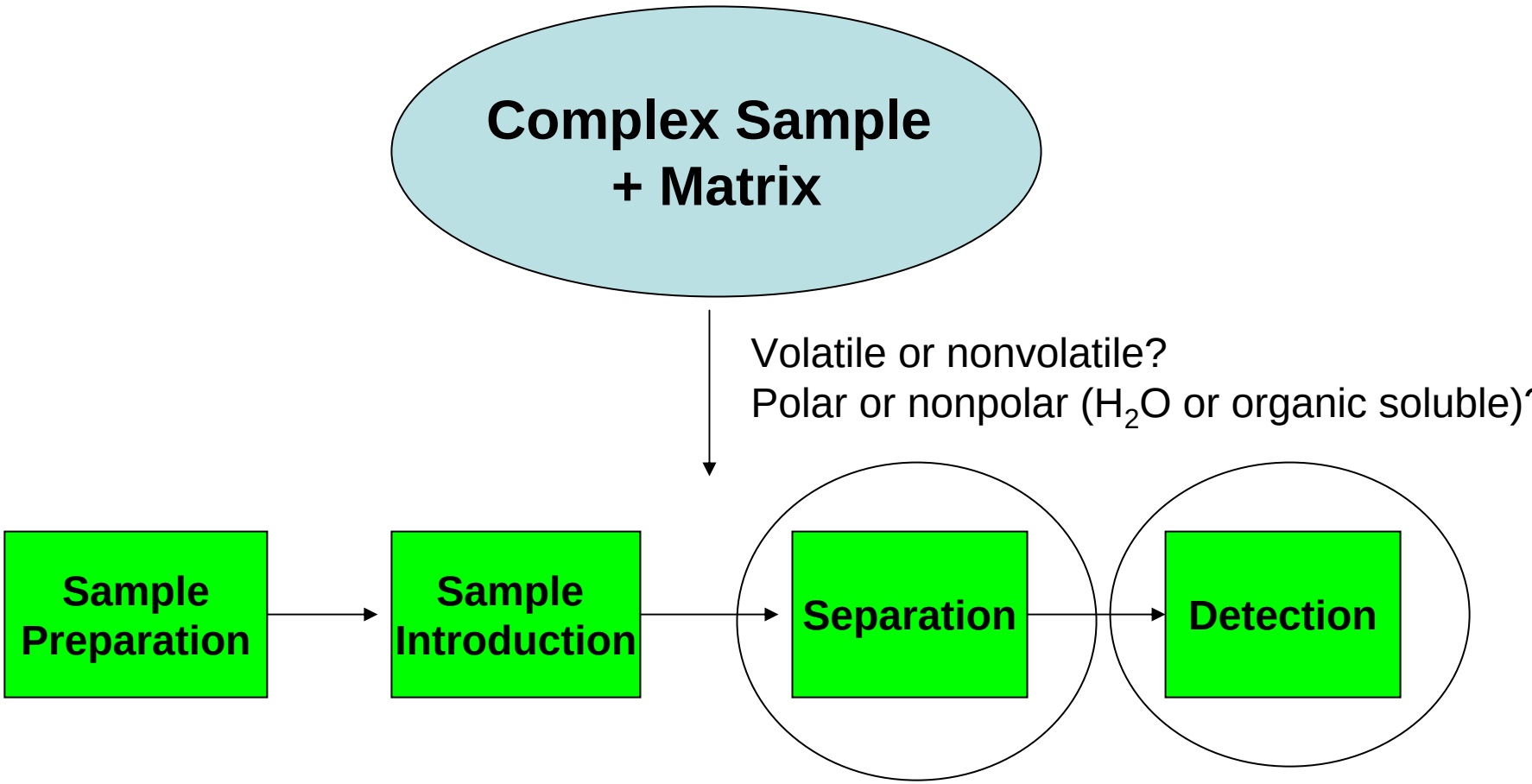
Chapter 28 – Introduction to High Performance Liquid Chromatography (HPLC)

Read: 816-837 Problems: 28-2,5,13, 20, 22



General Types of Liquid Chromatography

- ***Partition*** – most widely used…..stationary phase (liquid-bonded) is a sceond liquid immiscible with the liquid mobile phase. Useful for separating polar compounds of low molecular weight (< 3000 Da).
- ***Adsorption of Liquid-Solid*** – Silica (SiO_2) used as the stationary phase. Separations controlled by adsorption-displacement processes. Polar compounds strongly retained.
- ***Ion Exchange*** – based on ion-exchange processes between ions in solution and ions of like sign on the stationary phase.
- ***Size Exclusion*** – Silica or polymeric materials with specific pore sizes are used as the stationary phase. Molecules are effectively trapped and removed from the mobile phase depending on their size and the pore size.
- ***Affinity*** – involves covalent bonding of an affinity ligand.

Liquid Chromatography

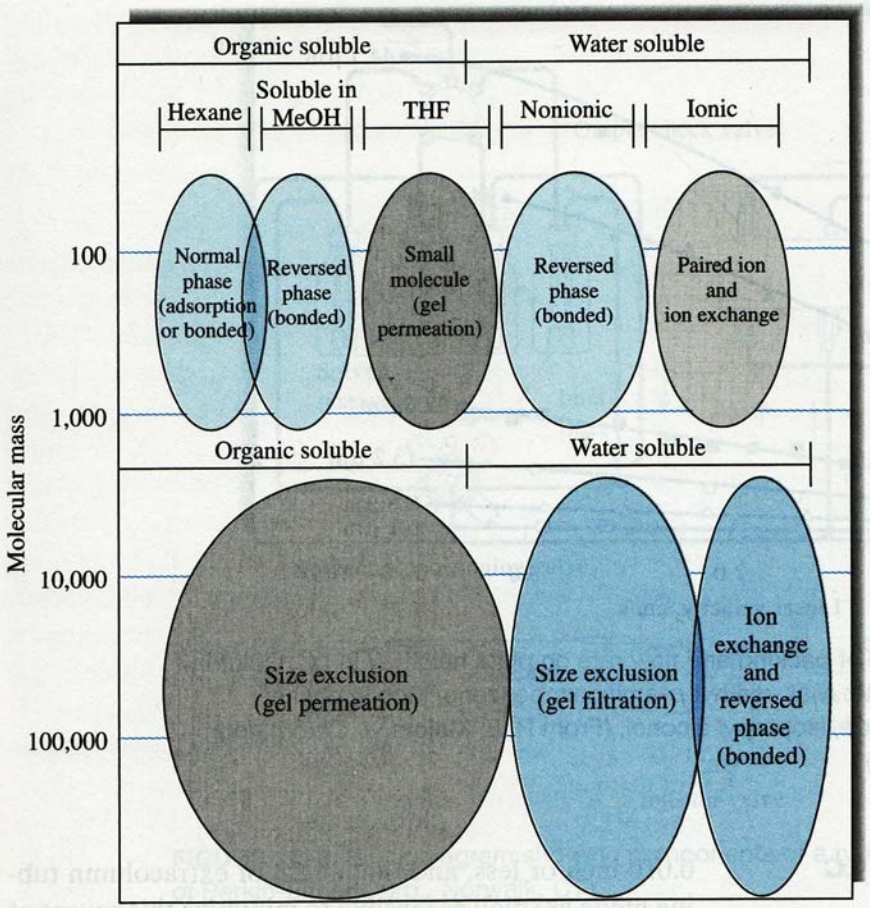


FIGURE 28-1 Selection of LC modes. Methods can be chosen based on solubility and molecular mass. In most cases for non-ionic small molecules ($\mathcal{M} < 2000$), reversed-phase methods are suitable. Techniques toward the bottom of the diagram are best suited for species of high molecular mass ($\mathcal{M} > 2000$). (Adapted from *High Performance Liquid Chromatography*, 2nd ed., S. Lindsay and J. Barnes, eds., New York: Wiley, 1992. With permission.)

