Information given in these slides are, either in part or all, recollection from the followings:

http://bionmr.unl.edu/courses/chem421-821/lectures/chapter-2...

http://faculty.atu.edu/abhuiyan/Course/Chem 4414/Chapter 27.ppt

Principles of Instrumental Analysis, 6th Ed. Holler, Skoog & Crouch, 2007

Chapter 28
High-Performance Liquid Chromatography

28A SCOPE OF HPLC

- The name *high-performance liquid chromatography (HPLC) was o*riginally used to distinguish new procedures from the original gravity-flow methods. Today, virtually all LC is done using pressurized flow and we use the abbreviations LC and HPLC interchangeably.
- LC is the most widely used of all of the analytical separation techniques. The reasons for the popularity of the method are:
  - its sensitivity
  - its ready adaptability to accurate quantitative determinations,
  - its ease of automation
  - its suitability for separating nonvolatile species or thermally fragile ones,
  - and its widespread applicability to substances that are important to Industry, to many fields of science, and to the public.
    - Examples of such materials include amino acids, proteins, nucleic acids, hydrocarbons, carbohydrates, drugs, terpenoids, pesticides, antibiotics, steroids, metal-organic species, and a variety of inorganic substances.

Types of high performance liquid chromatography (HPLC):

- Mobile Phase: Liquid
- Stationary Phase
  - Solid
  - Liquid Layer
  - Ion exchange resin
  - Microporous beads
  - Chemically modified resin

- Separation Mechanism
  - Adsorption
  - Partition
  - Ion exchange
  - Size Exclusion or gel
  - Affinity

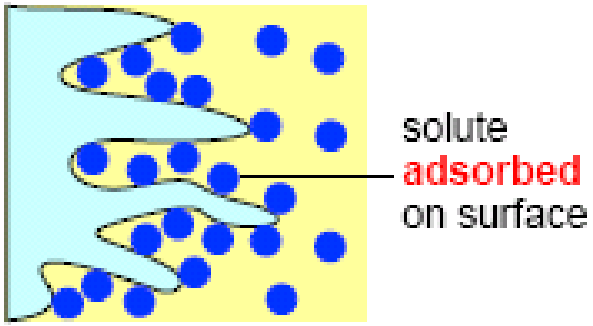
-affinity chromatography for isolation and preparation of biomolecules,

- chiral chromatography for separating enantiomers.

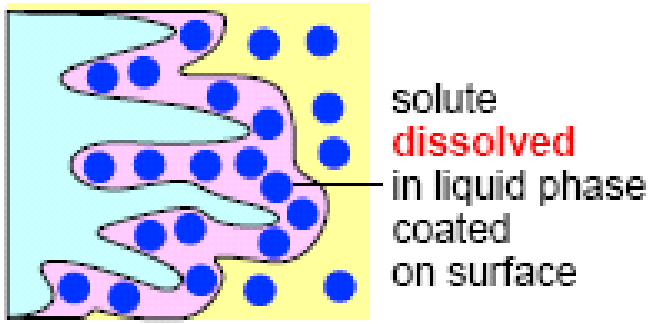
Types of high performance liquid chromatography (HPLC):

Techniques in LC are classified according to the method of solute separation

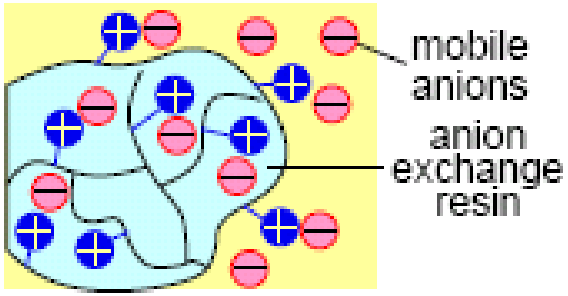
1) Adsorption chromatography
2) Partition chromatography
3) Ion-exchange chromatography
4) Affinity chromatography
5) Size-exclusion chromatography



