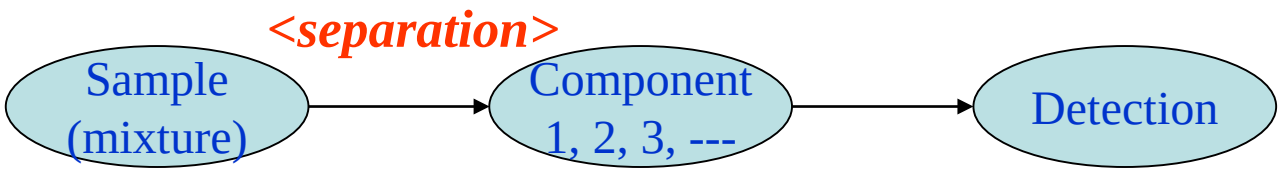
Chapter 26:

Introduction to Chromatographic Separations

In real analytical problems,
we must identify and quantitate one or more components from a complex mixture

Separation of mixture into each component is the first step in analysis

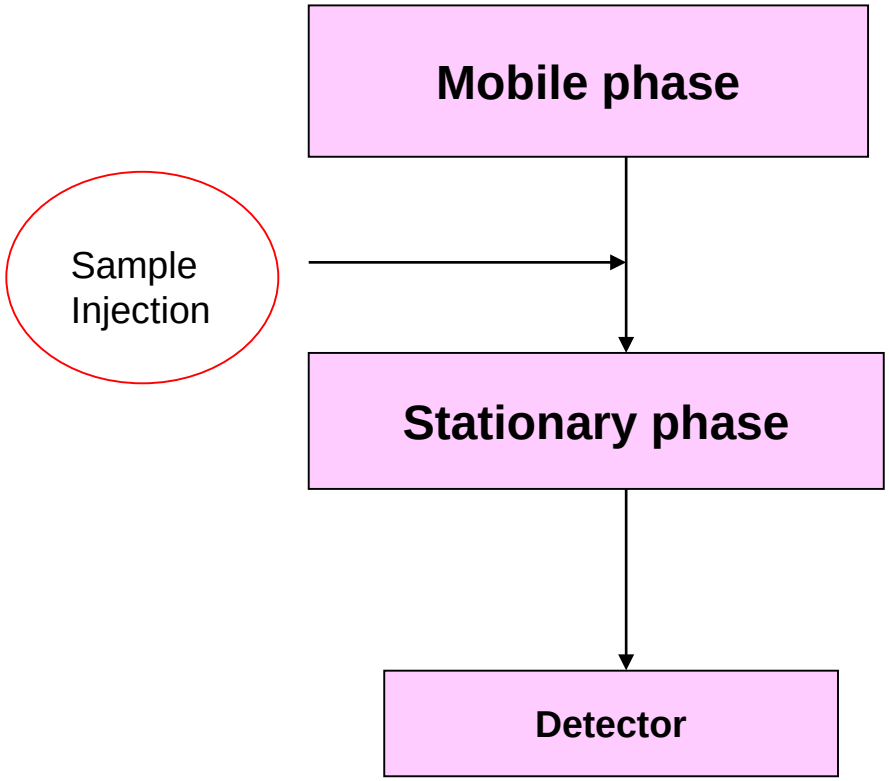


- Optical (absorbance, FL, CL)
- Electrochemical (voltammetry)
- Mass-to-charge

What is Chromatography?

Martin and Synge: Nobel Prize in 1952

-Chromatography operates on the same principle as extraction, but one phase is held in place while the other moves past it.



Gas: gas chromatography
Liquid: liquid chromatography

Solid: GSC, LSC
Liquid: GLC, LLC (partition chromatography)

TABLE 26-1 Classification of Column Chromatographic Methods

General Classification	Specific Method	Stationary Phase	Type of Equilibrium
1. Gas chromatography (GC)	a. Gas-liquid chromatography (GLC)	Liquid adsorbed or bonded to a solid surface	Partition between gas and liquid
	b. Gas-solid	Solid	Adsorption
2. Liquid chromatography (LC)	a. Liquid-liquid, or partition	Liquid adsorbed or bonded to a solid surface	Partition between immiscible liquids
	b. Liquid-solid, or adsorption	Solid	Adsorption
	c. Ion exchange	Ion-exchange resin	Ion exchange
	d. Size exclusion	Liquid in interstices of a polymeric solid	Partition/sieving
	e. Affinity	Group specific liquid bonded to a solid surface	Partition between surface liquid and mobile liquid
3. Supercritical fluid chromatography (SFC; mobile phase: supercritical fluid)		Organic species bonded to a solid surface	Partition between supercritical fluid and bonded surface