Mobile phase plays an integral role in separations (retention), unlike GC!

Partition Chromatography: Reversed-phase and normal phase

Instrumentation

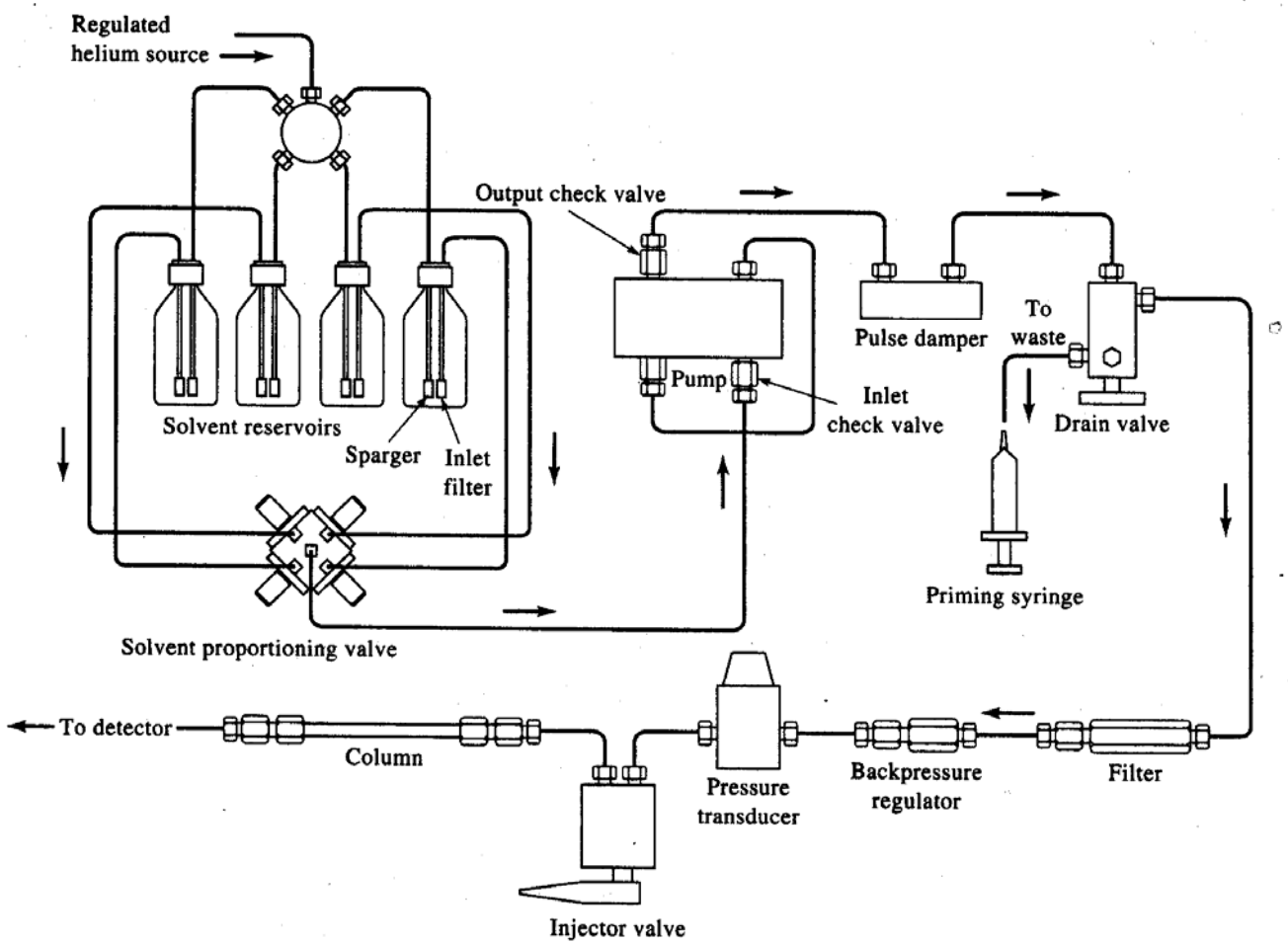
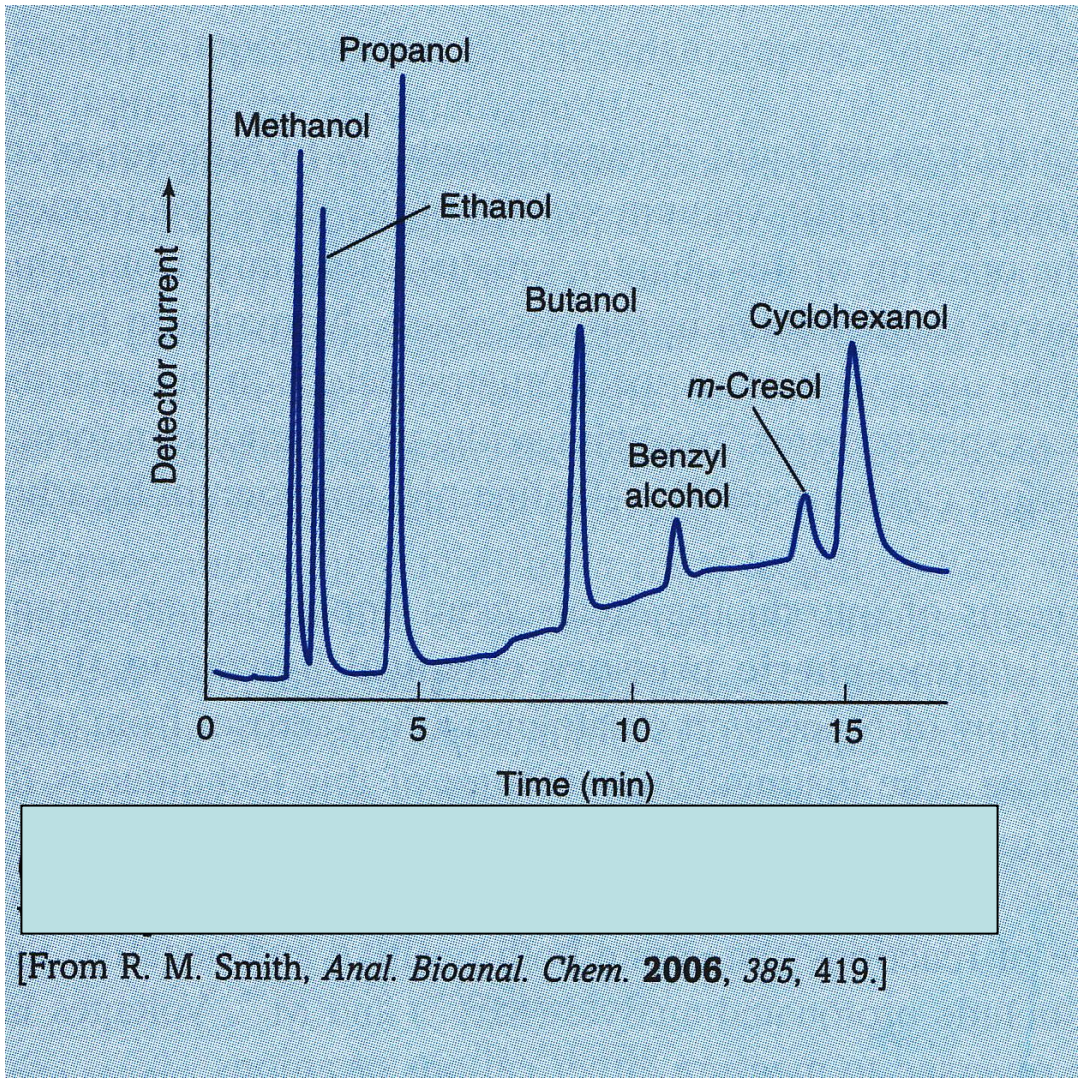


Figure 28-4 Schematic of an apparatus for HPLC. *(Courtesy of Perkin-Elmer Corporation, Norwalk, CT.)*

How would you achieve this separation and detection?



