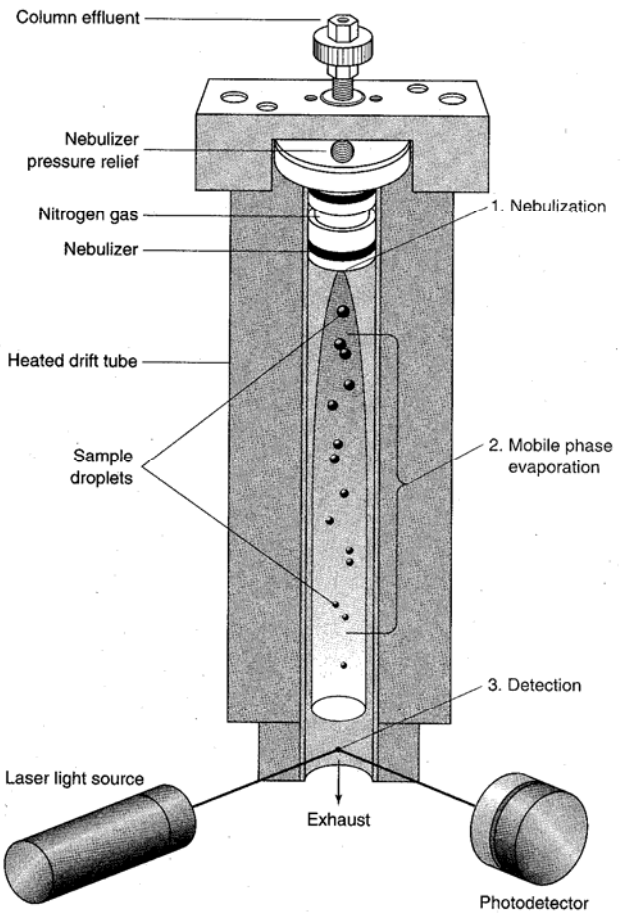

Figure 25-19 Operation of an evaporative light-scattering detector. [Courtesy Alltech Associates, Deerfield, IL.]

-LOD 500 ng

Electrochemical Detectors

-Based on amperometric response of analyte to electrode usually held at constant potential.

-If the analyte is electroactive, can be highly sensitive since response is based on a surface phenomenon rather than a solution bulk property (e.g. UV-vis absorbance)

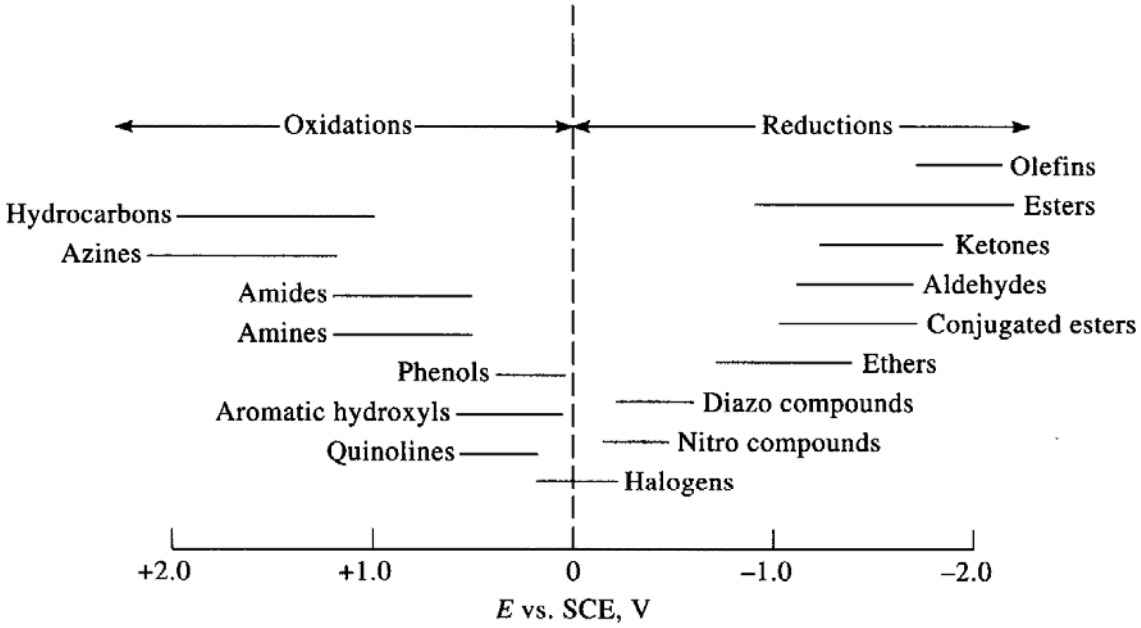

Figure 28-12 Potentially detectable organic functional groups by amperometric measurements. The horizontal lines show the range of oxidation or reduction potentials wherein compounds containing the indicated functional groups are electroactive.