Ion-exchange chromatography

Stat. phase:
- SO_3^-
- $N(CH_3)_3^+$

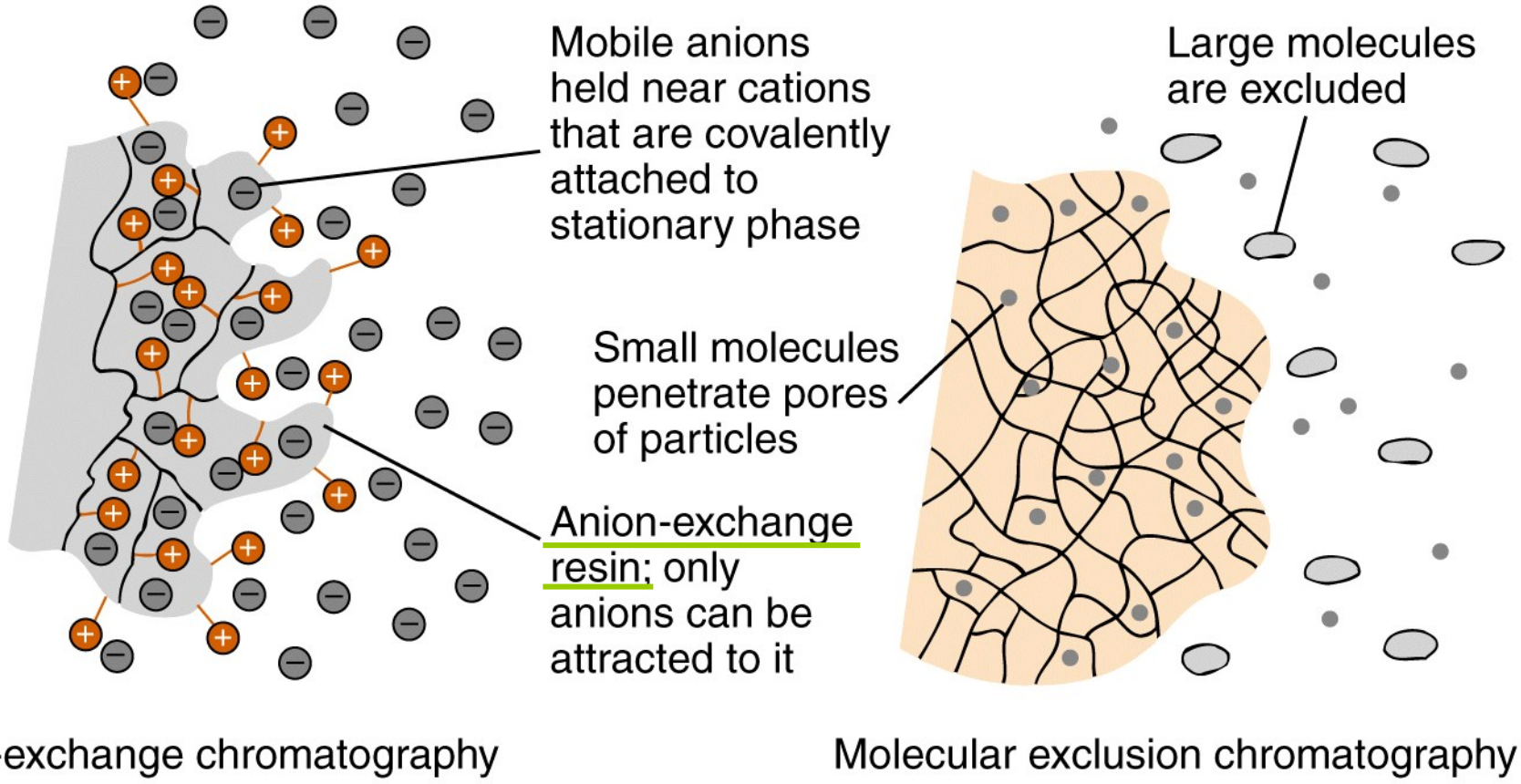
Mobile phase: liquid

Molecular exclusion chromatography

: Gel permeation c. (GPC), Gel filtration C

Stat. phase: gel

Mobile phase: liquid

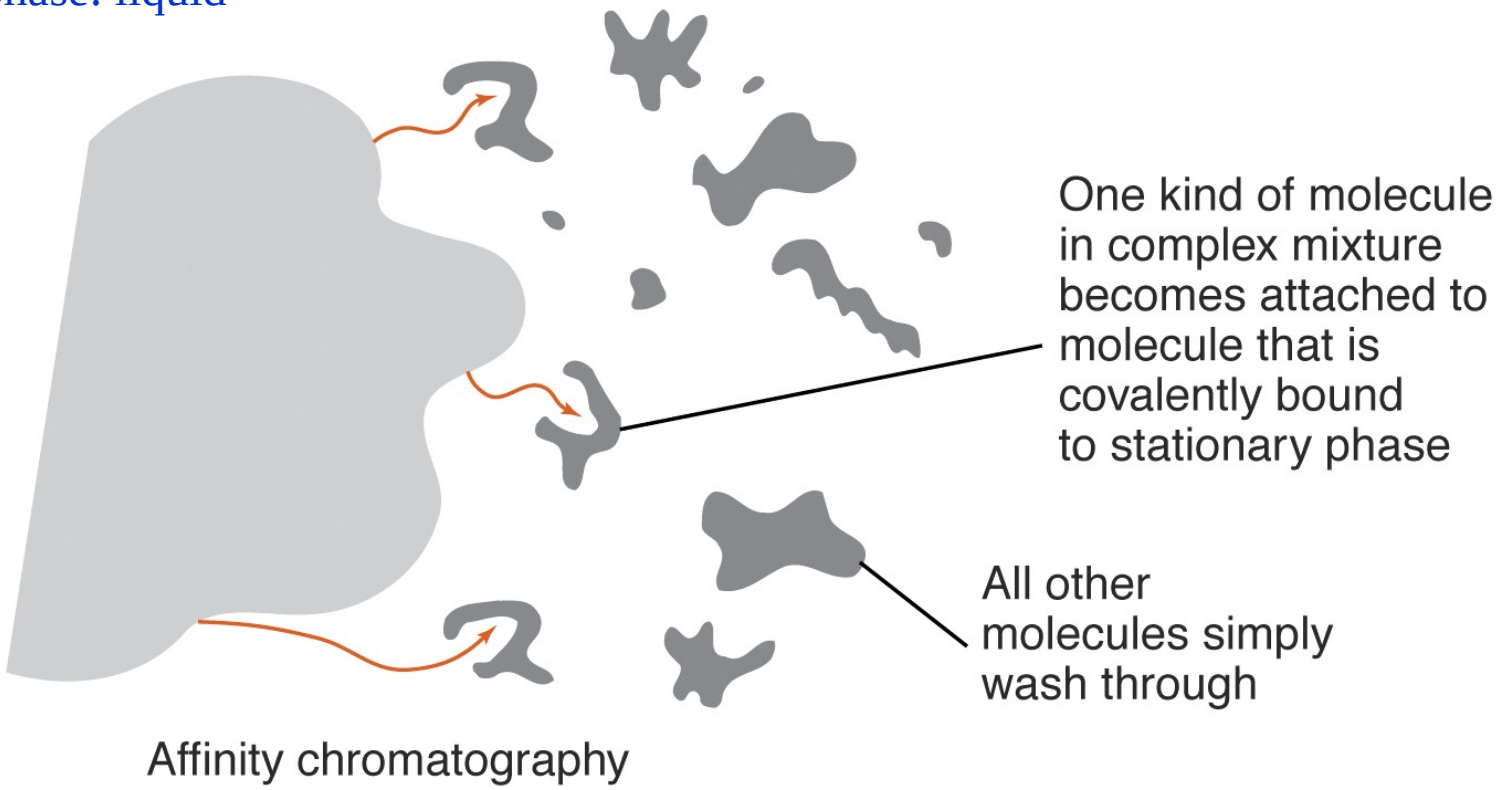


Affinity chromatography

Stat. phase:
- Immobilized antibody
- Specific protein

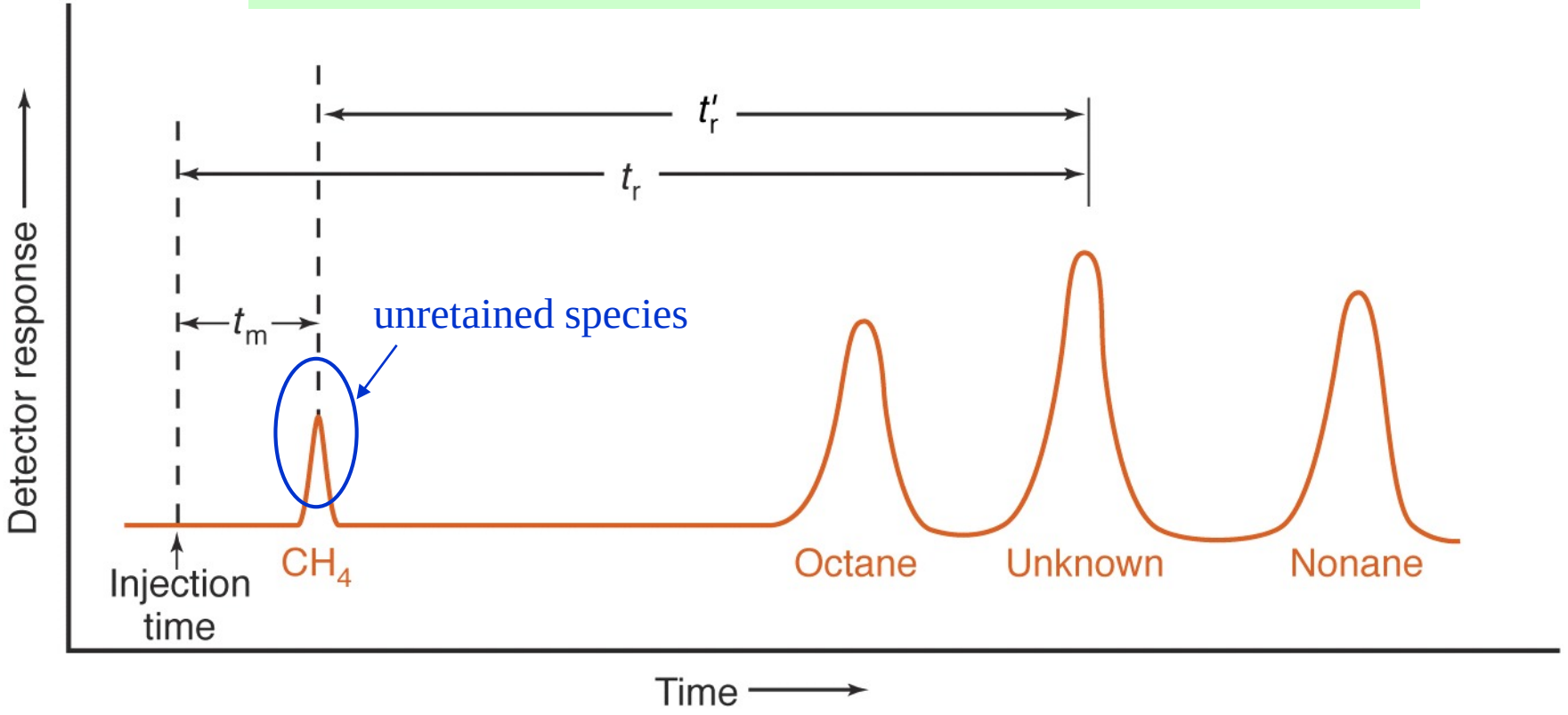
Mobile phase: liquid

The most selective kind of chromatography



Affinity chromatography

Chromatogram



- ***Retention time*** for each component: t_r
- ***Dead time*** for unretained species: t_m
- ***Adjusted retention time*** (t_r') = $t_r - t_m$
- ***Capacity factor*** (k') = $(t_r - t_m)/t_m = t_r'/t_m$
- ***Relative retention*** (α) for any two components (A, B) = $(t_r')_B / (t_r')_A = (k')_B / (k')_A$

Selectivity factor

$= K_B / K_A$ (partition coefficient)

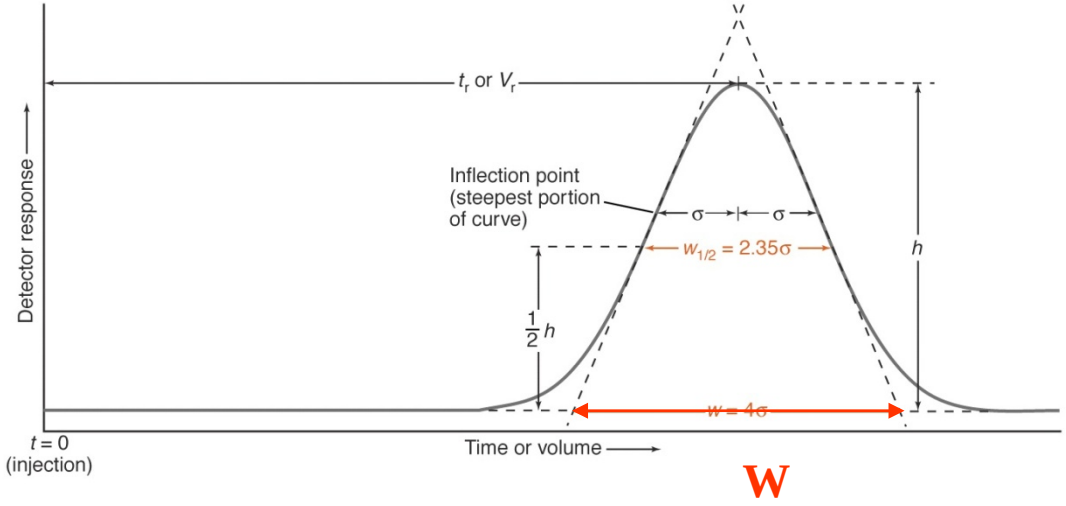
Efficiency of Separation

Two factors to determine separation efficiency:

- Difference in elution time between peaks (larger time difference → better separation)
- Broadness of the separated peaks (the wider the peaks → the poorer their separation)

- **Resolution** of two peaks from each other is defined as (R, R_s)

R_s = (distance between peaks)/(average base width)
= $(\Delta t_r)/[(W_1 + W_2)/2] \approx (\Delta t_r)/ W$ (in most case, $W_1 \approx W_2$)

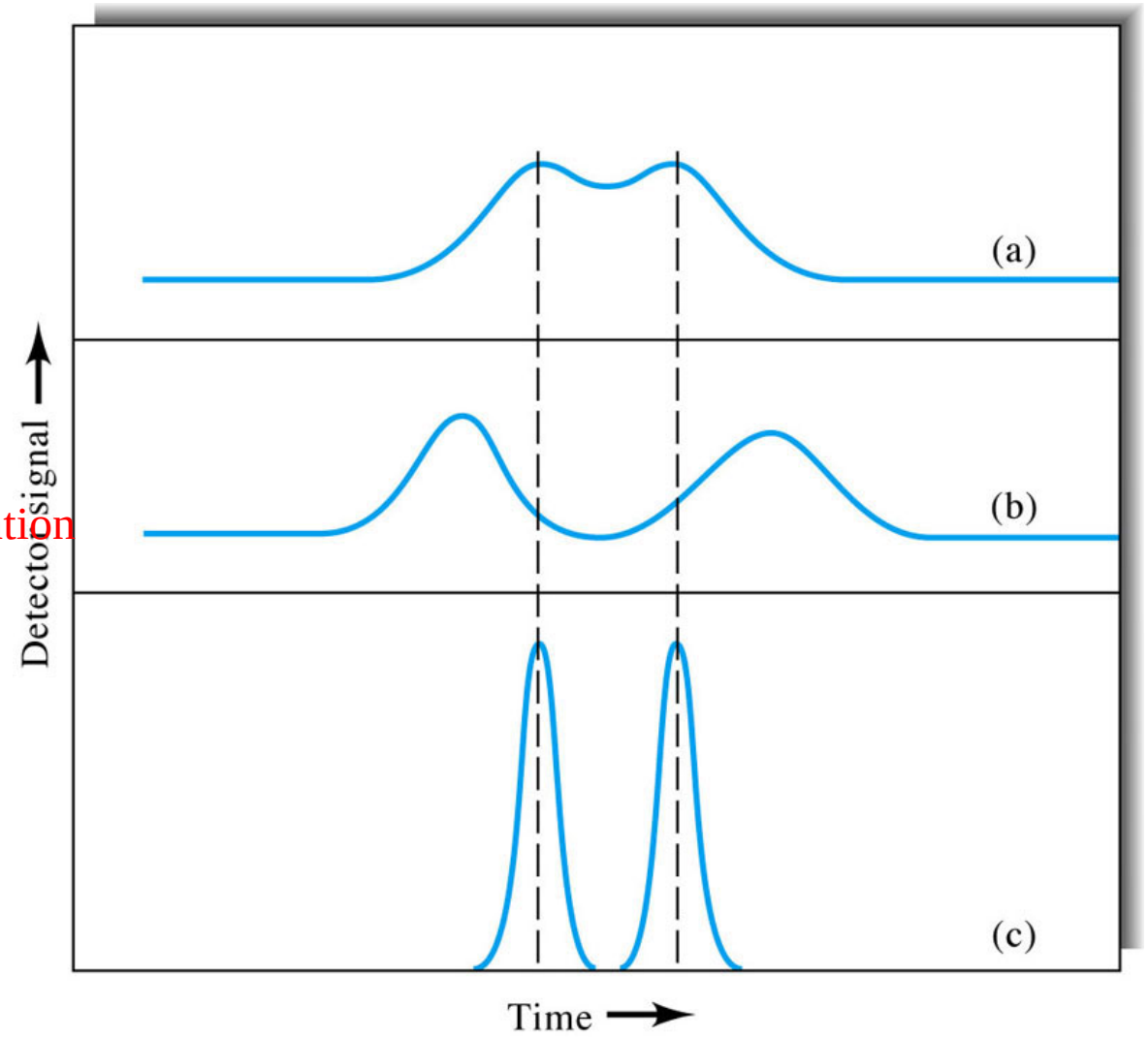


Efficiency of Separation

original

Increase in band separation

Decrease in band width



Gas Chromatography

Mobile phase (carrier gas): gas (He, N_2, H_2)

- do not interact with analytes
- only transport the analyte through the column

Analyte: volatile liquid or gas

Stationary phase:

- solid (GSC) or non-volatile liquid (GLC)

GSC (gas-solid adsorption chromatography)

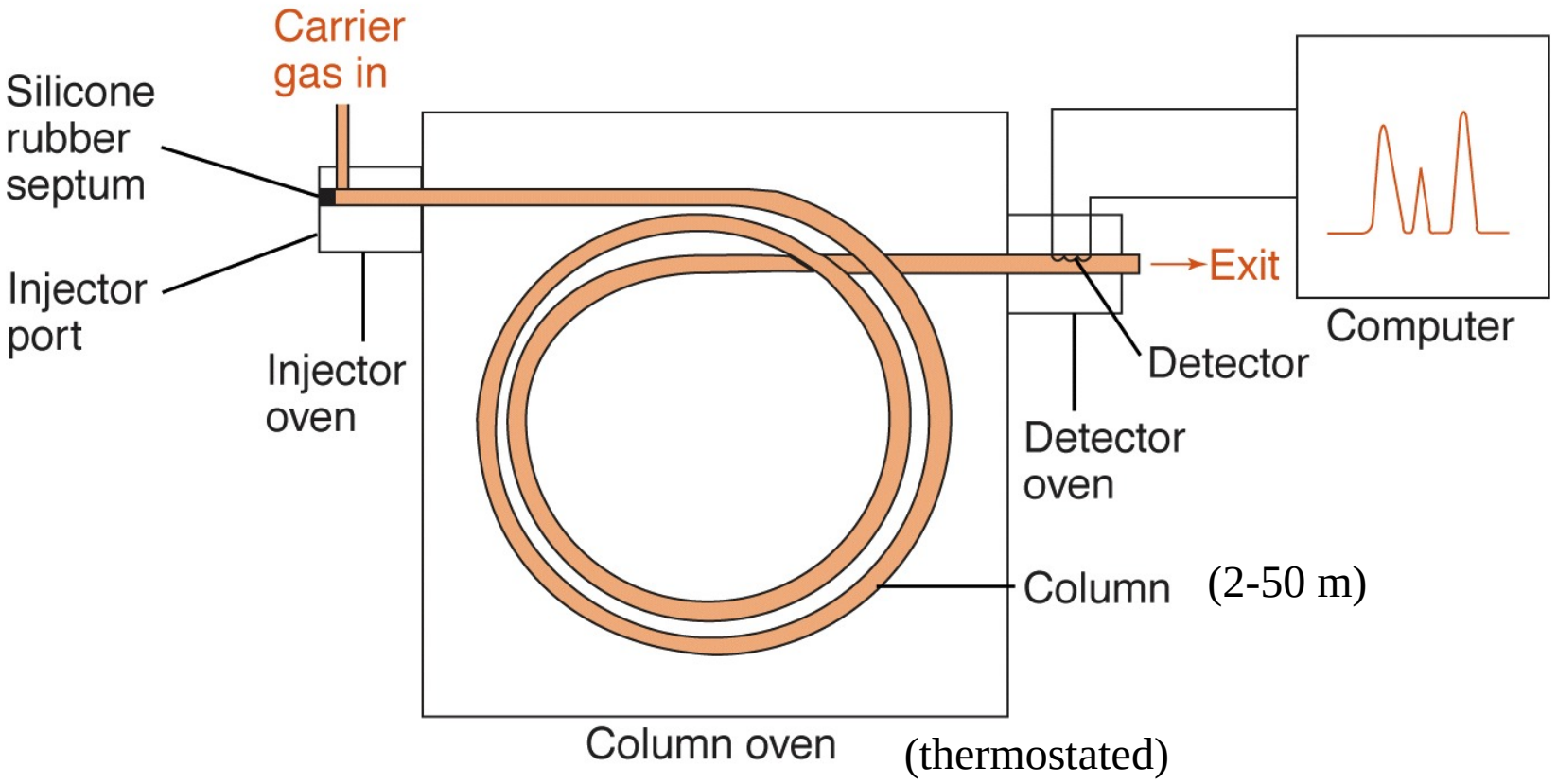
- semi-permanent retention of active or polar molecules
- severe tailing of elution peaks

GLC (gas-liquid partition chromatography)

- non-volatile liquid is coated on the inside of the column or on a fine solid support
- In 1955, the first commercial apparatus for GLC appeared on the market

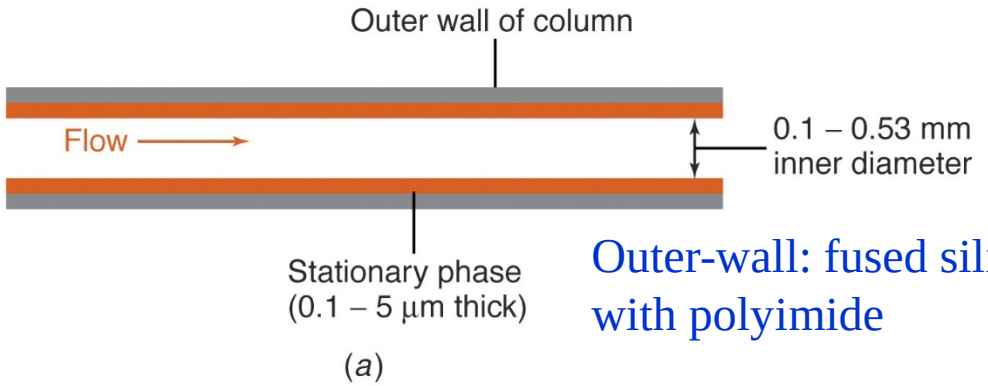
Instrument for gas chromatography

Temp of a sample injector port:
50 ^{o}C above the b.p. of least volatile component of the sample → rapidly evaporates



The column should be hot enough to provide sufficient vapor pressure for analyte to be eluted in a reasonable time.