[From R. M. Smith, *Anal. Bioanal. Chem.* **2006**, *385*, 419.]

Some General Facts

$$H = A + B/u + C_s u + C_{extra}$$

Differences in flow velocity due to drag against the wall.

- ***Isocratic*** vs. ***gradient*** elution (shortens separation time without sacrificing resolution of early eluting peaks.
- Columns: 5-25 cm length, 3-5 mm i.d., packed with 5µm diam.particles.
- Efficiency: 40,000-70,000 plates/m (10,000 plates/column).
- Packings: ***pellicular*** vs. ***porous***

Example of a Gradient Separation

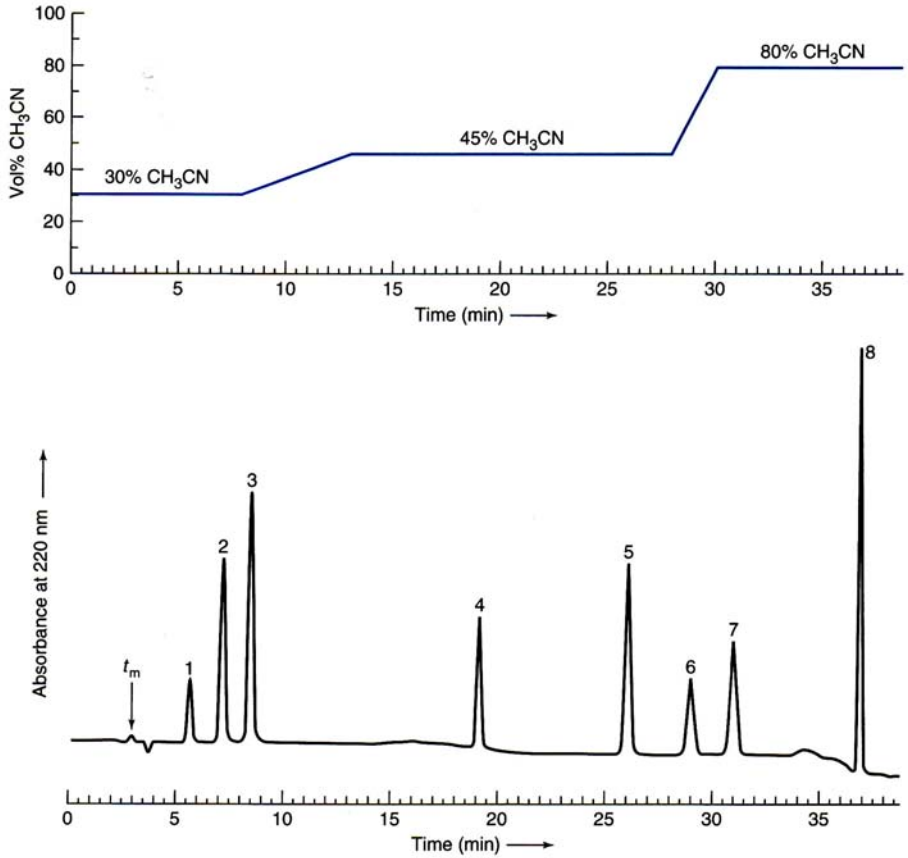


Figure 22-21 Gradient elution of the same mixture of aromatic compounds shown in Figure 22-20 with the same column, flow rate, and solvents. The upper trace is the *segmented gradient* profile, so named because it is divided into several different segments.

Detector Characteristics

Two types: bulk property of mobile phase and solute property

- Responsiveness to the analyte(s).
- Good reproducibility (< 5% RSD).
- Good calibration sensitivity.
- Wide linear dynamic range (3-5 orders of magnitude).
- Low limit of detection (mass or concentration) (pmol-nmol, or lower).
- Good reliability and non-destructive to the sample.
- Minimal internal volume to reduce zone broadening.
- No-need for responsiveness over a wide temperature range.

Detectors for HPLC

TABLE 28-1 Performance of HPLC Detectors

HPLC Detector	Commercially Available	Mass LOD* (typical)	Linear Range† (decades)
Absorbance	Yes	10 pg	3–4
Fluorescence	Yes	10 fg	5
Electrochemical	Yes	100 pg	4–5
Refractive index	Yes	1 ng	3
Conductivity	Yes	100 pg–1 ng	5
Mass spectrometry	Yes	<1 pg	5
FTIR	Yes	1 µg	3
Light scattering	Yes	1 µg	5
Optical activity	No	1 ng	4
Element selective	No	1 ng	4–5
Photoionization	No	<1 pg	4

Sources: From manufacturer's literature; *Handbook of Instrumental Techniques for Analytical Chemistry*, F. Settle, ed., Upper Saddle River, NJ: Prentice-Hall, 1997; E. S. Yeung and R. E. Synovec, *Anal. Chem.*, **1986**, *58*, 1237A.

*Mass LODs (limits of detection) depend on compound, instrument, and HPLC conditions, but those given are typical values with commercial systems when available.

†Typical values from the preceding sources.

Mass LOD = concentration (mol/L) x inj. vol. (L) x FW (g/mol)

UV/Vis and Fluorescence Detection

Z-cell design

For fluorescence detection, the detector would be positioned orthogonal (90^o) w.r.t. the source.

$A(\lambda) = \varepsilon(\lambda)bC$

$F(\lambda) = (\text{constant})\ \varepsilon(\lambda)bCP_o\phi$

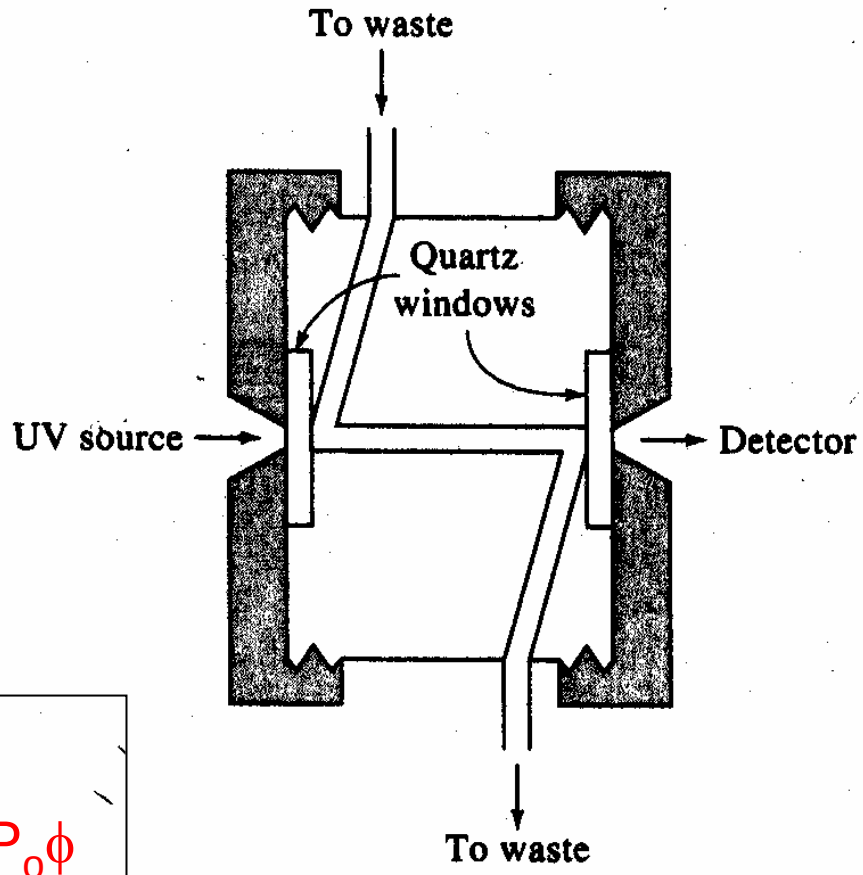


Figure 28-9 Ultraviolet detector cell for HPLC.