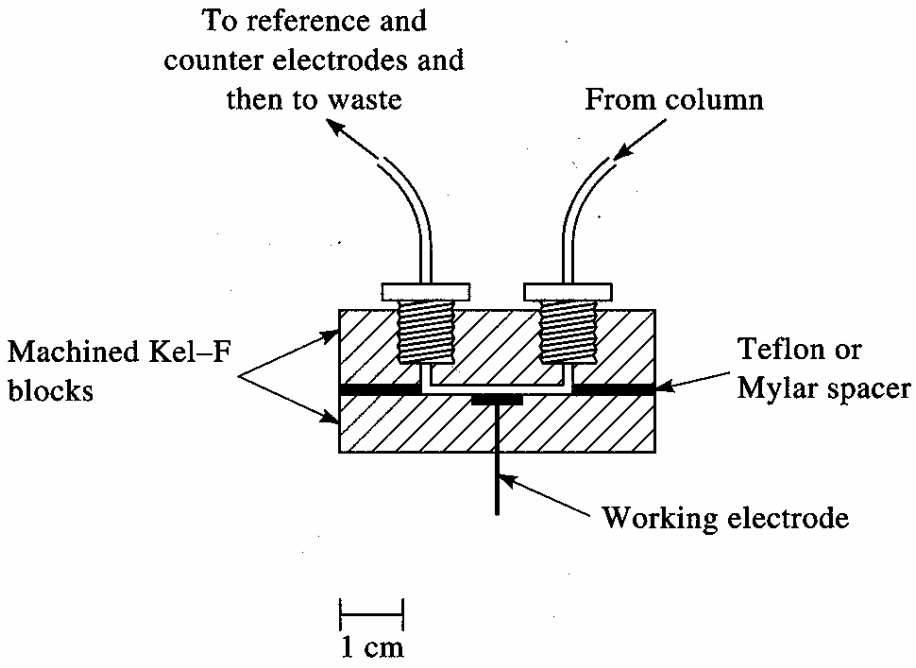

Figure 28-13 Amperometric thin-layer detector cell for HPLC.