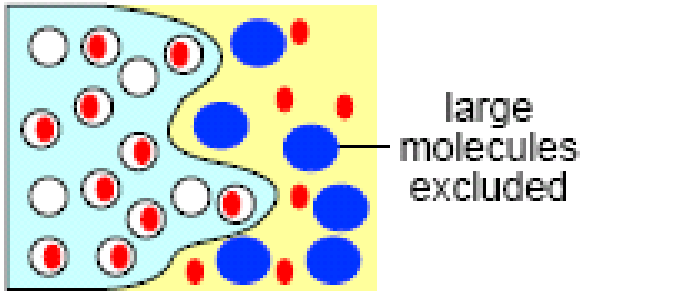
Adsorption Chromatography



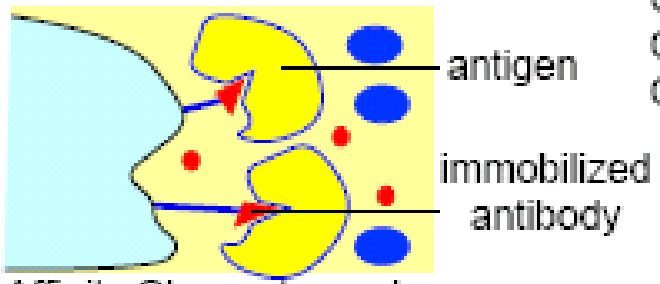
Partition Chromatography



Ion-Exchange Chromatography

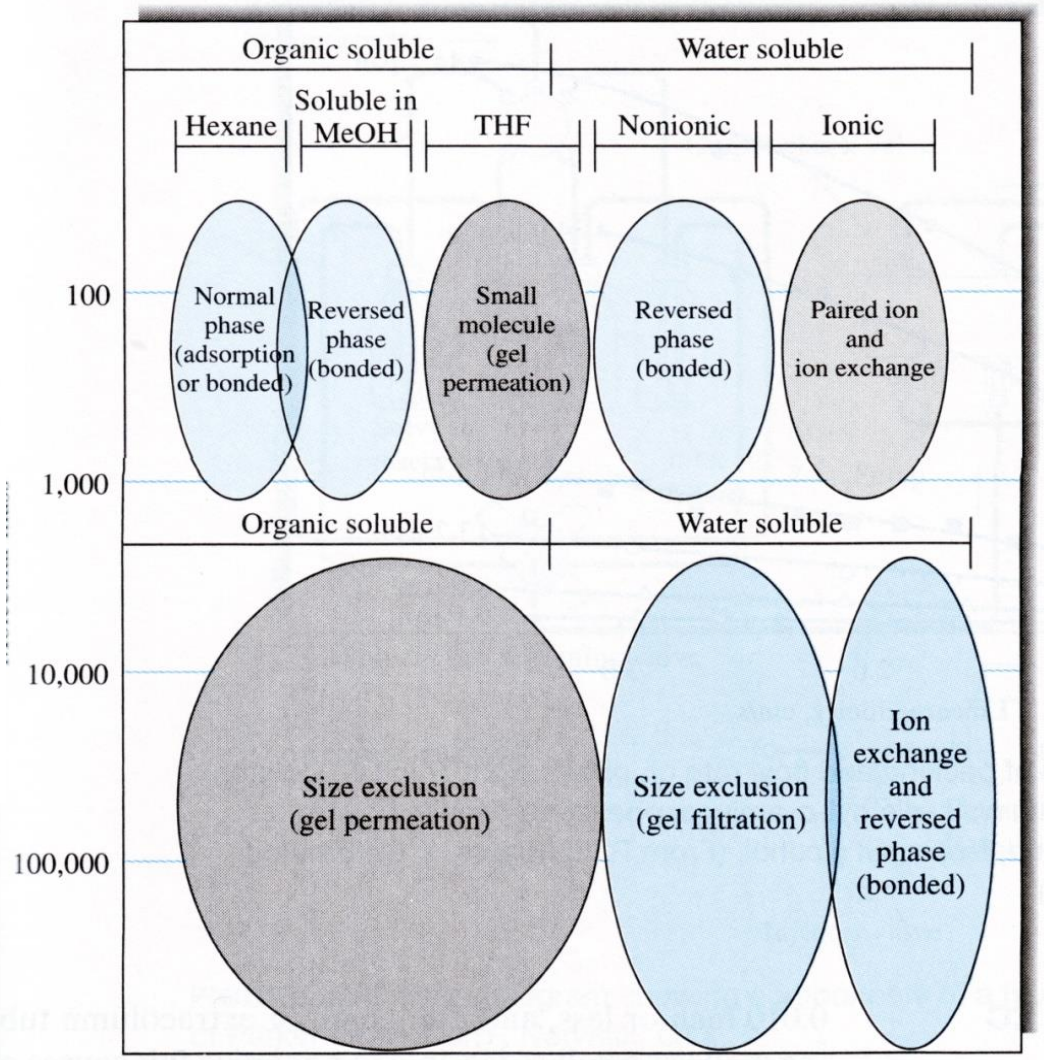


Molecular Exclusion Chromatography
Gel Permeation Chromatography
Gel-Filtration Chromatography
Gel Chromatography



Affinity Chromatography

Various liquid chromatographic procedures are complementary in their application



Methods can be chosen based on solubility and molecular mass.

molecular masses > 10,000
size-exclusion chromatography

lower-molecular-mass ionic
ion-exchange chromatography.

Small polar but nonionic species
reversed-phase methods

Adsorption chromatography
retention, reproducibility and
irrev. adsorption problems ⇒
has been largely replaced by
normal-phase (bonded-phase)

The specialized forms of LC,
affinity chromatography
for isolation and preparation
of biomolecules,
and chiral chromatography
for separating enantiomers.

28B Column Efficiency in LC

- The van Deemter equation $H = A + B/u + Cu$
- The discussion on band broadening is generally applicable to LC.
- An important effect of stationary-phase particle size and additional sources of zone spreading that are sometimes of considerable importance in LC