Electrochemical Detection

Amperometric detection = fixed potential and measure the current response.

$$i = (\text{constant})\ C$$

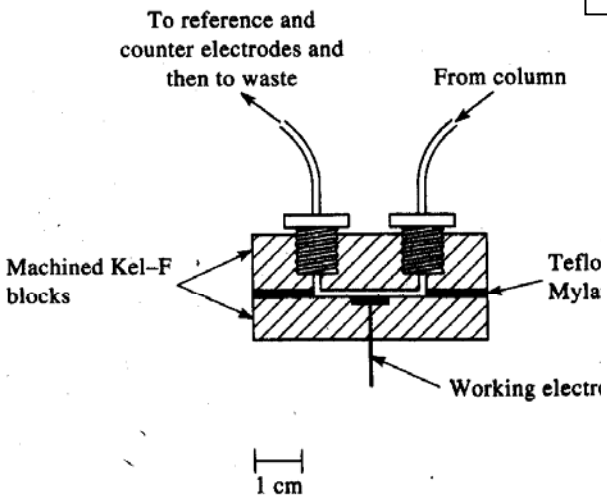


Figure 28-13 Amperometric thin-layer detector cell HPLC.

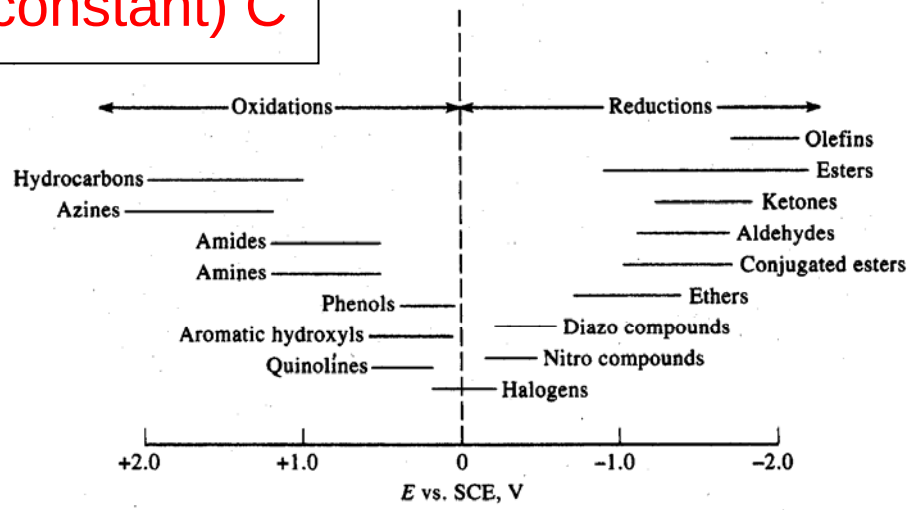
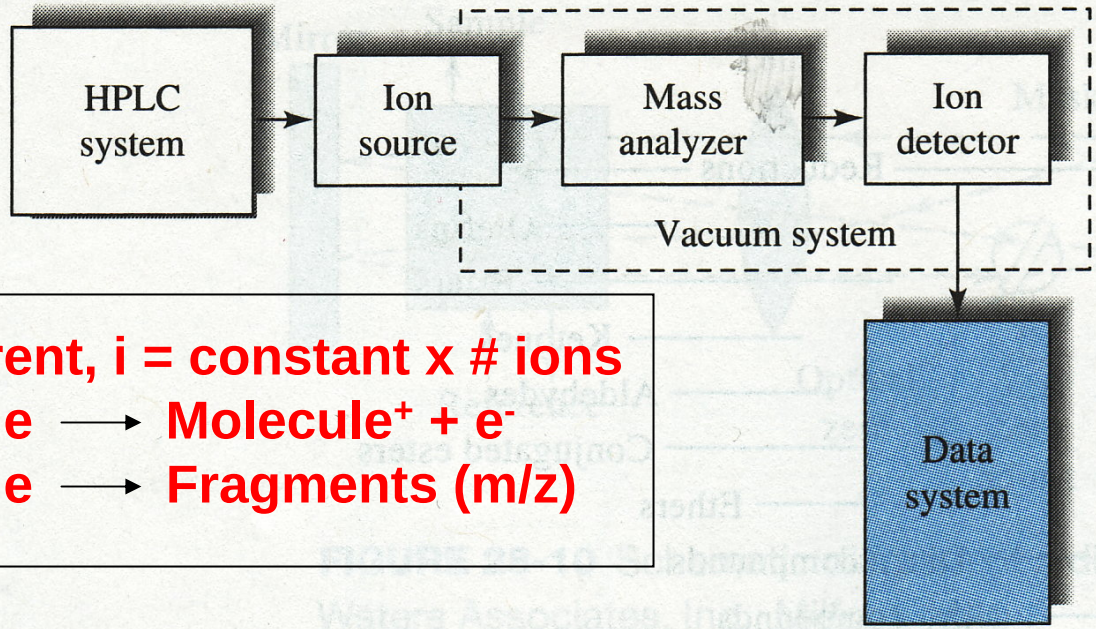


Figure 28-12 Potentially detectable organic functional groups by amperometric measurements. The horizontal lines show the range of oxidation or reduction potentials wherein compounds containing the indicated functional groups are electroactive.

Often a fixed or constant potential is applied, which is sufficient to oxidize or reduce the solute at a mass transport limited rate. As solute zone elutes off the column, the zone passes across the electrode surface and the reaction occurs.

Mass Spectrometric Detection



Ion current, i = constant x # ions

Molecule → Molecule$^+$ + e$^-$

Molecule → Fragments (m/z)

FIGURE 28-13 Block diagram of an LC/MS system. The effluent from the LC column is introduced to an atmospheric pressure ionization source, such as an electrospray or a chemical ionization source. The ions produced are sorted by the mass analyzer and detected by the ion detector.