LC-MS

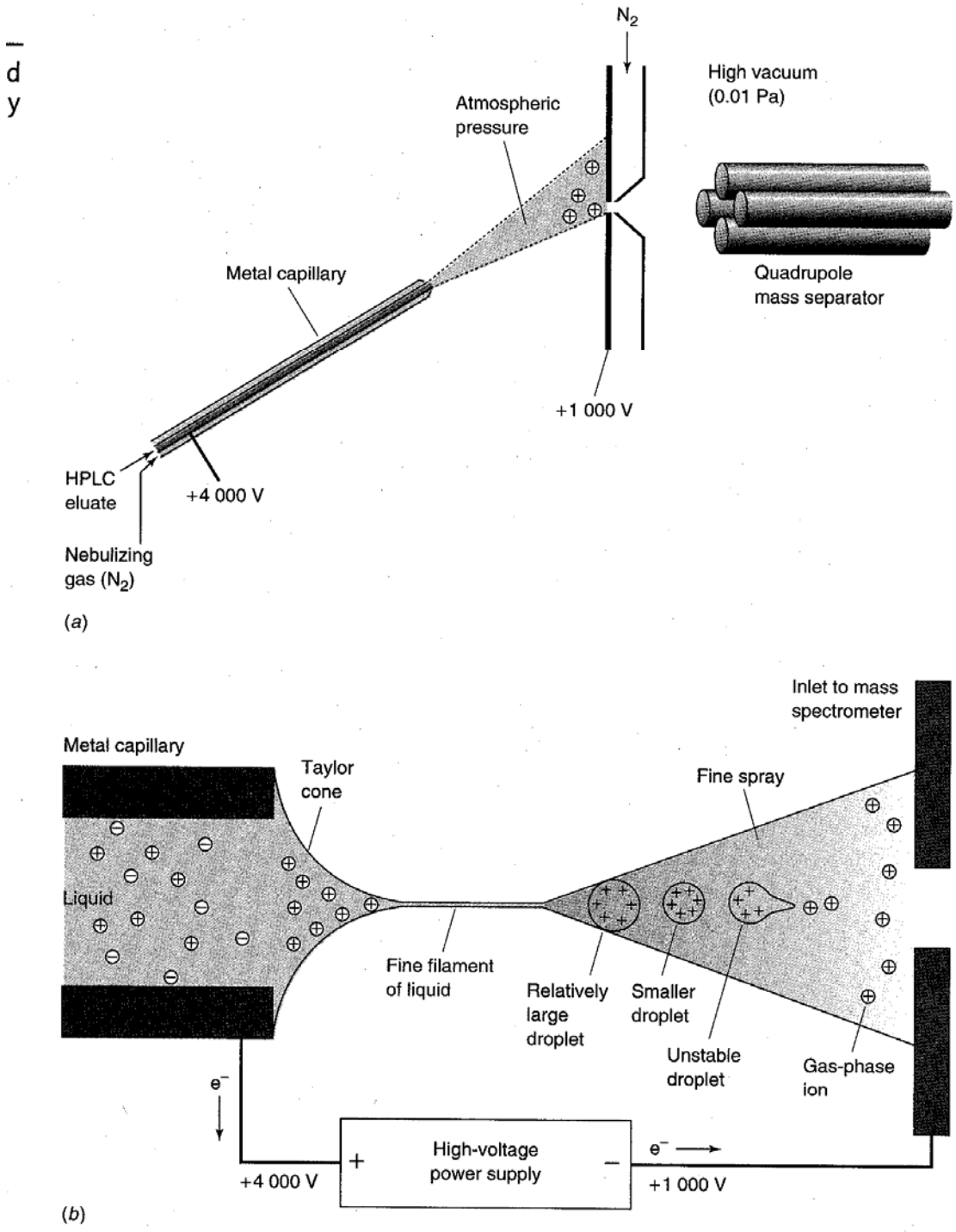

Figure 25-30 (*a*) Pneumatically assisted electrospray interface for mass spectrometry. See Figure 26-25 for a photograph of an electrospray aerosol. (*b*) Detailed view of gas-phase ion formation. Analyte ions are derived from those that were already present in the liquid phase during chromatography. [Adapted from E. C. Huang, T. Wachs, J. J. Conboy, and J. D. Henion, *Anal. Chem.* **1990,** *62,* 713A; and P. Kebarle and L. Tang, *Anal. Chem.* **1993,** *65,* 972A.]

-Selected ion-monitoring focuses the mass spec onto one particular m/e ratio. S/N enhancement occurs when scanning mode is off.