28B-1 Effects of Particle Size of Packings

The mobile-phase mass-transfer coefficient reveals that C_M is directly related to the square of the diameter d_p *of the particles* making up a packing. Because of this, the efficiency of an LC column should improve dramatically as the particle size decreases. Figure 28-2, it is seen that a reduction of particle size from 45 to 6 micrometer results in a tenfold or more decrease in plate height.

TABLE 26-3 Processes That Contribute to Band Broadening

Process	Term in Equation 26-23	Relationship to Column* and Analyte Properties
Multiple flow paths	A	$A = 2\lambda d_p$
Longitudinal diffusion	B/u	$\frac{B}{u} = \frac{2\gamma D_M}{u}$
Mass transfer to and from stationary phase	$C_S u$	$C_S u = \frac{f(k)d_f^2}{D_S}u$
Mass transfer in mobile phase	$C_M u$	$C_M u = \frac{f'(k)d_p^2}{D_M}u$

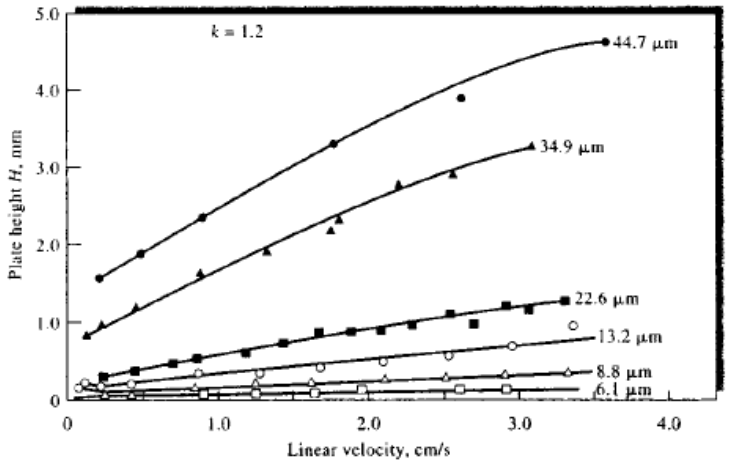


FIGURE 28-2 Effect of particle size of packing and flow rate on plate height H in LC. Column dimensions: 30 cm × 2.4 mm. Solute: *N,N'*-diethyl-*p*-aminoazobenzene. Mobile phase: mixture of hexane, methylene chloride, isopropyl alcohol. (From R. E. Majors, *J. Chromatogr. Sci.*, **1973**, *11*, 88. With permission.)