Mass Spectrometric Detection

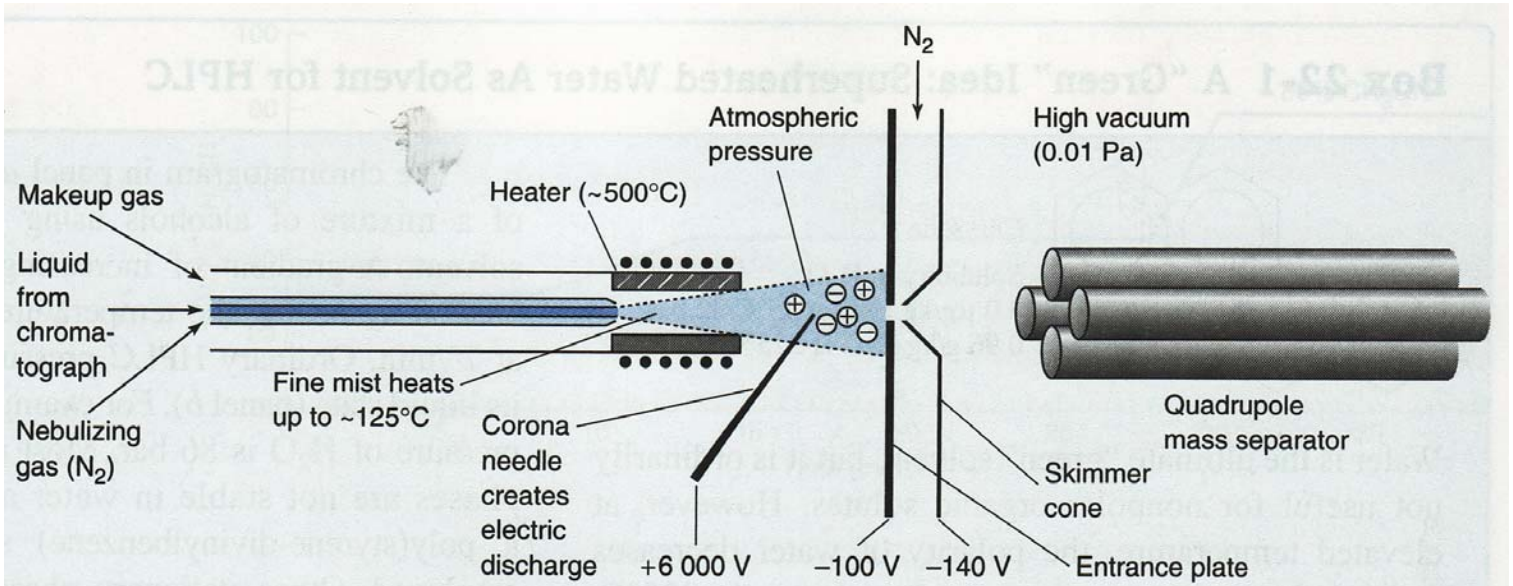


Figure 22-23 Atmospheric pressure chemical ionization interface between liquid chromatography column and mass spectrometer. Aerosol is produced by the nebulizing gas flow and the heater. Electric discharge from the corona needle creates gaseous ions from analyte. [Adapted from E. C. Huang, T. Wachs, J. J. Conboy, and J. D. Henion, *Anal. Chem.* **1990**, *62*, 713A.]

Refractive Index Detection

n (refractive index) = c/ν

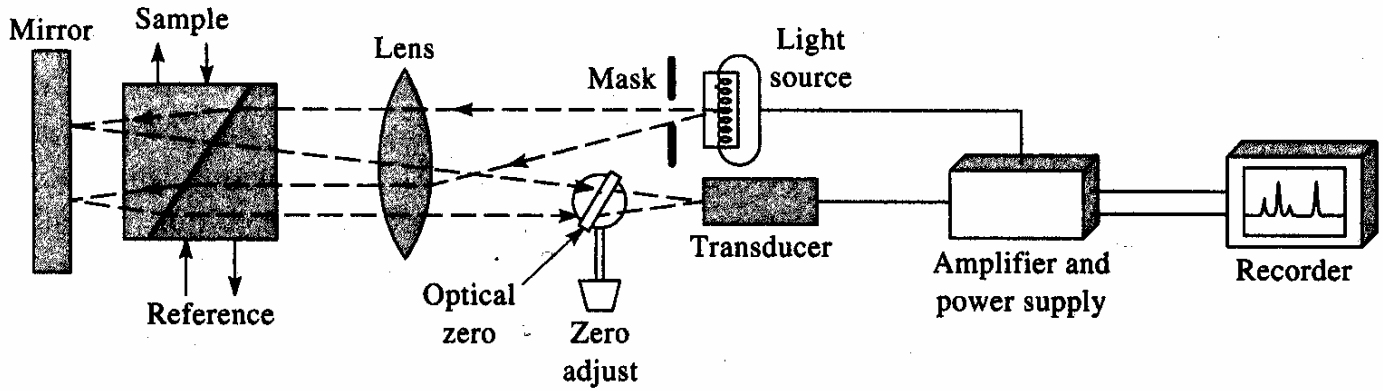


Figure 28-11 Schematic of a differential refractive-index detector. *(Courtesy of Waters Associates, Inc., Milford, MA 91757.)*

Mobile phase passes through one-half of the cell and the mobile-phase + solute passes through the other half. Presence of solute causes the light to be bent more and less intensity reaches the fixed-position detector. Solutes have electron density and therefore are more optically dense. This lead to a greater refractive index.

Plate Height and Efficiency

N (plate number) = L/H

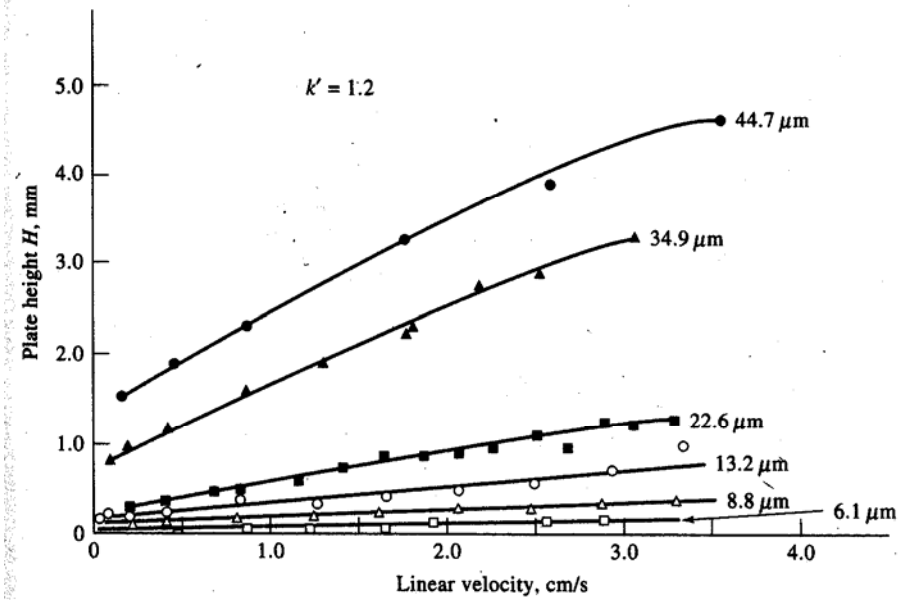


Figure 28-2 Effect of particle size of packing and flow rate upon plate height H in liquid chromatography. Column dimensions: 30 cm × 2.4 mm. Solute: N,N-diethyl-*n*-aminoazobenzene. Mobile phase: mixture of hexane, methylene chloride, isopropyl alcohol. *(From R. E. Majors,* J. Chromatogr. Sci., ***1973, 11, 92. With permission.)***

$$H(\text{in column}) = A + B/u + Cu$$

Diffusion in liquids is much slower!