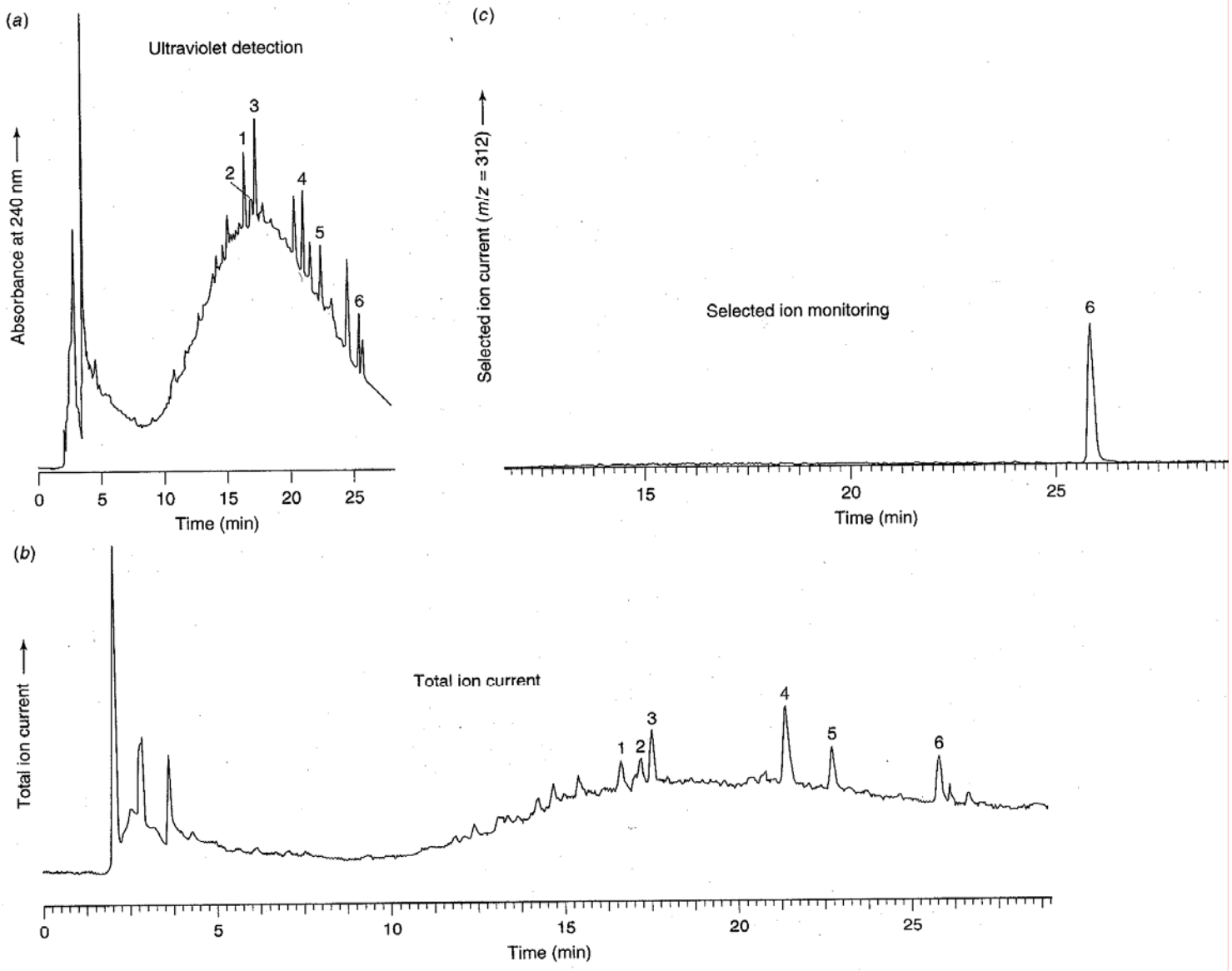

Figure 25-31 Chromatograms of herbicides (designated 1–6) spiked into river water a a level near 1 ppb demonstrate increased signal-to-noise ratio in selected ion monitoring. (*a*) Ultraviolet detection at 240 nm. (*b*) Electrospray total ion current detection with a mas spectrometer. (*c*) Electrospray selected ion monitoring at $m/z = 312$. [From A. Laganà, G. Fago, and A. Marino, *Anal. Chem.* **1998,** *70,* 121.]

-LOD 1 pg

Summary of LC Detectors

TABLE 28-1 Performances of LC Detectors

LC Detector	Commercially Available	Mass LOD (commercial detectors)[a]	Mass LOD (state of the art)[b]
Absorbance	Yes[c]	100 pg–1 ng	1 pg
Fluorescence	Yes[c]	1–10 pg	10 fg
Electrochemical	Yes[c]	10 pg–1 ng	100 fg
Refractive index	Yes	100 ng–1 μg	10 ng
Conductivity	Yes	500 pg–1 ng	500 pg
Mass spectrometry	Yes[d]	100 pg–1 ng	1 pg
FT–IR	Yes[d]	1 μg	100 ng
Light scattering[e]	Yes	10 μg	500 ng
Optical activity	No	—	1 ng
Element selective	No	—	10 ng
Photoionization	No	—	1 pg–1 ng

[a]Mass LOD is calculated for injected mass that yields a signal equal to five times the σ noise, using a mol wt of 200 g/mol, 10 μL injected for conventional or 1 μL injected for microbore LC.
[b]Same definition as *a*, above, but the injected volume is generally smaller.
[c]Commercially available for microbore LC also.
[d]Commercially available, yet costly.
[e]Including low-angle light scattering and nephelometry.
(From E. S. Yeung and R. E. Synovec, *Anal. Chem.*, **1986**, *58*, 1238. With permission.)

Table 25-2

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
Electrochemical	0.01–1	No
Fluorescence	0.001–0.01	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, *Anal. Chem.* **1986**, *58*, 1237A.