Open Tubular Columns



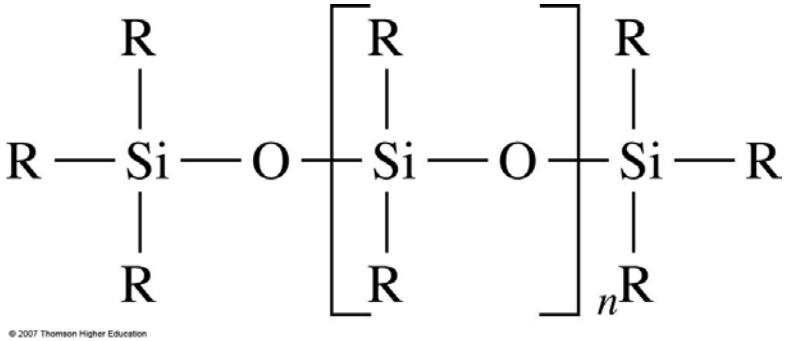
Outer-wall: fused silica coated with polyimide

- Higher N, smaller H → Higher resolution
  (H = A + B/U + CU, no A term in OTC)
- Higher flow rate → shorter analysis time

Thin coating: small C-term (decreased H) :

Compared with packed columns,
OTC offers higher resolution, shorter analysis time, greater sensitivity, lower sample capacity

Liquid Sta. Phase



Choice of liquid phase for a given problem:
"like dissolves like"

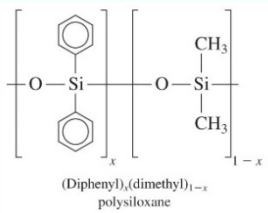
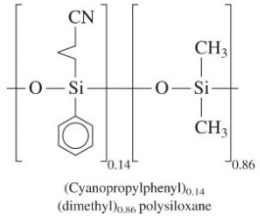
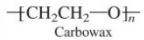
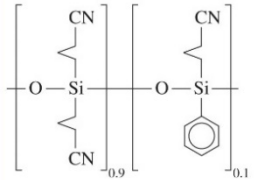
- Nonpolar columns: best for nonpolar solutes
- Polar columns for polar solutes

- As a column ages,

→ stationary phases bakes off
→ surface silanol groups (Si-OH) are exposed
→ peak tailing (polar analyte)

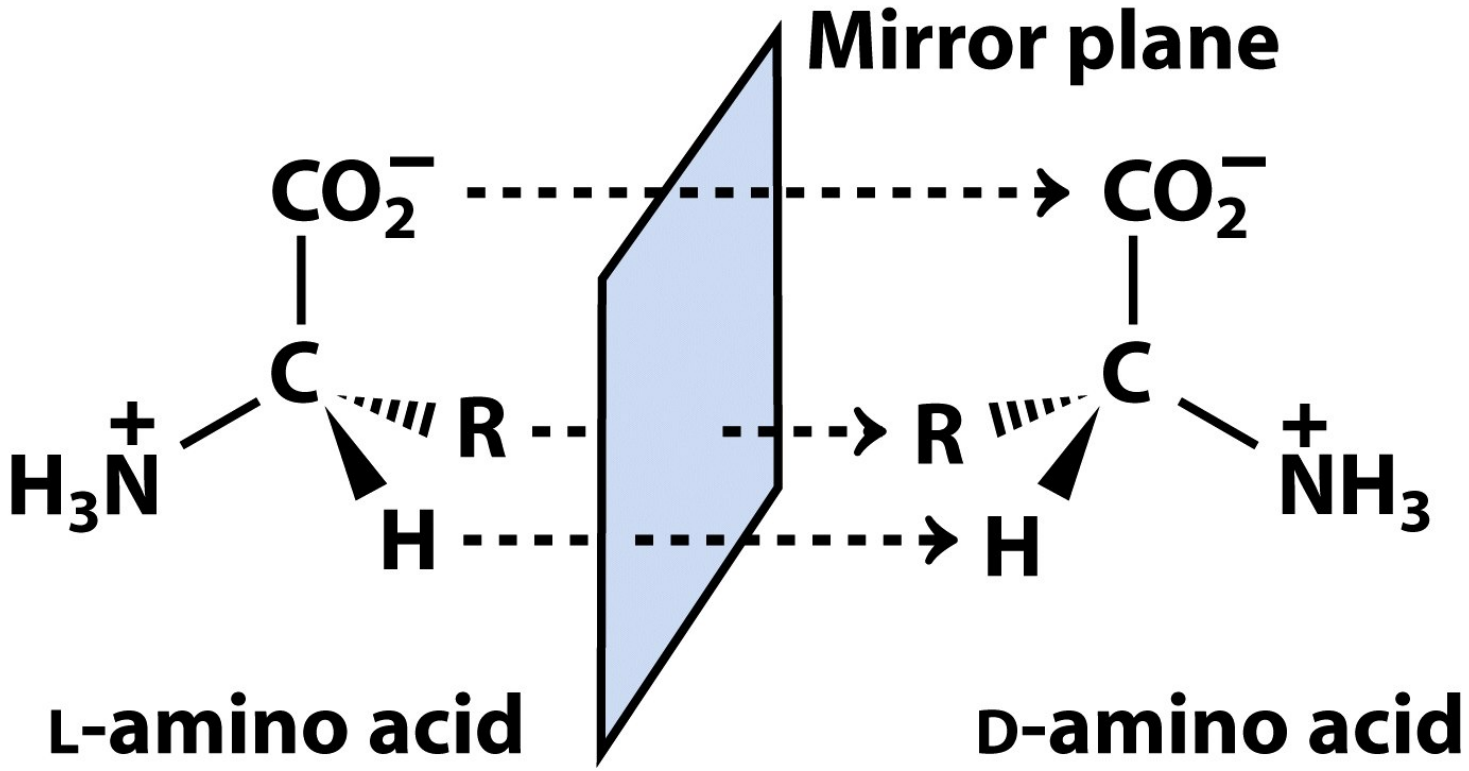
Therefore, stationary phase is covalently attached to silica surface

TABLE 23-1 Common stationary phases in capillary gas chromatography

Structure	Polarity		Temperature rang
 (Diphenyl)$_x$(dimethyl)$_{1-x}$ polysiloxane	$x = 0$	Nonpolar	−60°–320°
	$x = 0.05$	Nonpolar	−60°–320°
	$x = 0.35$	Intermediate polarity	0°–300°
	$x = 0.65$	Intermediate polarity	50°–370°
 (Cyanopropylphenyl)$_{0.14}$ (dimethyl)$_{0.86}$ polysiloxane	Intermediate polarity		−20°–280°
 Carbowax (poly(ethylene glycol))	Strongly polar		40°–250°
 (Biscyanopropyl)$_{0.9}$ (cyanopropylphenyl)$_{0.1}$ polysiloxane	Strongly polar		0°–275°

Harris, *Quantitative Chemical Analysis*, 8e

Chiral Separation



Unnumbered figure pg 533a
Quantitative Chemical Analysis, Seventh Edition

Quantitative and Qualitative Analysis by GC

Qualitative analysis:

- retention time (GC-FID, TCD, ECD…): comparison with authentic sample
- mass (GC-MS)

Quantitative analysis:

- peak area or peak height

TABLE 27-1 Typical Gas Chromatographic Detectors

Type	Applicable Samples	Typical Detection Limit
Flame ionization	Hydrocarbons	1 pg/s
Thermal conductivity	Universal detector	500 pg/mL
Electron capture	Halogenated compounds	5 fg/s
Mass spectrometer (MS)	Tunable for any species	0.25 to 100 pg
Thermionic	Nitrogen and phosphorous compounds	0.1 pg/s (P), 1 pg/s (N)
Electrolytic conductivity (Hall)	Compounds containing halogens, sulfur, or nitrogen	0.5 pg Cl/s, 2 pg S/s, 4 pg N/s
Photoionization	Compounds ionized by UV radiation	2 pg C/s
Fourier transform IR (FTIR)	Organic compounds	0.2 to 40 ng

Flame Ionization Detector (FID)

- Most widely used and generally applicable detector
- Column eluate is mixed with H_2/air and then burned in flame.

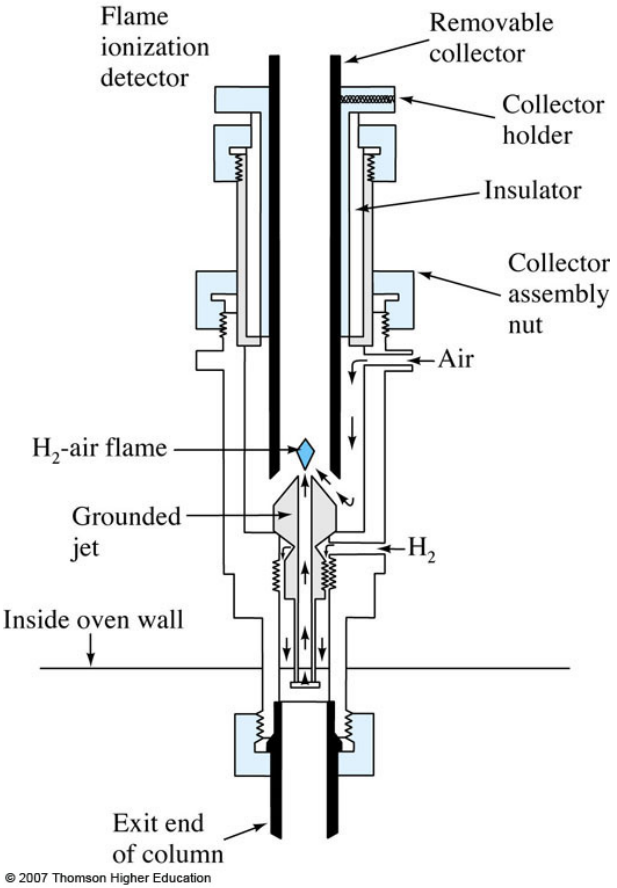
$$CH + O \rightarrow CHO^+ + e^-$$

- Most organic compounds, when pyrolyzed at the temp of H_2/air flame, produce ions (~$1/10^5$) and electrons that can conduct electricity through the flame

- FID responds to the # carbons entering the detector per unit time
: mass sensitive rather than concentration-sensitive

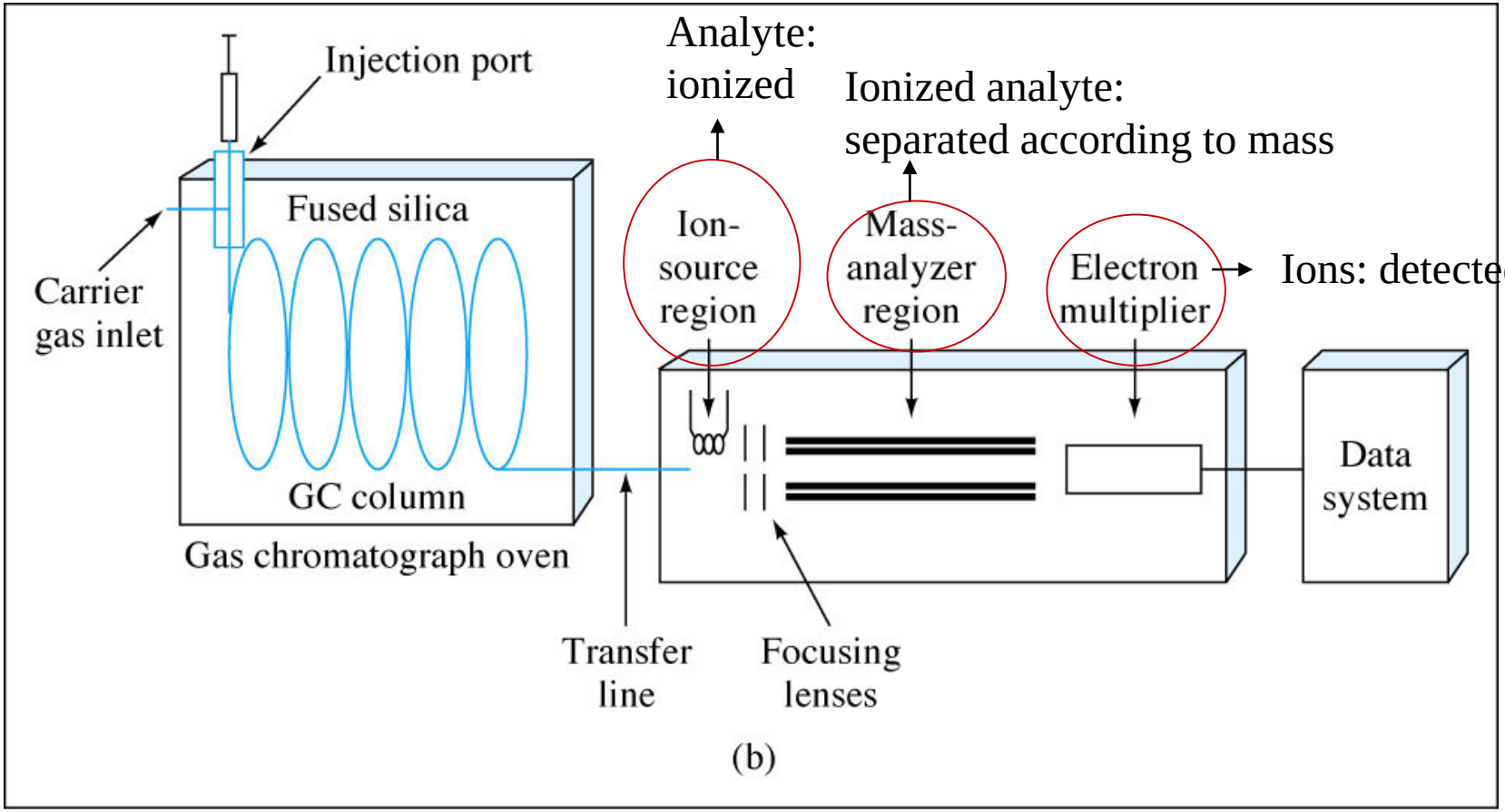
- Functional groups (carbonyl, carboxyl, halogen) yield fewer ions

- FID is insensitive to noncombustible gases (H_2O, CO_2, O_2, N_2, SO_2, and NOx)



GC-Mass Spectrometer

Mass spectrometer is a powerful detector for both qualitative and quantitative analysis of analyte in gas or liquid chromatography