28B-2 Extracolumn Band Broadening in LC

- In LC significant band broadening occurs outside the column packing itself.
- This *extracolumn band broadening occurs as the solute is carried through open tubes* such as those found *in the injection system, the detector region, and the piping connecting the various components of the syste*m. Here, broadening arises from differences in flow rates between layers of liquid adjacent to the wall and the center of the tube. As a result, the center part of a solute band moves more rapidly than the peripheral part.
- In GC, extracolumn spreading is largely offset by diffusion. Diffusion in liquids, however, is significantly slower, and band broadening of this type often becomes noticeable. It has been shown that the contribution of extracolumn effects H_{ex} *to the total plate height is given by*

$$H_{ex} = \frac{\pi r^2 u}{24 D_M}$$

u is the linear-flow velocity (cm/s),
r is the radius of the tube (cm),
D_M is the diffusion coefficient of the solute in the mobile phase *(cm^2/s).*

• Extracolumn broadening can become quite serious when small-bore columns are used. Here, the radius of the extracolumn components should be reduced to 0.01 inch or less, and the length of extra column tubing made as small as feasible to minimize this source of broadening.

HPLC Advantages vs GC

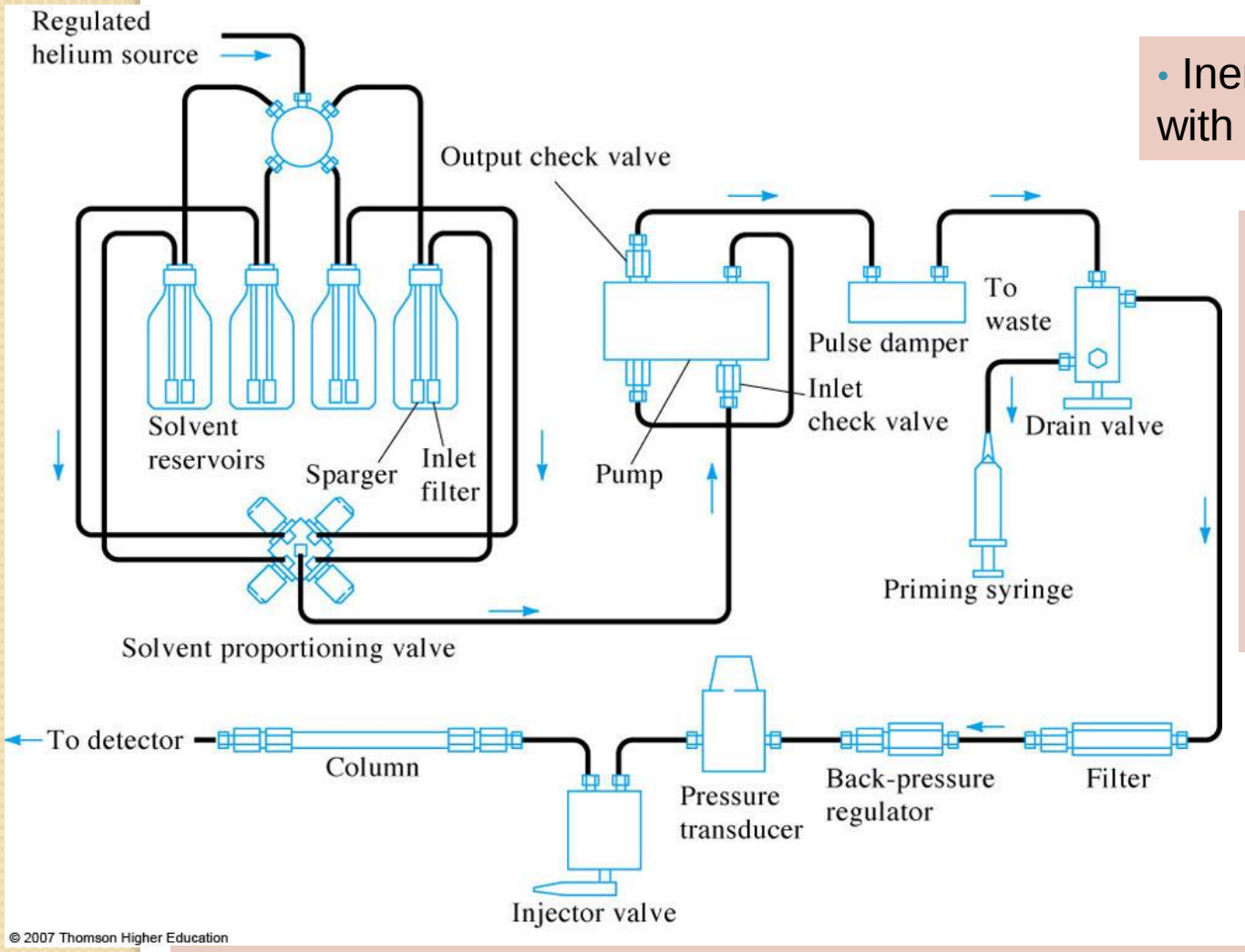
- Not limited by sample volatility or thermal stability
- Two interacting phases
- Room temperature analysis
- Ease of sample recovery