$$H(\text{extra column}) = \pi r^2 u / 24 D_M$$

r = tube radius, cm
u = flow velocity, cm/s
D_M = diff. Coeff., cm^2/s

General Elution Problem

Isocratic versus gradient elution

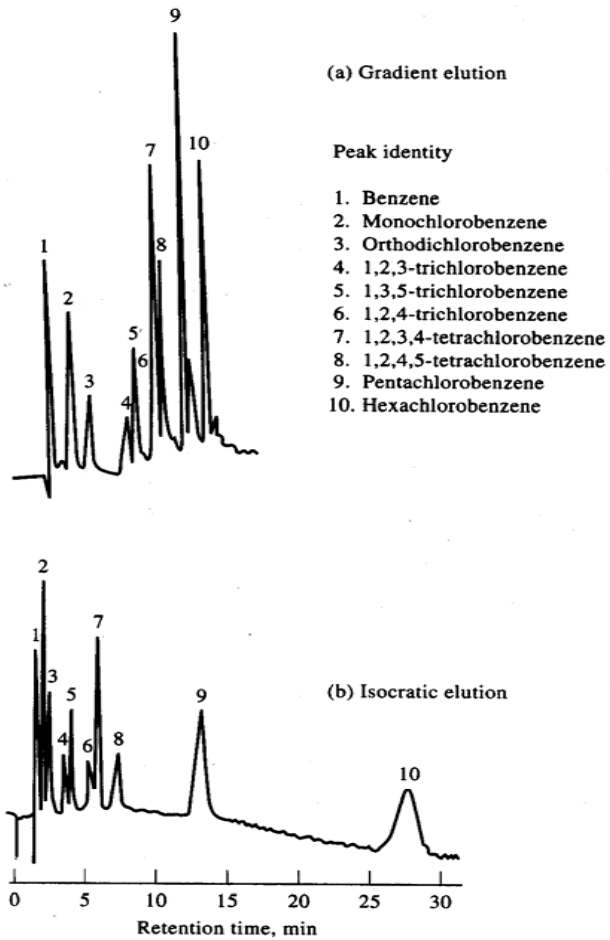


Figure 28-5 Improvement in separation efficiency by gradient elution. Column: 1 m × 2.1 mm i.d., precision-bore stainless; packing: 1% Permaphase® ODS. Sample: 5 μL of chlorinated benzenes in isopropanol. Detector: UV photometer (254 nm). Conditions: temperature, 60°C, pressure, 1200 psi. *(From J. J. Kirkland,* Modern Practice of Liquid Chromatography, *p. 88. New York: Interscience, 1971. Reprinted by permission of John Wiley & Sons, Inc.)*

Long retention times mean the solute is interacting strongly with the stationary phase material on the column. In order to elute more quickly, the mobile phase composition must be adjusted to give the solute more affinity for the liquid phase.

The opposite for short retention times.

Types of Partition Chromatography

There are two general types of partition methods: reversed-phase and normal-phase.

In reversed-phase LC, the stationary phase is non-polar (chemically modified silica) and the mobile phase is polar (water + organic modifier). Excellent for solubilizing and separating polar solutes.

In normal-phase LC, the stationary phase is polar (silica or chemically modified surface) and the mobile phase is non-polar (hexane, ether).

Balance average concentration in mobile phase versus concentration in the stationary phase.

$$K_{eq} = C_{stat}/C_{mobile}$$

Detectors for Liquid Chromatography

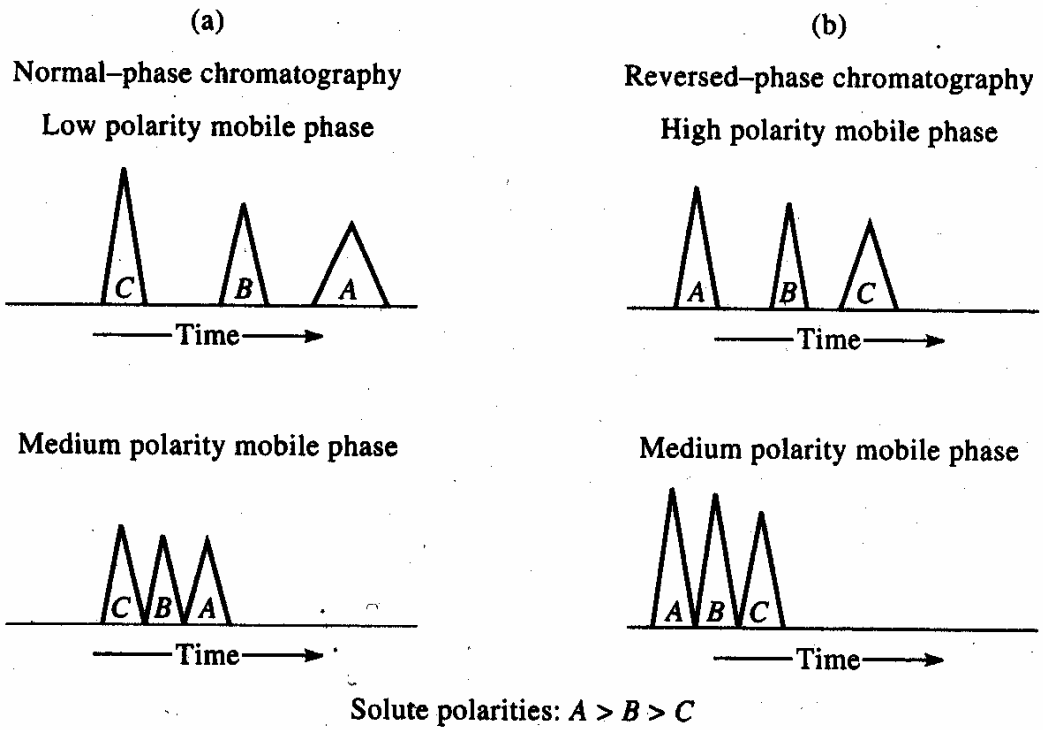


Figure 28-14 The relationship between polarity and elution times for normal-phase and reversed-phase chromatography.