GC-Mass Spectrometer

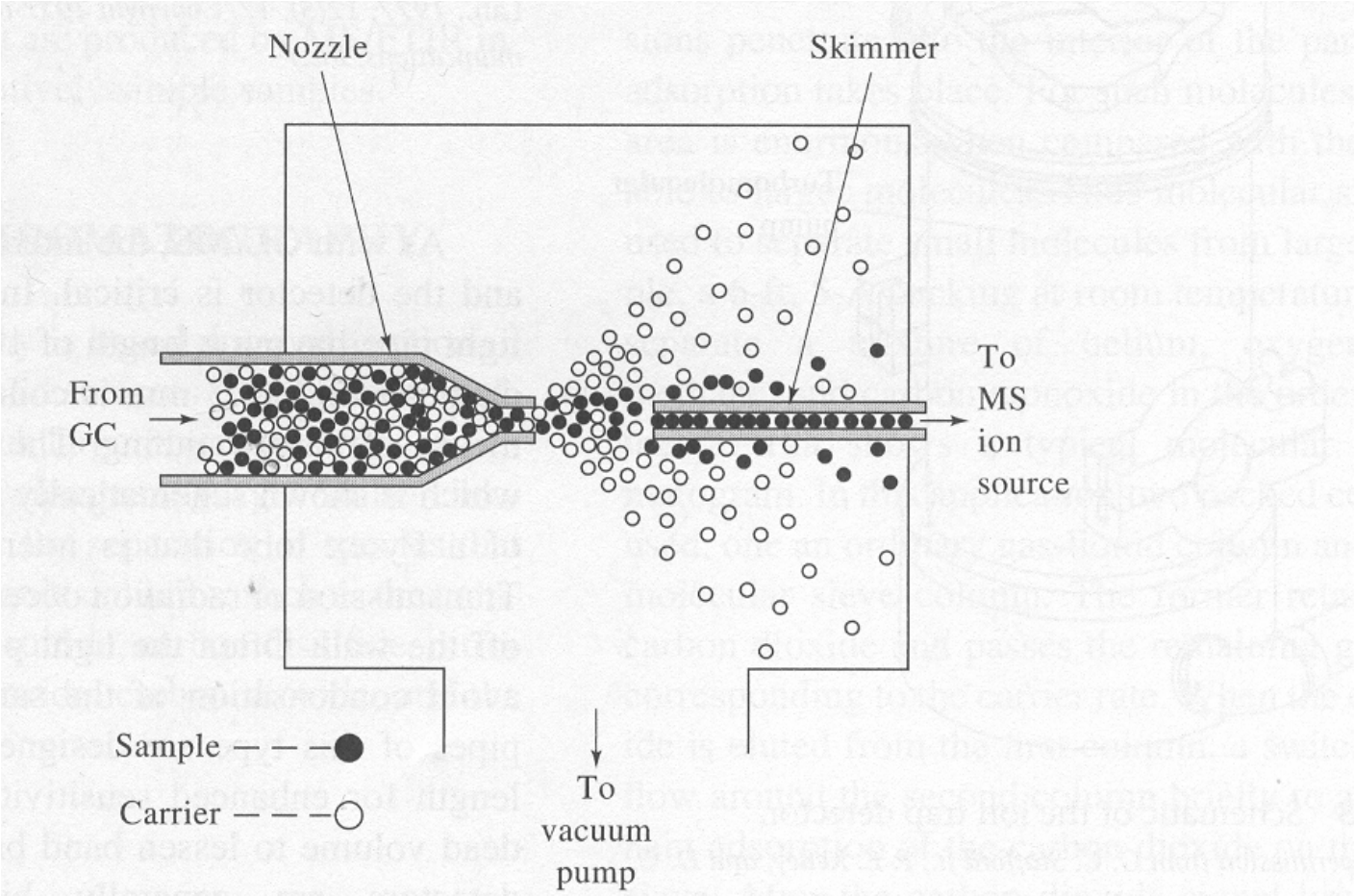


Figure 27-14 Schematic of a jet separator. *(Courtesy of DuPont Instrument Systems, Wilmington, DE.)*

HPLC

Mobile phase: liquid

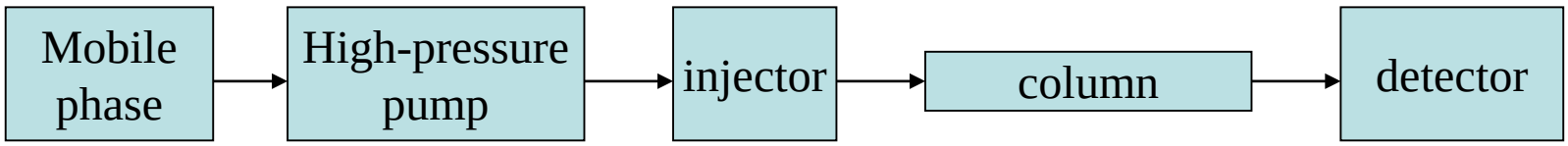
Analyte: non-volatile liquid

Stationary phase:

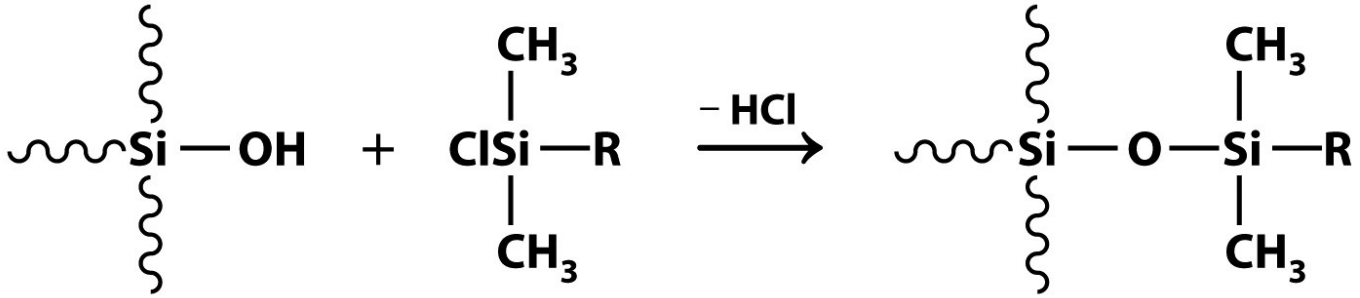
- solid (GSC) or non-volatile liquid (GLC)

HPLC; uses high-pressure pump to deliver liquid mobile phase

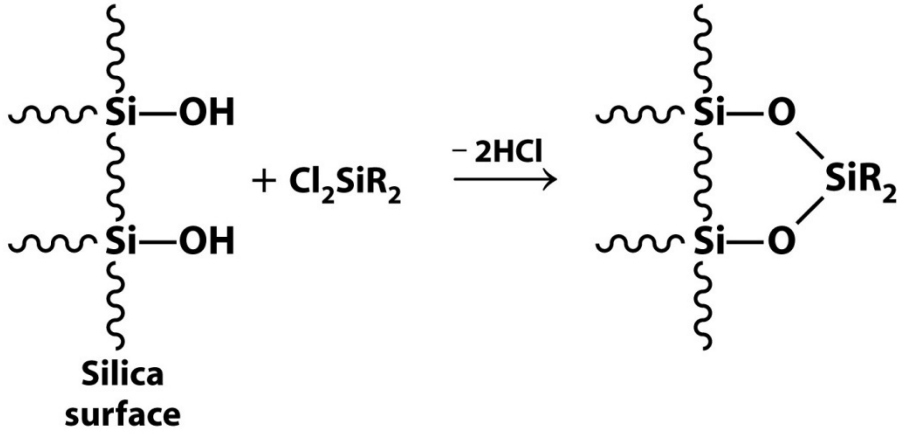
<HPLC system>



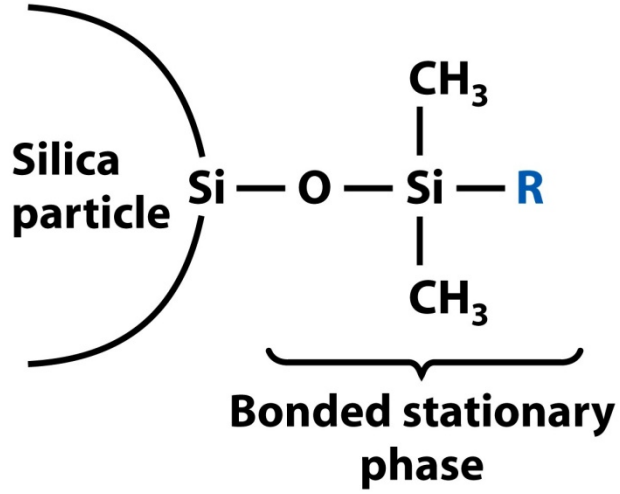
Bonded Stationary Phase for Partition Chromatography



Unnumbered figure pg 561a
Quantitative Chemical Analysis, Seventh Edition



Unnumbered figure pg 561b
Quantitative Chemical Analysis, Seventh Edition



Unnumbered figure pg 561c
Quantitative Chemical Analysis, Seventh Edition

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

Bonded Stationary Phase

TABLE 24-5 Selection of bonded stationary phases for HPLC

Bonded group	Polarity	Retention mechanisms	Comments
C_{18}, C_8, C_4	Nonpolar	van der Waals	C_8 does not retain hydrophobic compounds as strongly as C_{18}
Phenyl	Nonpolar	Hydrophobic and pi-pi	
Cyano	Intermediate	Hydrophobic, dipole-dipole, and pi-pi	Resolves polar organic compounds by reversed-phase or normal-phase chromatography
Amino	Polar (—NH_2) or ionic (—NH_3^+)	Dipole-dipole and H-bonding	Normal-phase or ion-exchange separations; separates carbohydrates, polar organic compounds, and inorganic ions; reacts with aldehydes and ketones
Bare silica	Very polar	H-bonding	Normal-phase separations

For a free column selection tool, see http://www.usp.org/USPNF/columnsDB.html.

SOURCE: *C. S. Young and R. J. Weigand, "An Efficient Approach to Column Selection in HPLC Method Development," LCGC* ***2002,*** *20, 464.*

Detectors in HPLC

- Ultraviolet detector: most common
- Refractive index (universal)
- Fluorescence
- Electrochemical
- Conductivity (ion-exchange C)
- Mass spectrometry
- Chemi-(electrochemi-)luminescence

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

UV Detector

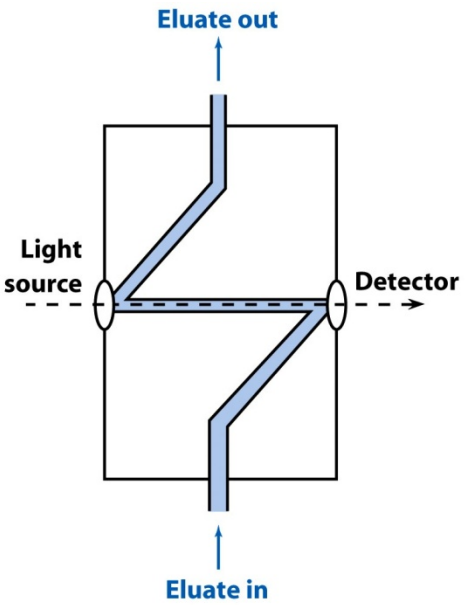


Figure 25-19
Quantitative Chemical Analysis, Seventh Edition

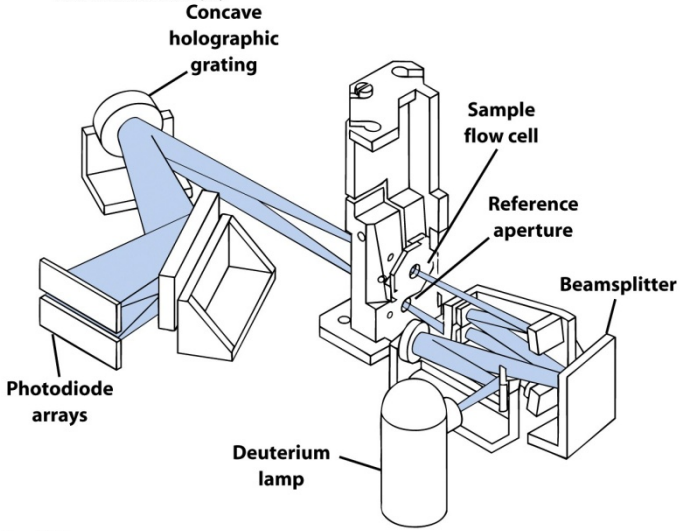


Figure 25-20a
Quantitative Chemical Analysis, Seventh Edition

PDA Detector

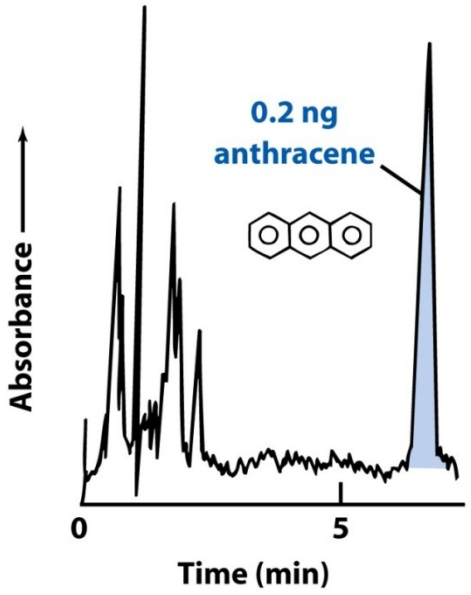


Figure 25-20b
Quantitative Chemical Analysis, Seventh Edition

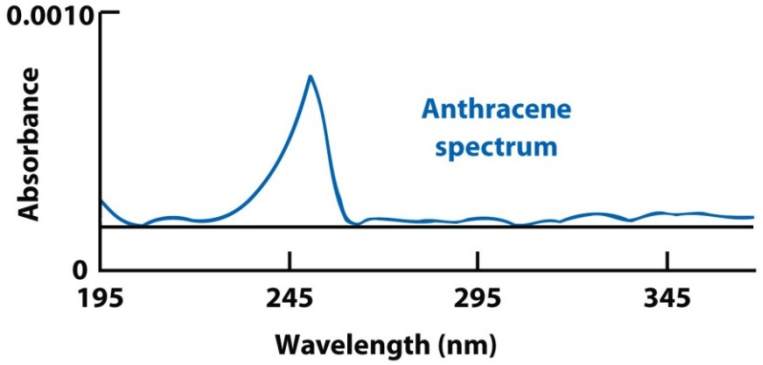
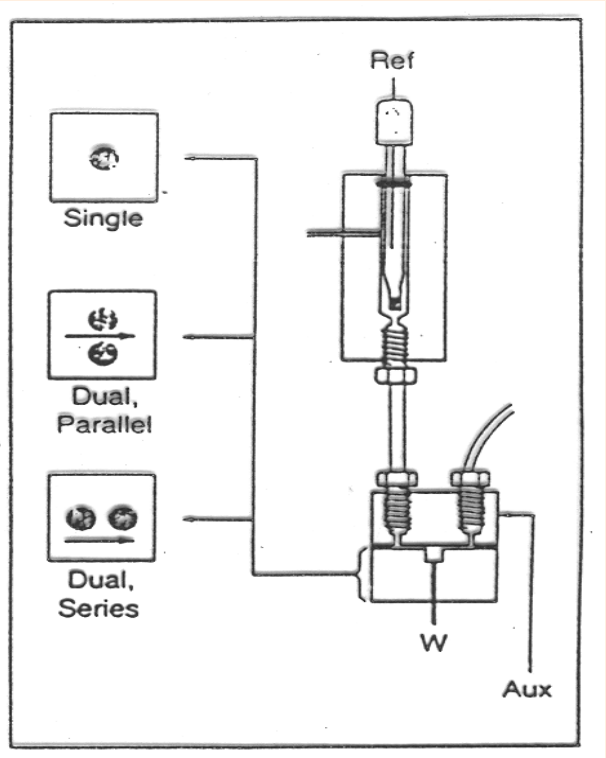