28C LC Instrumentation

Pumping pressures of several hundred atmospheres are required to achieve reasonable flow rates with packings of 3 to 10 μm,which are common in modern LC.
Because of these high pressures, the equipment for HPLC tends to be more elaborate and expensive than equipment for other types of chromatography.

- Solvent Reservoirs
- Pump
- Sample Injector
- Column(s)
- Detector
- Data System

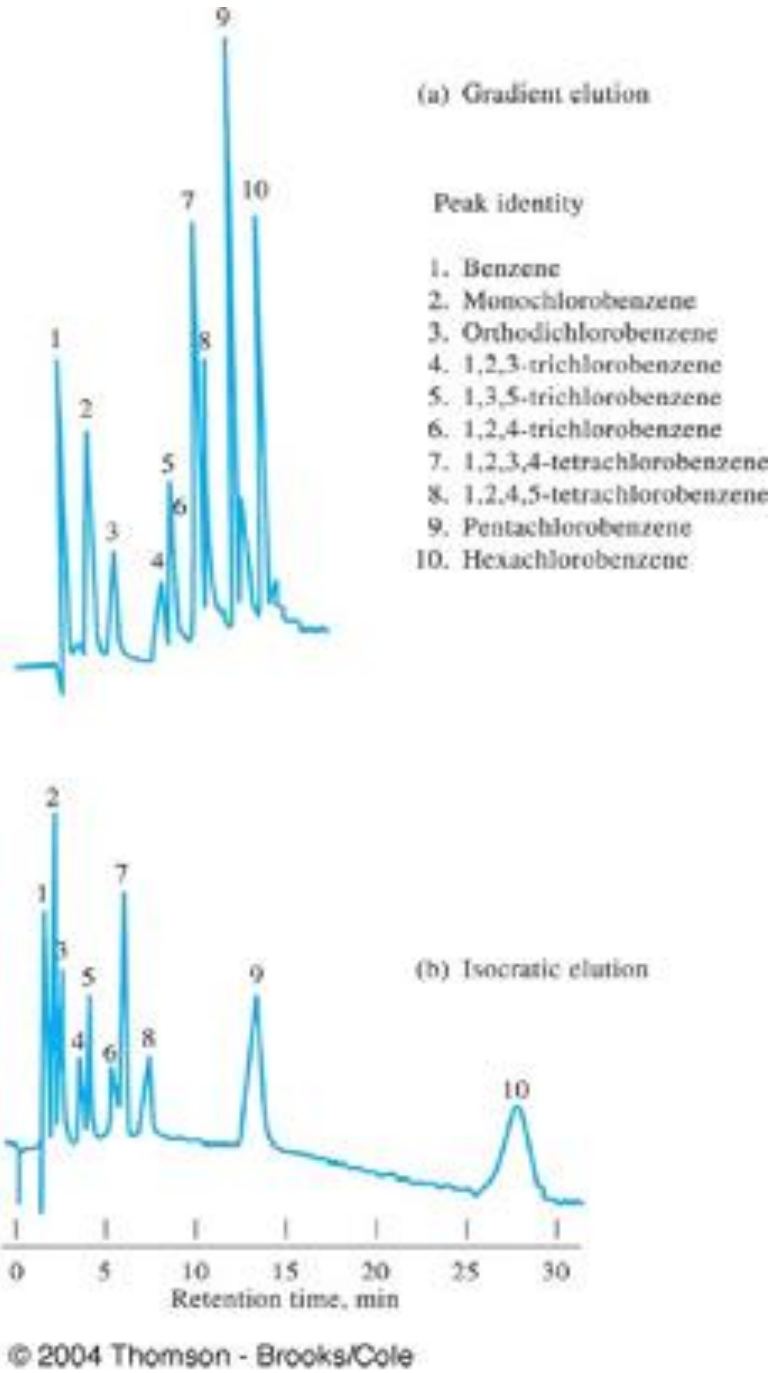
28C-1 Mobile-Phase Reservoirs and Solvent Treatment Systems