Effect of Stationary Phase Material

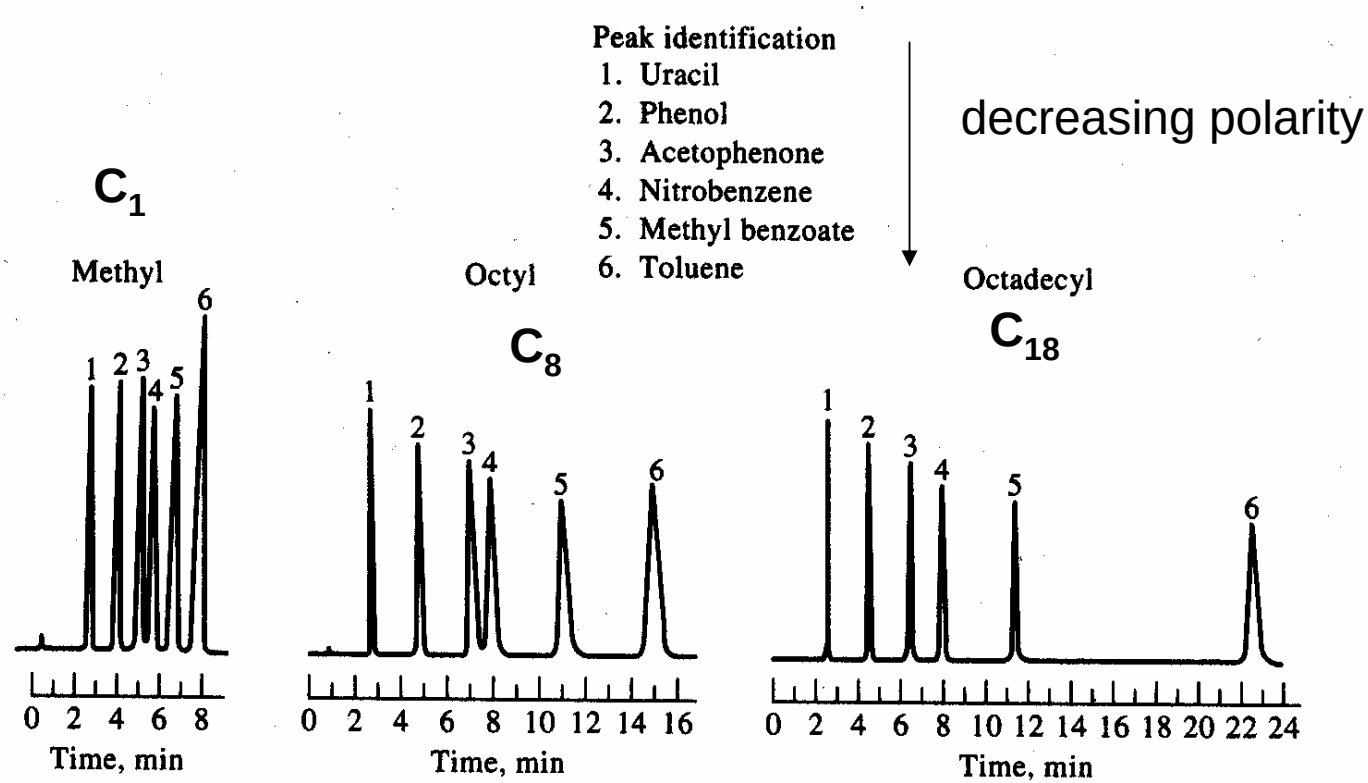


Figure 28-15 Effect of chain length on performance of reversed-phase siloxane columns packed with 5-μm particles. Mobile phase: 50/50 methanol/water. Flow rate: 1.0 mL/min.

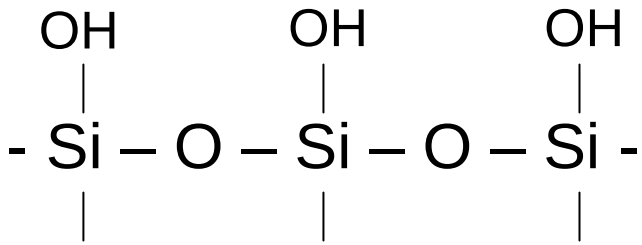
Reversed-phase

Separations

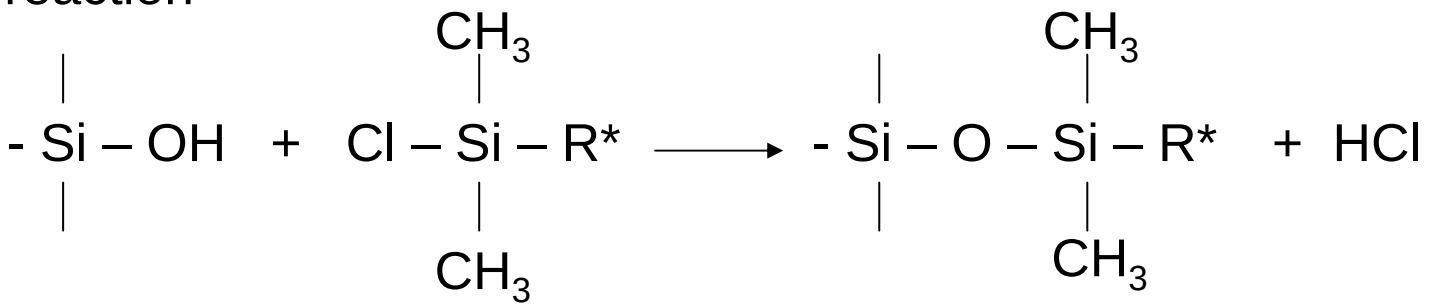
Successful chromatography with interactive mobile phases requires a proper balance of intermolecular forces among the *three* active participants in the process – the solute, the mobile phase and the stationary phase.

Stationary Phases for Reversed-Phase LC

The supports for the majority of the bonded-phase packings (bonded is more stable than simple coated phases) are prepared from rigid silica. Uniform particle size of 2-10 μm is typical. The surface of fully hydrolyzed silica contains *reactive* silanol groups at the surface.

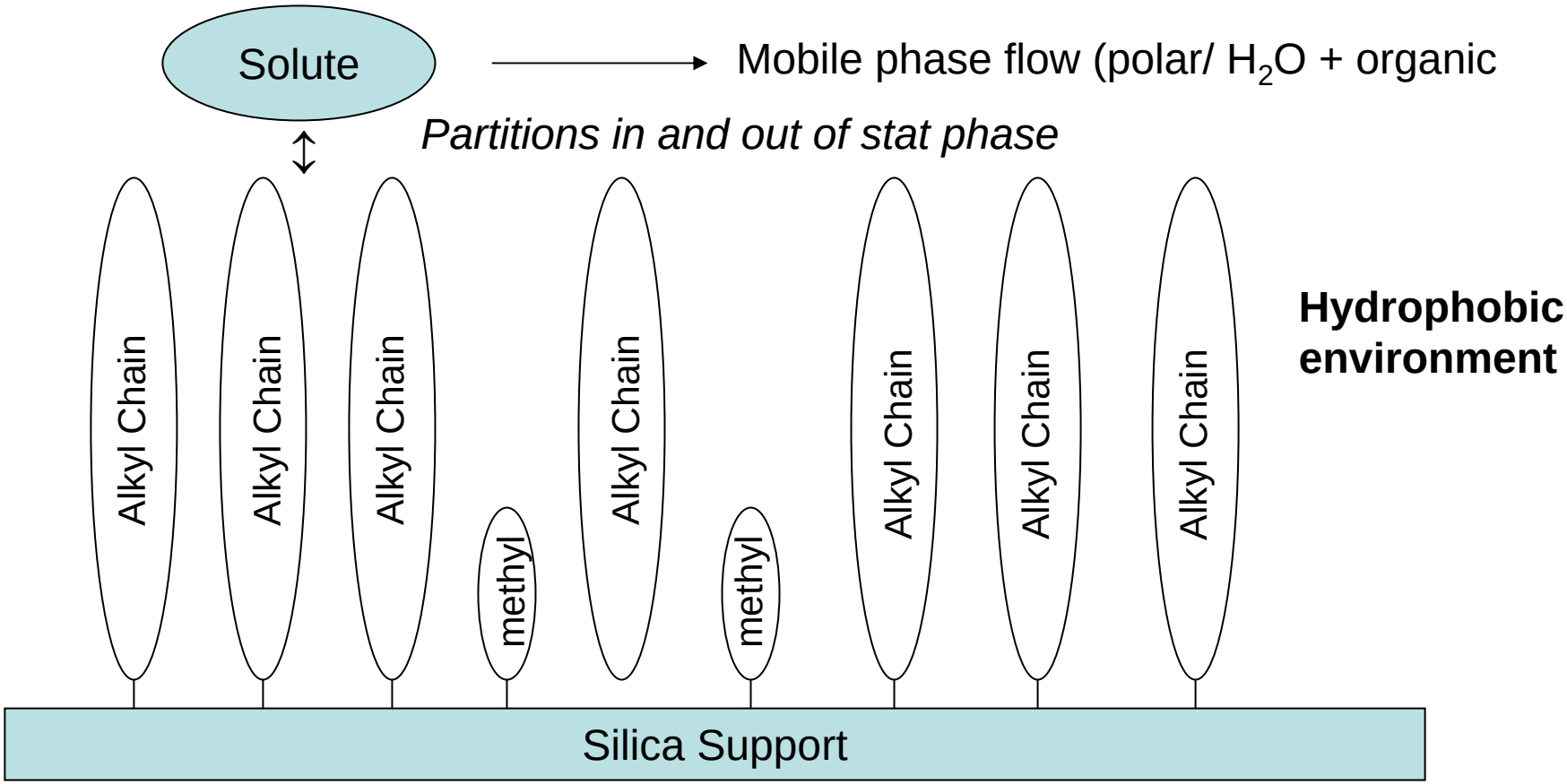


Silanization reaction



Stationary Phases for Reversed-Phase LC

Commonly, the R group is a C8 (n-octyl), C12 (n-octyl) or C18 (n-octyldecyl). Mobile phase is H_2O + a miscible organic solvent (acetonitrile, methanol, ethanol, isopropanol).