Figure 25-20c
Quantitative Chemical Analysis, Seventh Edition

Electrochemical Detector

An electrochemical detector responds to analytes that can be oxidized or reduced such as *phenols, aromatic compounds, peroxides, mercaptans, ketones, aldehydes, conjugated nitriles, aromatic halogen compounds, and aromatic nitro compounds*



Pulsed electrochemical detection for carbohydrate

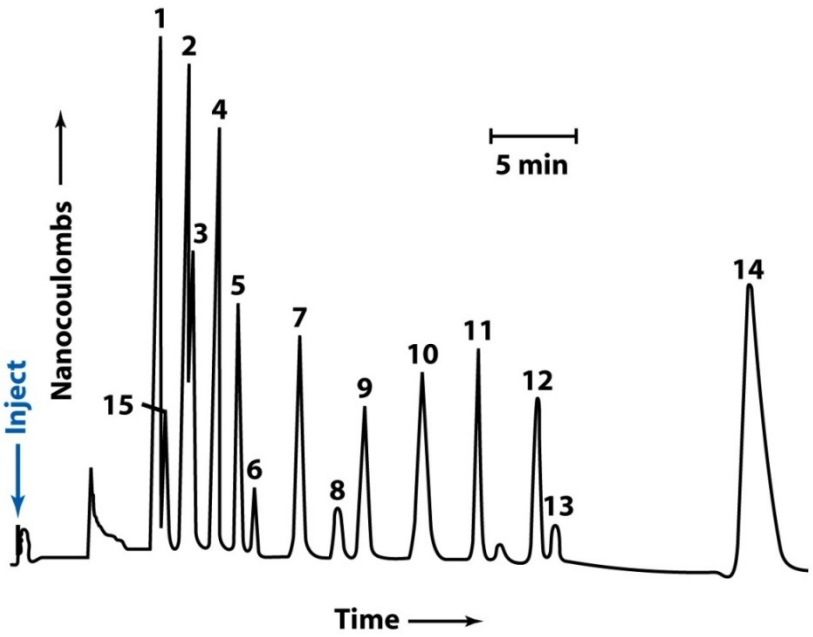
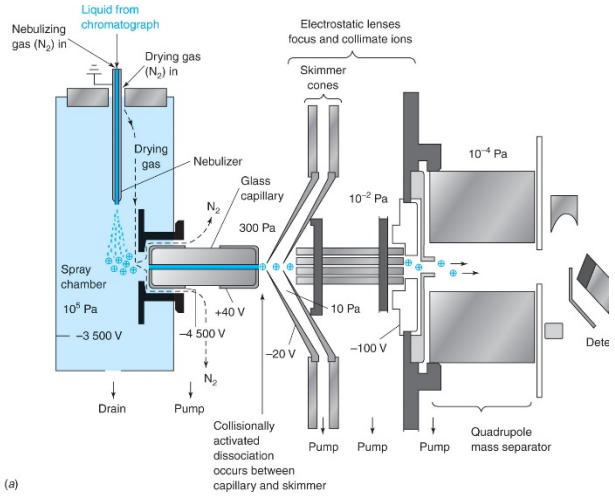


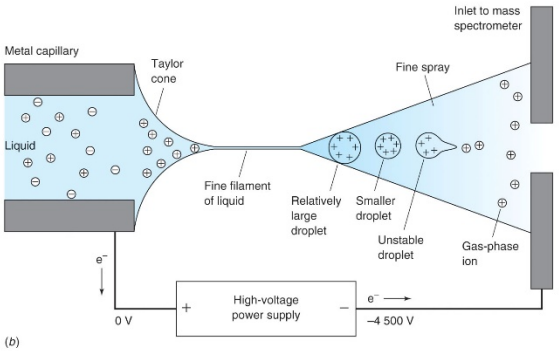
Figure 25-23
Quantitative Chemical Analysis, Seventh Edition

LC-Mass

Mass spectrometry requires high vacuum to prevent molecular collisions during ion separation

LC creates a huge volume of gas when solvent evaporizes at the interface between column and mass spectrometer
→ Most of this gas must be removed prior to ion separation





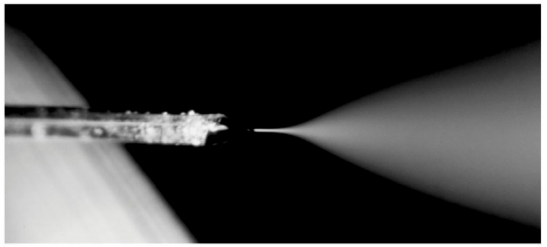
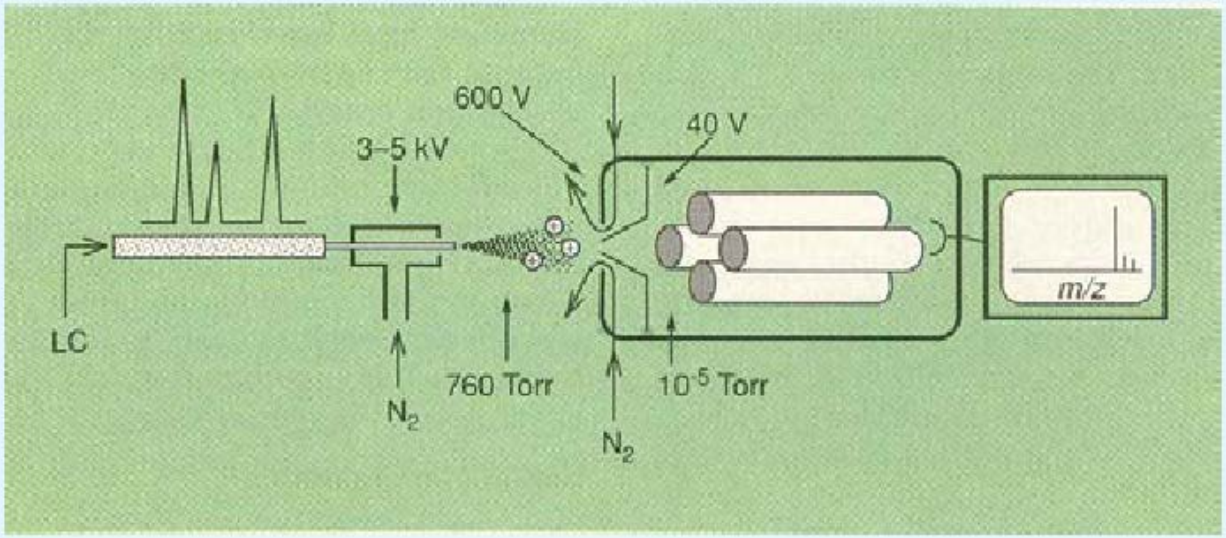


FIGURE 21-21 (*a*) Pneumatically assisted electrospray interface for mass spectrometry. (*b*) Gas-phase ion formation. [Adapted from E. C. Huang, T. Wachs, J. J. Conboy, and J. D. Henion, "Atmospheric Pressure Ionization Mass Spectrometry," *Anal. Chem.* **1990**, *62*, 713A and P. Kebarle and L. Tang, "From Ions in Solution to Ions in the Gas Phase," *Anal. Chem.* **1993**, *65*, 972A.] (*c*) **Electrospray from a silica capillary.** [Courtesy R. D. Smith, Pacific Northwest Laboratory, Richland, WA.]

Incompatibilities between HPLC and MS

- MS cannot accept HPLC solvent volume (HPLC 500-4,000 mL/min of gas MS pumps about 10-50 mL/min)

- Conventional gas phase ionization in MS not suitable for compounds separated by HPLC which are thermally labile, polar, or high molecular weight



Two most dominant ionization methods in LC-MS

: pneumatically assisted electrospray and atmospheric pressure chemical ionizaion (APCI)

Electrospray Ionization

- ESI takes places under atmospheric pressures and temperatures
- Sample is pumped through a SS capillary needle at a rate of few mL/min
- The needle is maintained at ~ kV against a cylindrical electrode that surround the needle
- Charged spray of fine droplet pass through a desolvating capillary

→ evaporation of solvent and formation of charged analyte

SS capillary needle (~kV)

2002 Nobel Prize: John B. Fenn

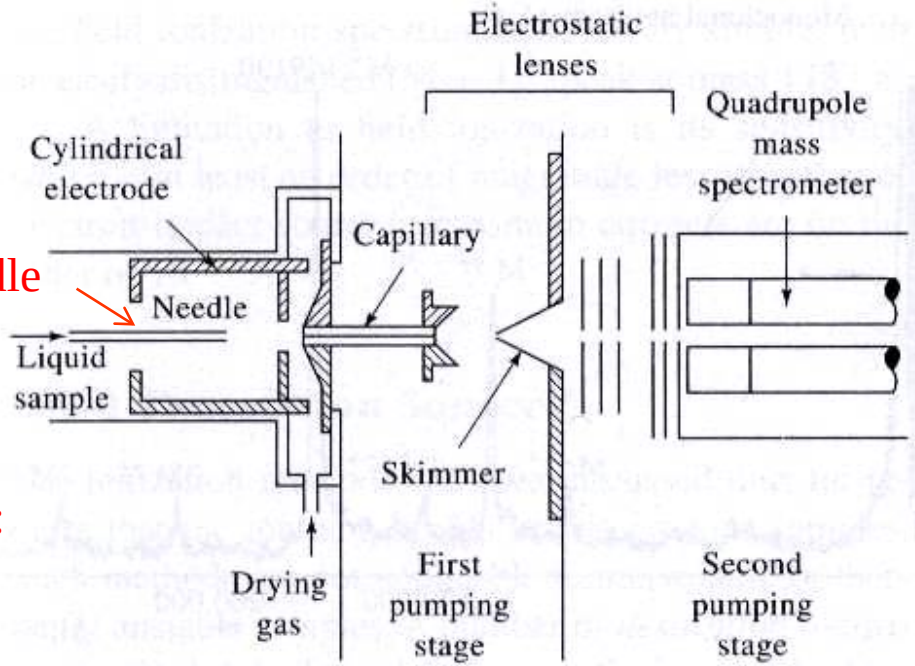


Figure 20-8 Apparatus for electrospray ionization. *(From J. B. Fenn et al., Science, 1989, 246, 65.)*

Chapter 30:

Capillary Electrophoresis

Electrophoresis

Electrophoresis: separation method based on differential rate of migration of charged species in a buffer solution under the influence of an electric filed

First developed by the Swedish chemist Arne Tiselius in the 1930s: 1948 Nobel Prize

Analytes: - inorganic anions, cations
- amino acids
- carbohydrates
- peptides, proteins
- nucleic acids, polynucleotides

Special strength of electrophoresis: separation of charged macromolecules
- proteins (enzymes, hormons, antibodies)
- nucleic acids (DNA, RNA)

Basis for Electrophoresis

$v = \mu_e E$

v: migration velocity of an ion (cm s^{-1})
μ_e: electrophoretic mobility (cm^2 V^{-1} s^{-1})
E: electric field (V cm^{-1})

Electrophoretic mobility(μ_e)

- Proportional to the ionic charge on the analytes
- Inversely proportional to frictional retarding force determined by
    (1) size and shape of the ion
    (2) the viscosity of the medium in which analyte migrates

For the same size:
The greater the charge → the greater the driving force → faster migration

For the ions of the same charge:
Smaller ion → smaller frictional force → faster migration

Therefore, the ion's charge-to-size ratio determines the electrophoretic mobility

Capillary Electrophoresis (CE)

Capillary Electrophoresis:

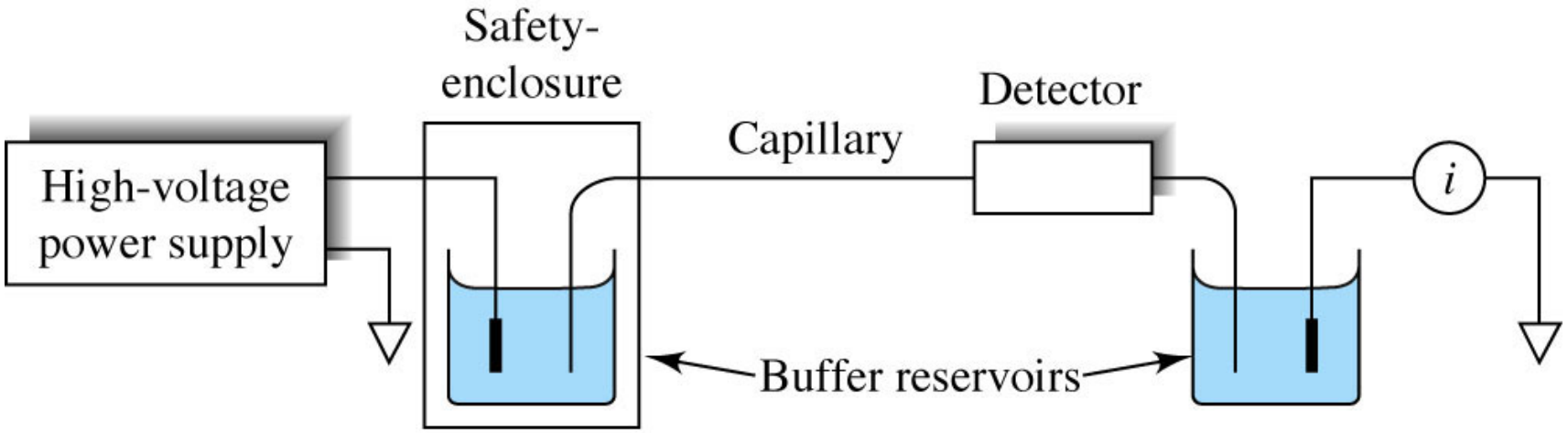
- Use of fused silica (SiO_2) capillary tube (50 cm long, inner diameter: 25-75 μm)
- Electric field: 30 kV
- High speed, high-resolution separations
- Exceptionally small sample volumes (0.1 – 10 nL)

Migration rate:

$v = \mu_e \bullet (V/L)$

v: migration rate
V: applied voltage in volts
L: length over which the voltage is applied

Higher voltage → higher separation speed



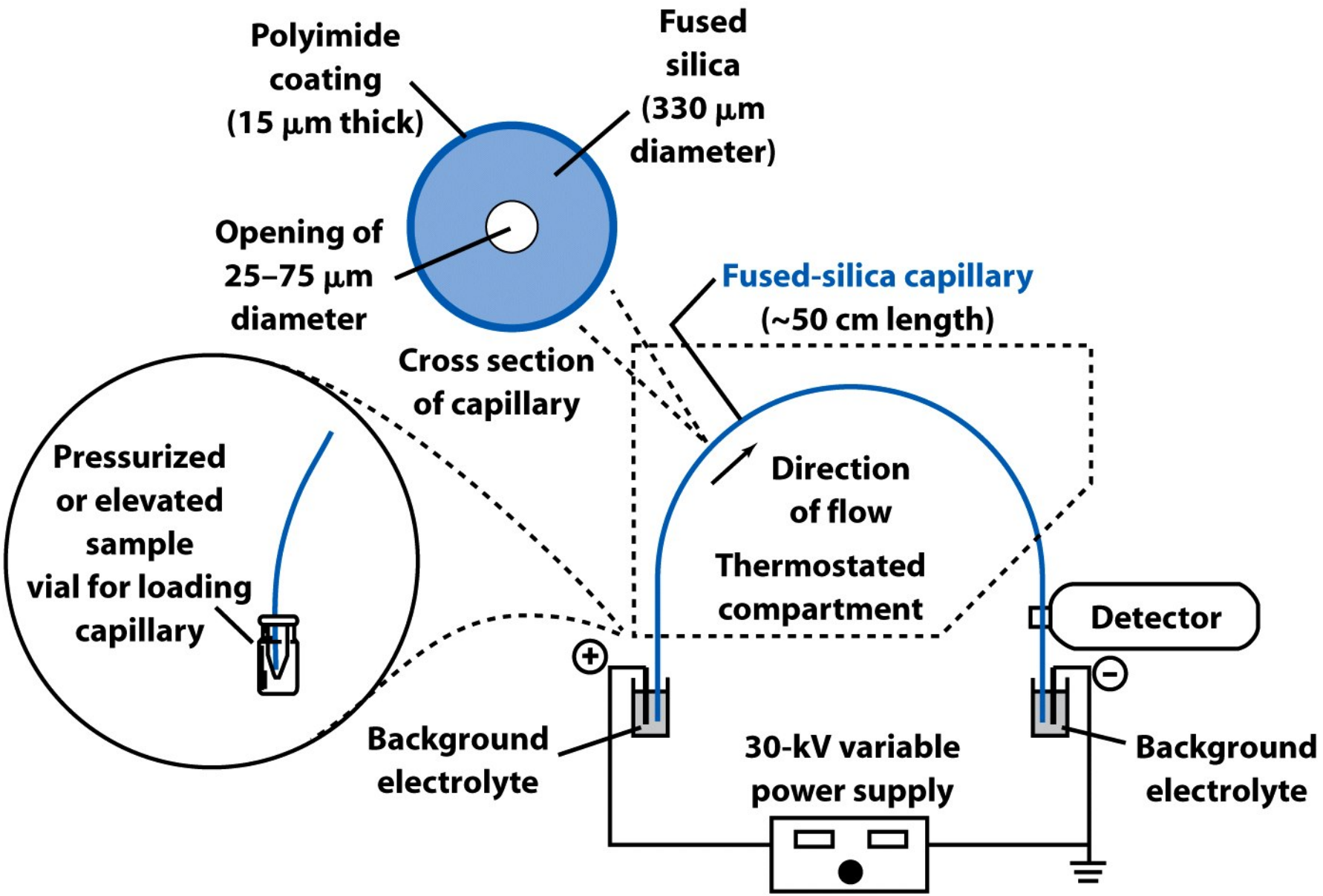


Figure 26-17
Quantitative Chemical Analysis, Seventh Edition

Electroosmosis

The inside of a fused silica wall is covered with silanol (Si-OH) groups with a negative charge ($Si\text{-}O^-$ above pH =2)

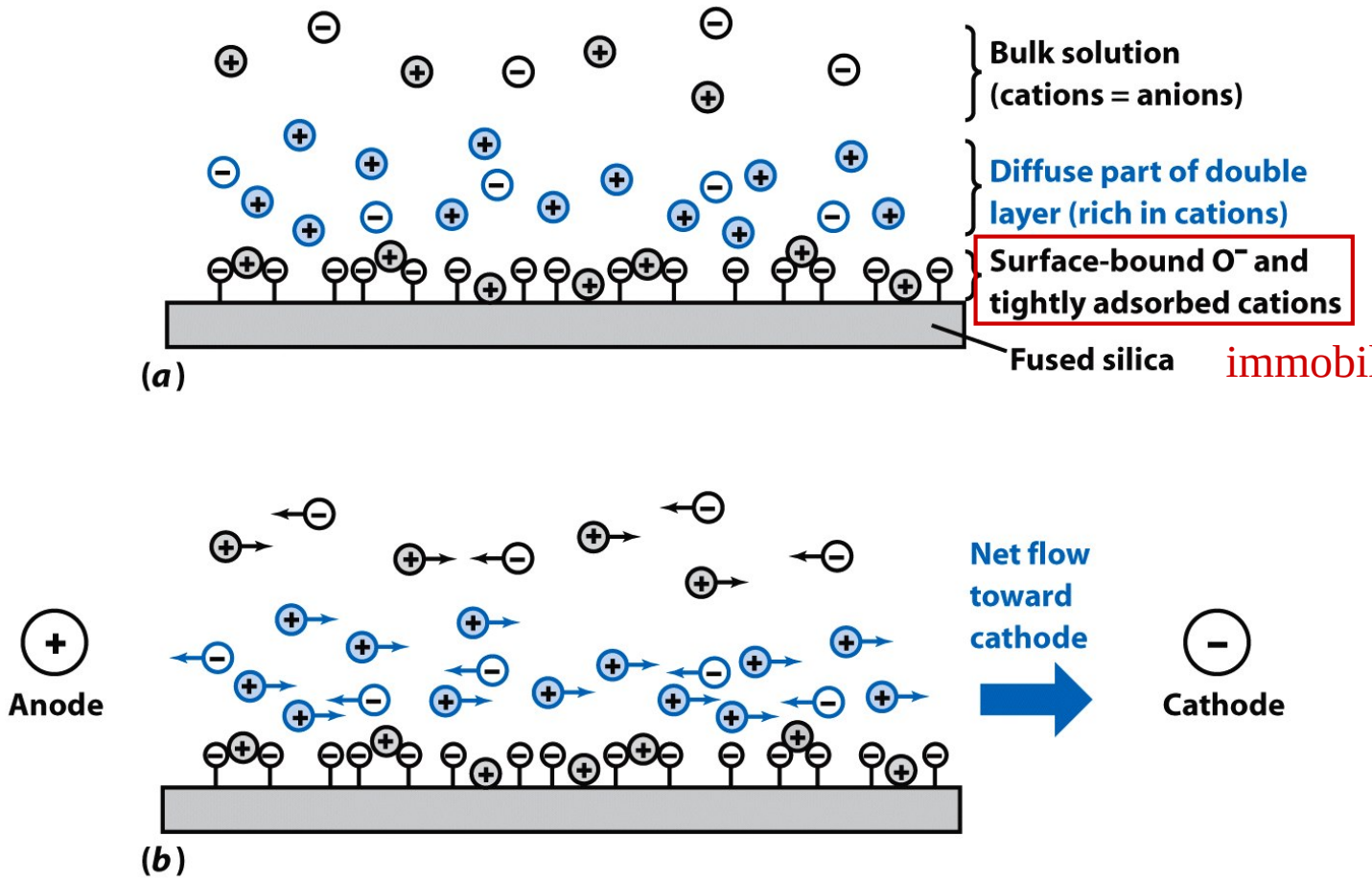


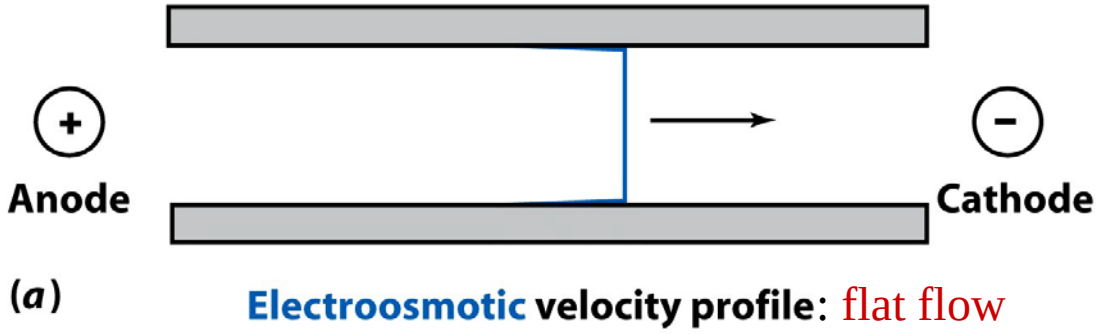
Figure 26-20
Quantitative Chemical Analysis, Seventh Edition

Electroosmosis

Electroosmotic velocity: $v_{eo} = \mu_{eo} \bullet E$

electroosmotic mobility: proportional to surface charge density (higher pH → faster)
inversely proportional to the square root of ionic strength

Electroosmotic flow



Hydrodynamic flow:
Parabolic velocity profile

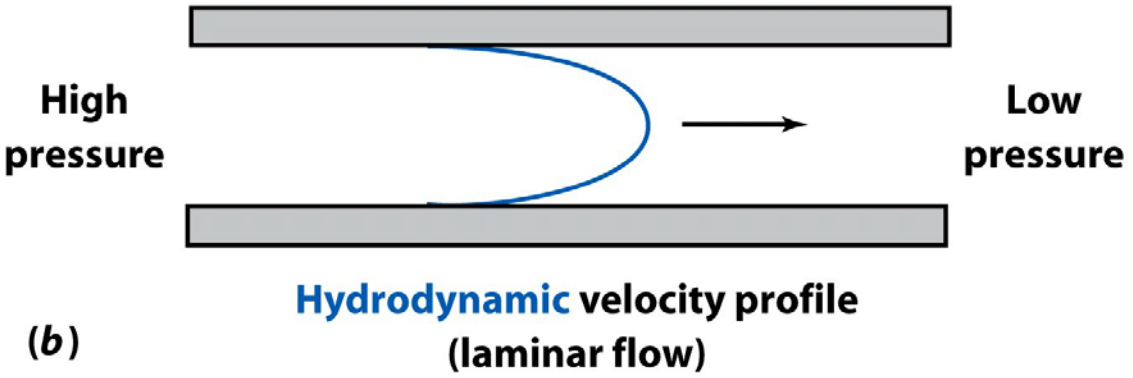


Figure 26-21
Quantitative Chemical Analysis, Seventh Edition

Variations of Capillary Electrophoresis

Capillary Zone Electrophoresis (CZE)

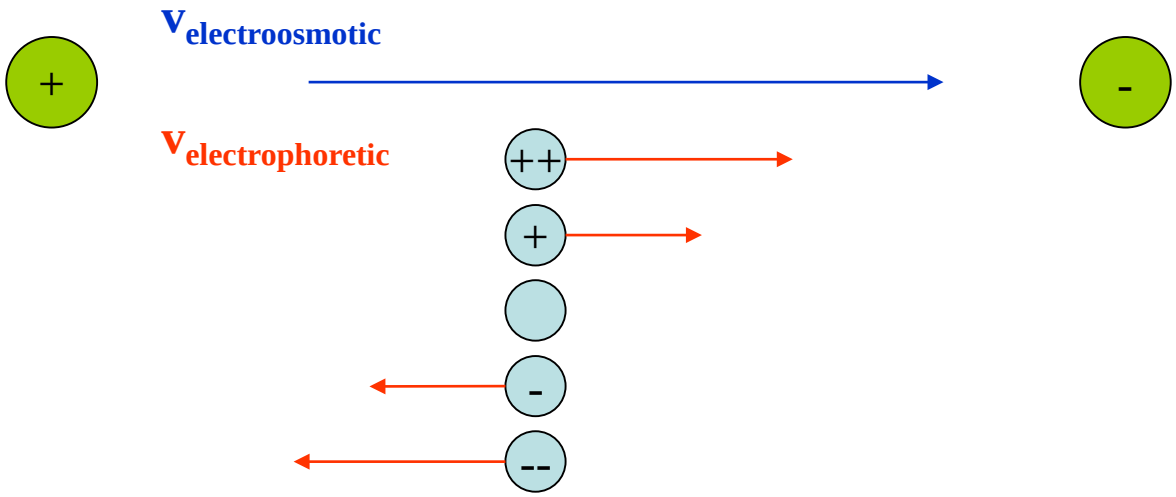
Capillary Gel Electrophoresis (CGE)