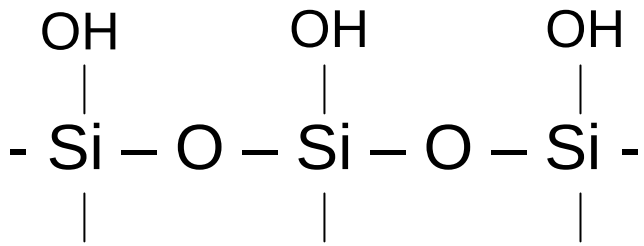


Separations

- In *reversed-phase* chromatography, the stationary phase is nonpolar, often a hydrocarbon, and the mobile phase is relatively polar (such as water, methanol or acetonitrile).
- The most polar component elutes first and *increasing* the polarity of the mobile phase increases the elution time.

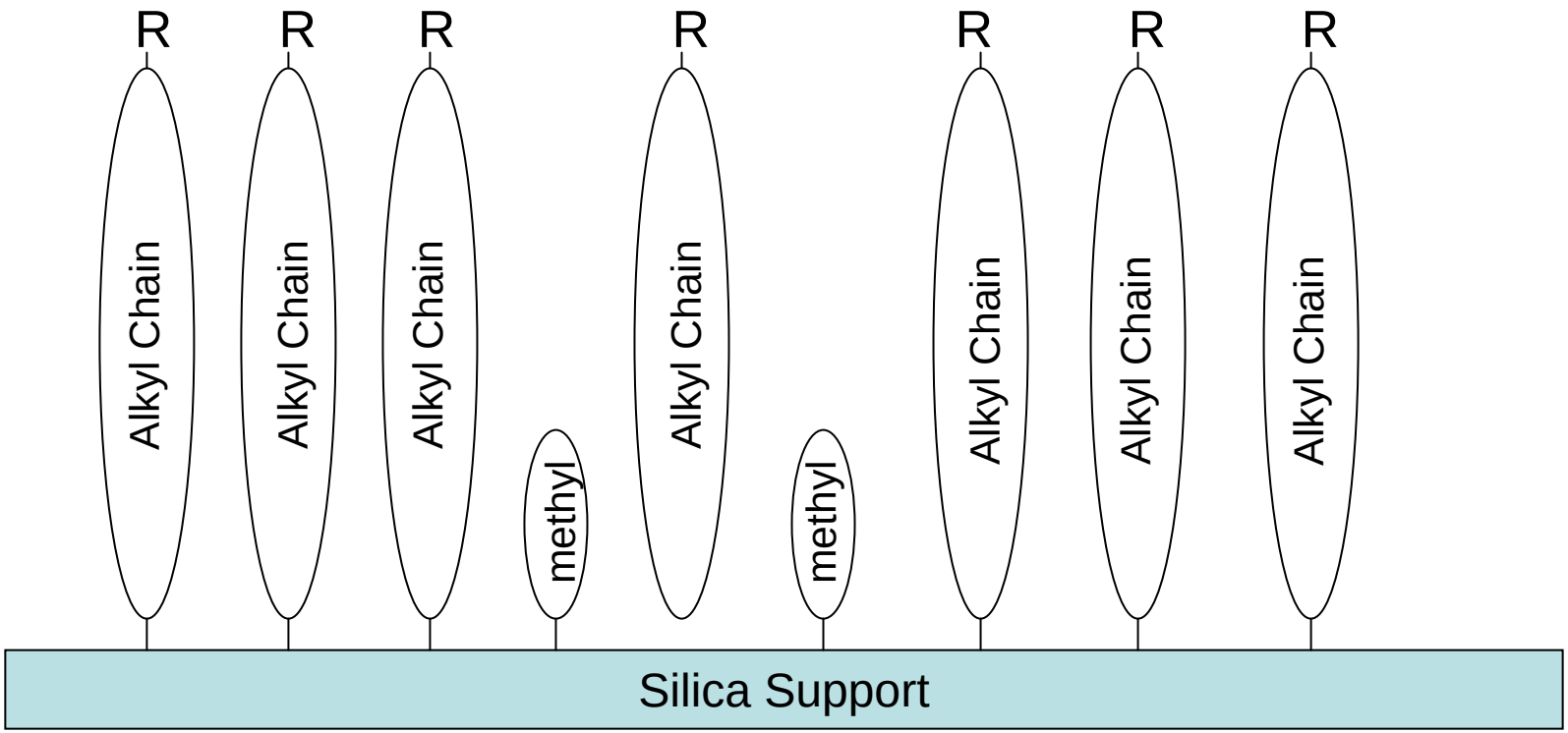
- In *normal-phase* chromatography, the stationary phase is polar, often the silica itself, and the mobile phase is relatively nonpolar (such as hexane, i-propylether, toluene, etc.).
- The least polar component elutes first (it is most soluble in the mobile phase) and increasing the polarity of the mobile phase *decreases* the elution time.

Stationary Phases for Normal-Phase LC



The polar silica surface can be used without modification.

R = cyano (-C_2H_4-CN), amino (-$C_2H_6NH_2$), diol (-$C_3H_6OCH_2CHOHCH_2OH$)



Solvents and Properties

TABLE 28-2 Properties of Common Chromatographic Mobile Phases

Solvent	Refractive Index[a]	Viscosity, cP[b]	Boiling Point, °C	Polarity Index, P'	Eluent Strength,[c] ϵ^0
Fluoroalkanes[d]	1.27–1.29	0.4–2.6	50–174	<−2	−0.25
Cyclohexane	1.423	0.90	81	0.04	−0.2
n-Hexane	1.372	0.30	69	0.1	0.01
l-Chlorobutane	1.400	0.42	78	1.0	0.26
Carbon tetrachloride	1.457	0.90	77	1.6	0.18
i-Propyl ether	1.365	0.38	68	2.4	0.28
Toluene	1.494	0.55	110	2.4	0.29
Diethyl ether	1.350	0.24	35	2.8	0.38
Tetrahydrofuran	1.405	0.46	66	4.0	0.57
Chloroform	1.443	0.53	61	4.1	0.40
Ethanol	1.359	1.08	78	4.3	0.88
Ethyl acetate	1.370	0.43	77	4.4	0.58
Dioxane	1.420	1.2	101	4.8	0.56
Methanol	1.326	0.54	65	5.1	0.95
Acetonitrile	1.341	0.34	82	5.8	0.65
Nitromethane	1.380	0.61	101	6.0	0.64
Ethylene glycol	1.431	16.5	182	6.9	1.11
Water	1.333	0.89	100	10.2	Large

[a] At 25°C.
[b] The centipoise is a common unit of viscosity; in SI units, 1 cP = 1 $mN \cdot s \cdot m^{-2}$.
[c] On Al_2O_3. Multiplication by 0.8 gives ϵ^0 on SiO_2.
[d] Properties depend upon molecular weight. Range of data given.

Application Areas

TABLE 28-3 Typical Applications of Partition Chromatography

Field	Typical Mixtures
Pharmaceuticals	Antibiotics, Sedatives, Steroids, Analgesics
Biochemical	Amino acids, Proteins, Carbohydrates, Lipids
Food products	Artificial sweeteners, Antioxidants, Aflatoxins, Additives
Industrial chemicals	Condensed aromatics, Surfactants, Propellants, Dyes
Pollutants	Pesticides, Herbicides, Phenols, PCBs
Forensic chemistry	Drugs, Poisons, Blood alcohol, Narcotics
Clinical medicine	Bile acids, Drug metabolites, Urine extracts, Estrogens