Capillary Electrochromatography

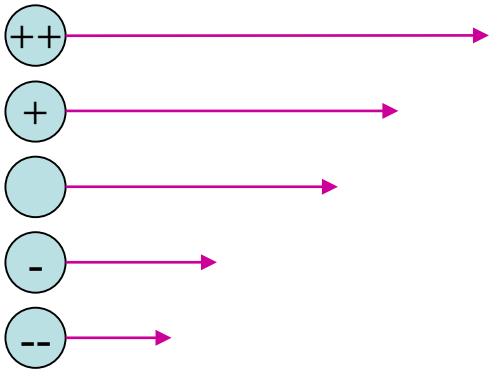
Micellar Electrokinetic Capillary Chromatography (MECC)

Capillary Isoelectric Focusiong

Capillary Zone Electrophoresis



$v_{total} = v_{electroosmotic} + v_{electrophoretic}$



Capillary Gel Electrophoresis

Macromolecules are separated in a gel by sieving:

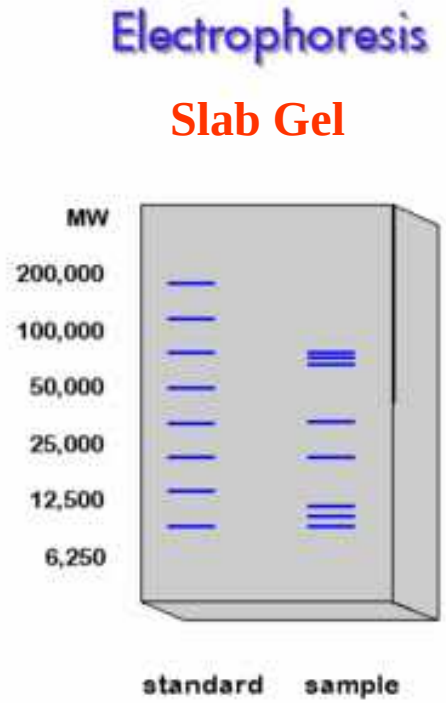
Smaller molecules → migrate faster than larger molecules through gel

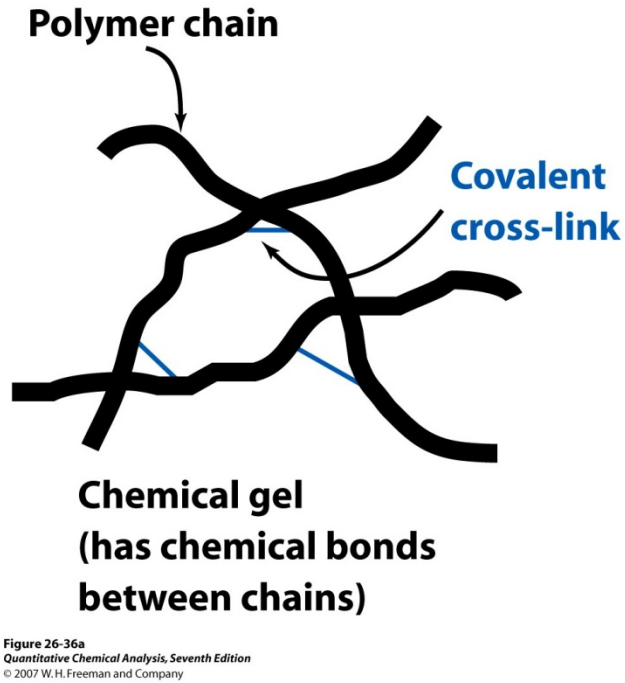
Analytes: proteins, DNA fragments, oligonucleotides)

Qualitative analysis can be conducted by comparing the patterns produced to standards.

This example is a molecular weight determination of proteins but other materials can be evaluated.

This approach is used in genetic 'fingerprinting.'





Polyacrylamide gel

Capillary Gel Electrophoresis

-Proteins are first denatured by reducing their disulfide (-S-S-) bonds with excess 2-mecaptoethanol and adding SDS($C_{12}H_{25}OSO_3^-Na^+$)
- Dodecyl sulfate anion coats hydrophobic regions and gives the protein a large negative charge that is approximately proportional to the length of protein

Sodium dodecyl sulfate (SDS)- Capillary gel electrophoresis

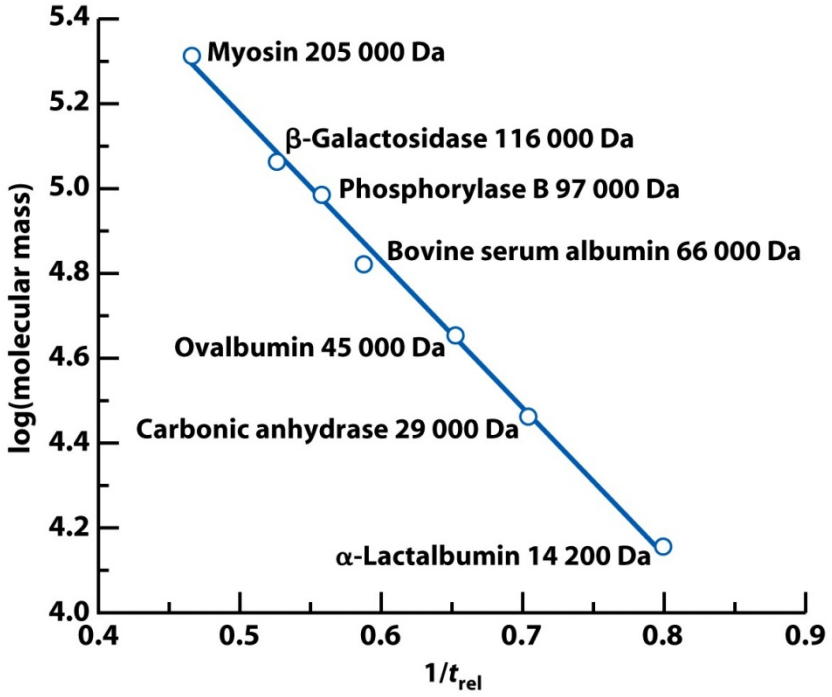


Figure 26-38
Quantitative Chemical Analysis, Seventh Edition

Detectors in Capillary Electrophoresis

Highly sensitive detector is required in CE (extremely small sample amount)

- UV detector : most general
- Fluorescence detector (laser-induced fluorescence): good sensitivity
- Amperometric detector
- Conductivity detector
- Chemiluminescence detector
- Mass spectrometer

Detectors in Capillary Electrophoresis

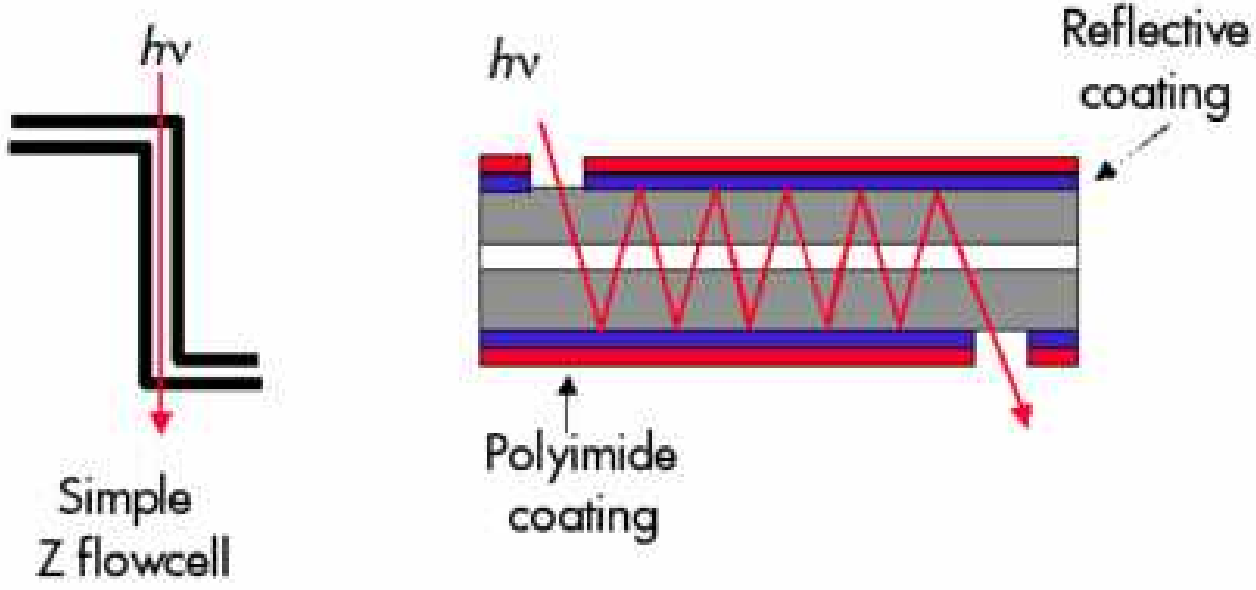
TABLE 30-1 Detectors for CE

Type of Detector	Representative Detection Limit* (attomoles detected)
Spectrometry	
Absorption†	1–1000
Fluorescence	1–0.01
Thermal lens†	10
Raman†	1000
Chemiluminescence†	1–0.0001
Mass spectrometry	1–0.01
Electrochemical	
Conductivity†	100
Potentiometry†	1
Amperometry	0.1

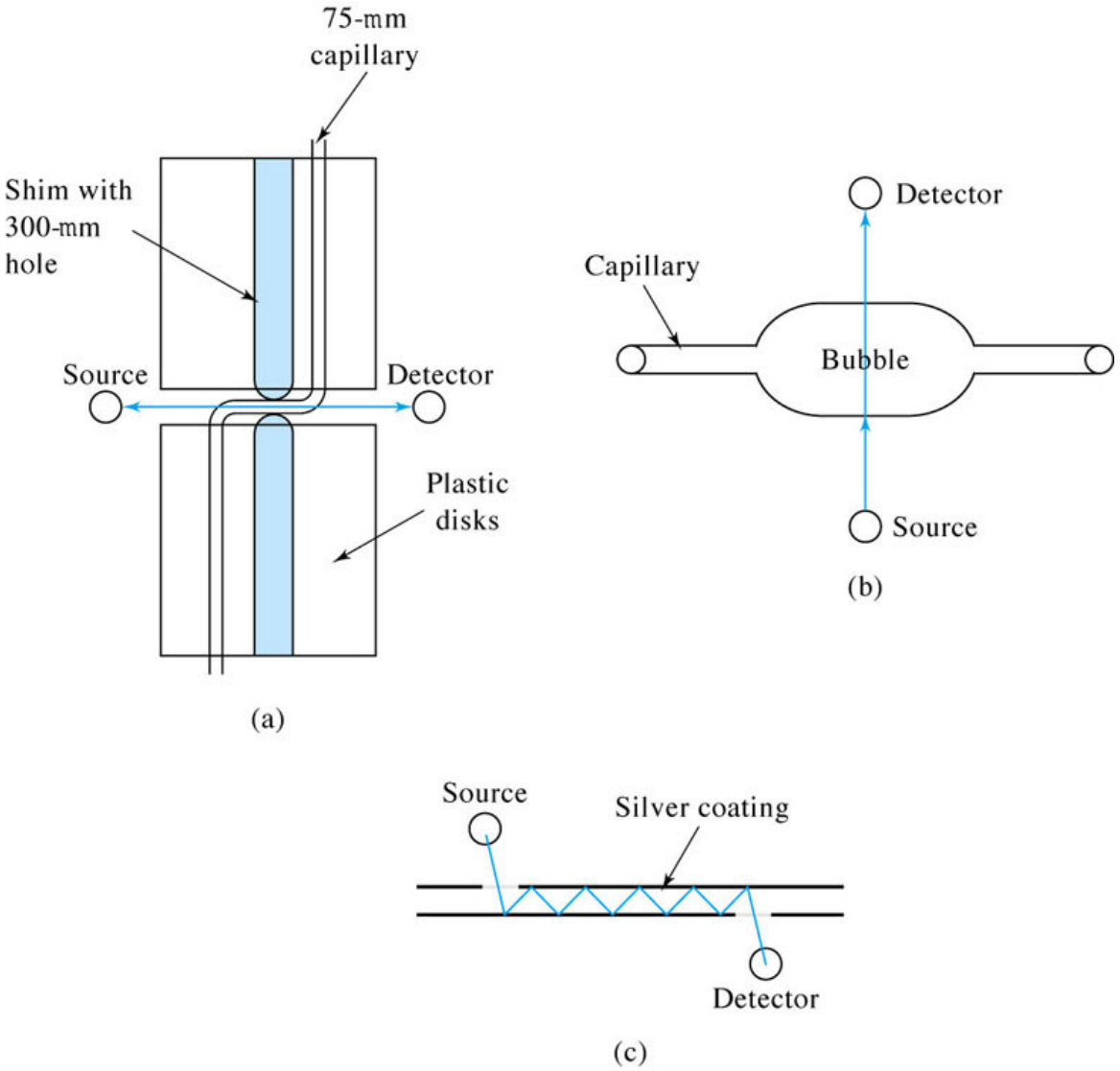
Detectors in Capillary Electrophoresis

UV/Vis

Because of the small volumes, one must get 'creative' to obtain a measurable response -- while still in the capillary tube.



Detectors in Capillary Electrophoresis



Anal. Chem. **1996,** *68,* 697−700

Detection of Attomolar Concentrations of Alkaline Phosphatase by Capillary Electrophoresis Using Laser-Induced Fluorescence Detection

Douglas B. Craig, Jerome C. Y. Wong, and Norman J. Dovichi*

Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada

Alkaline phosphatase can be assayed by monitoring the conversion of the fluorogenic substrate AttoPhos into the highly fluorescent product AttoFluor. We have used capillary electrophoresis with laser-induced fluorescence detection to monitor this reaction. The concentration limit of detection (3σ) of alkaline phosphatase is 1.5×10^{-17} M (2.1 fg/mL), which corresponds to a mass limit of detection of nine molecules (1.5×10^{-23} mol) contained within a 1-μL sample volume.

Anal. Chem. **2000,** *72,* 1872−1878

Separation and Detection of Explosives on a Microchip Using Micellar Electrokinetic Chromatography and Indirect Laser-Induced Fluorescence

Susanne R. Wallenborg and Christopher G. Bailey*

Sandia National Laboratories, P.O. Box 969/ms9671, Livermore, California 94551

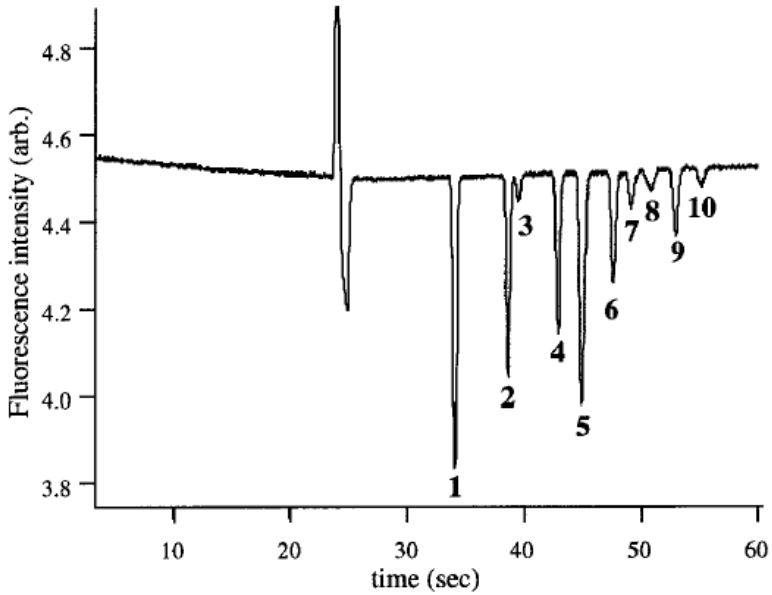


Figure 1. Chip-based MEKC−IDLIF electropherogram of the EPA 8330 mixture of nitroaromatics and nitramines. Analytes: 20 ppm of each TNB (1), DNB (2), NB (3), TNT (4), tetryl (5), 2,4-DNT (6), 2,6-DNT (7), 2-, 3-, and 4-NT (8), 2-Am-4,6-DNT (9), and 4-Am-2,6-DNT (10). Conditions: MEKC buffer, 50 mM borate, pH 8.5, 50 mM SDS, 5 μM Cy7, separation voltage 4 kV, separation distance 65 mm.

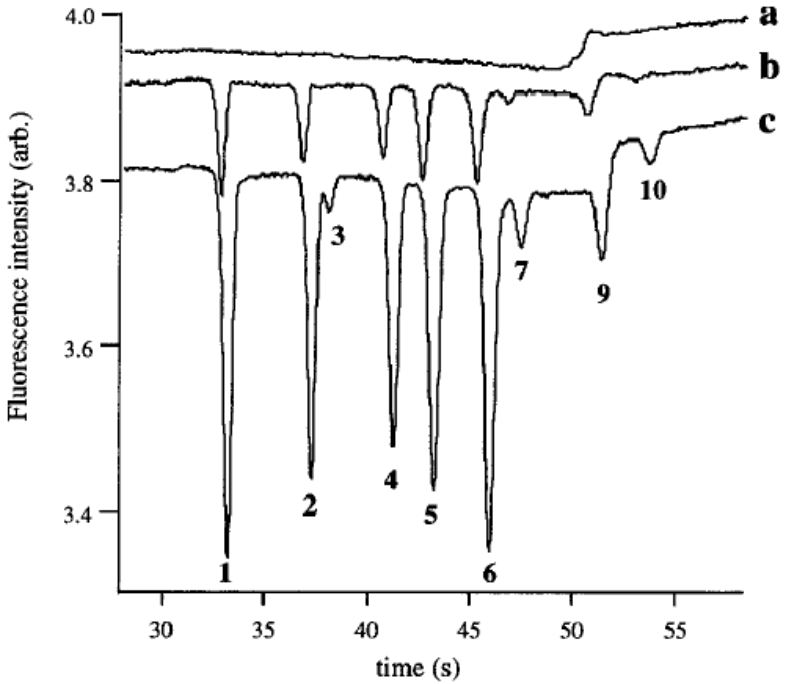


Figure 7. MEKC−IDLIF analysis of extracts from spiked soil samples: soil blank (a), soil containing 1 ppm of each analyte (b), and soil containing 5 ppm of each analyte (c). Peak identifications and conditions were as for Figure 1.

Detectors in Capillary Electrophoresis

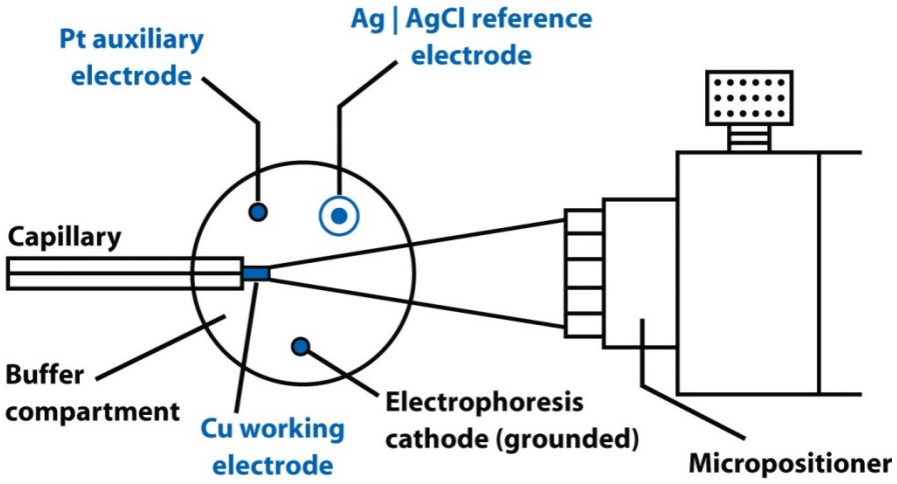


Figure 26-29a
Quantitative Chemical Analysis, Seventh Edition

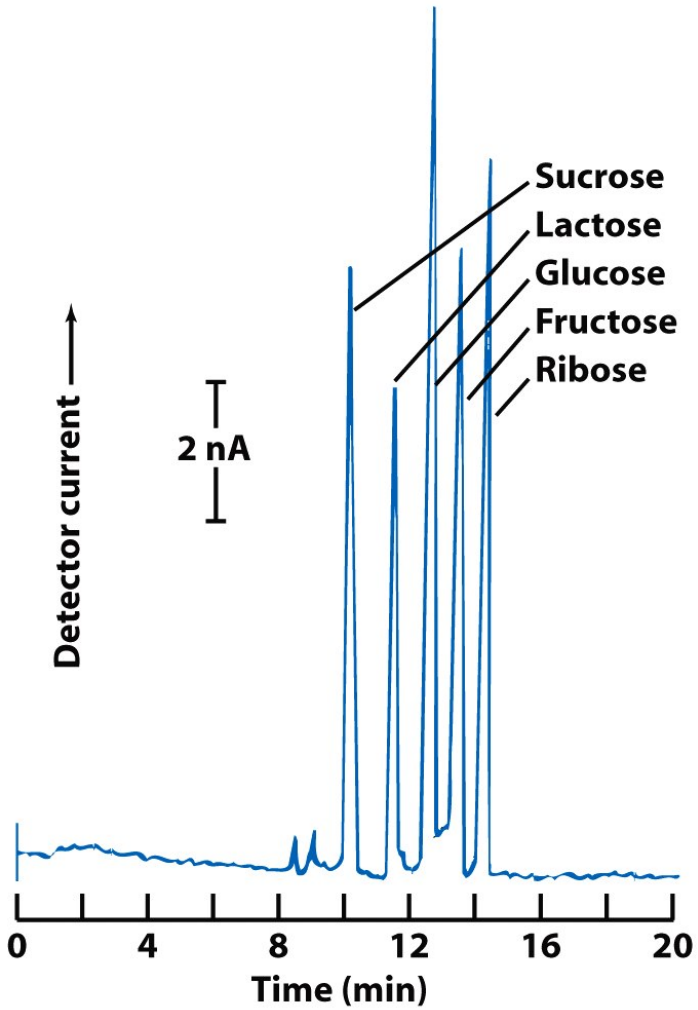
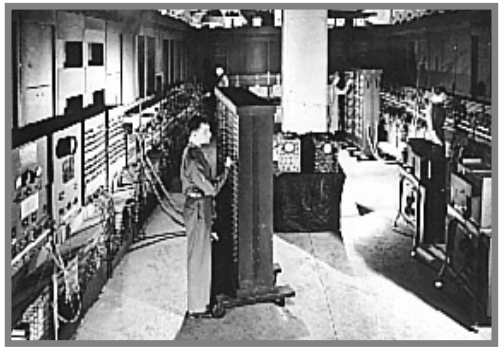
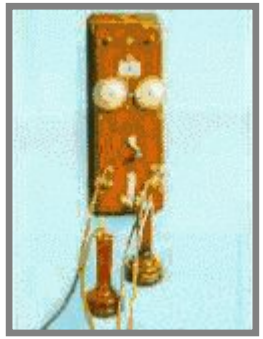


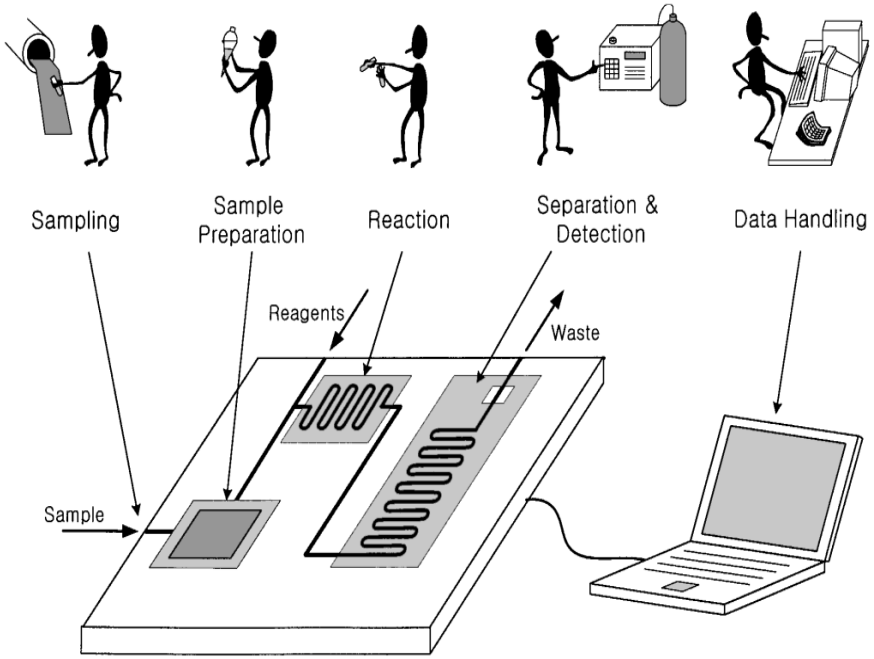
Figure 26-29b
Quantitative Chemical Analysis, Seventh Edition

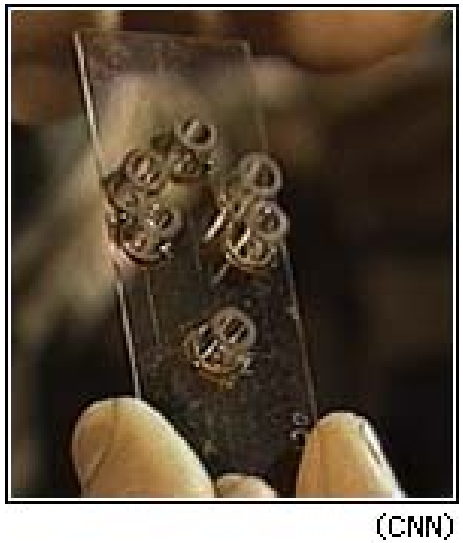
Lab-on-a-Chip





Lab-on-a-Chip

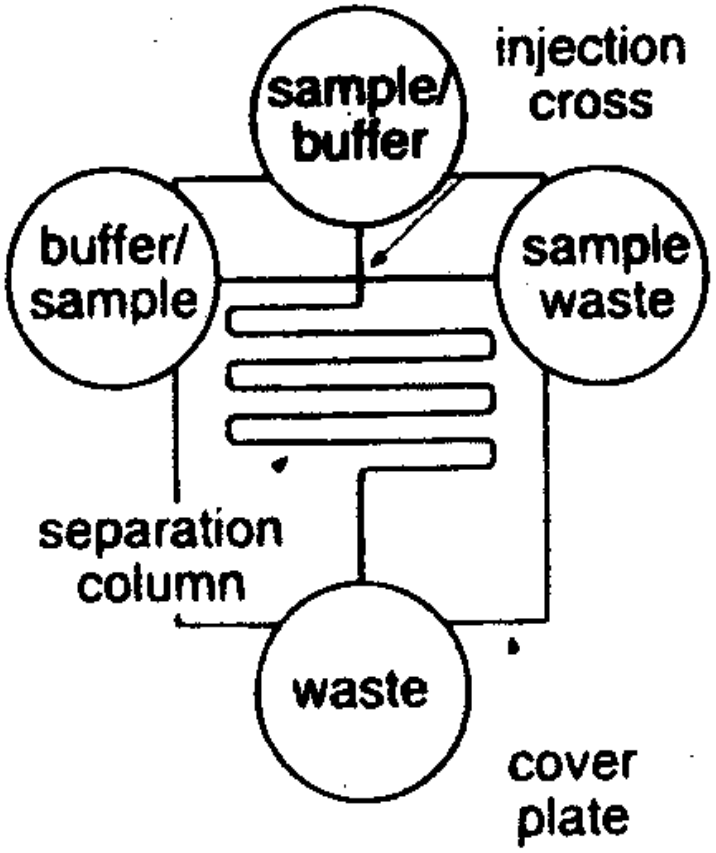



(CNN)

D. J. Harrison, et. al., *Science*, **1993**, *261*, 895-897

- Highly integrated system (sample pretreatment, reaction, separation, and detection all on-a-chip)
- High speed analysis (few second or few minutes)
- Ultra-low volume - minimal reagent or sample consumption (1-10 pL)
- Highly parallel - many samples at once: high-throughput
- Small and possibly portable system
- Potentially disposable assay systems

Lab-on-a-Chip



Agilent 2100

DNA
RNA
Protein