- Inert container (500 mL) with inert lines → pump

- to remove dissolved gases and dust from the liquids, degassing
  - Vacuum filtration
  - Sparge with inert gas (N_2 or He)
  - Ultrasonic under vacuum

- Reservoir filters (2-10 mm) at reservoir end of solvent delivery lines for Filtering dust and particulate matter from the solvents toprevent these particles from damaging the pumping or injection systems or clogging the column.



Isocratic elution: A separation that employs a single solvent or solvent mixture of constant composition.

Gradient elution: Here two or more solvent systems that differ significantly in polarity are employed. After elution is begun; the ratio of the solvents is varied in a programmed way, sometimes continuously and sometimes in a series of steps. Separation efficiency is greatly enhanced by gradient elution.

HPLC Pump Criteria

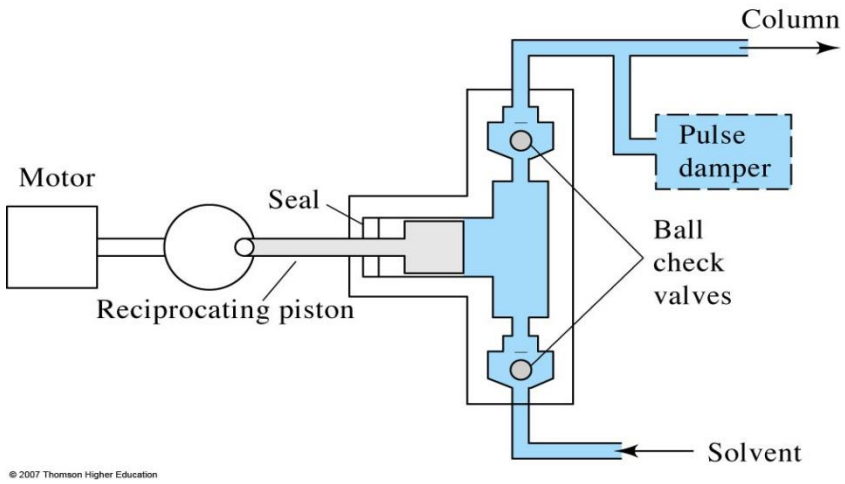
- Constructed of materials inert toward solvents to be used
- Deliver high volumes (flow rates) of solvent (0.1 to 10 mL/min)
- Deliver precise and accurate flow (<0.5% variation)
- Deliver high pressure (to 6000 psi=414 bar)
- Deliver pulse free flow
- Have low pump-head volume
- Be resistive to corrosion by a variety of solvents.

The high pressures generated by liquid chromatographic pumps are not an explosion hazard because liquids are not very compressible. Thus rupture of a component results only in solvent leakage. However. such leakage may constitute a fire or environmental hazard with some solvents.

HPLC Pumps: Types

1) Reciprocating pumps
2) Screw driven Syringe pumps

Reciprocating Pumps



- Small internal volumes (50 - 250μL)
- high output pressures (up to 10.000 psi).
- adaptability to gradient elution.
- large solvent capacities and constant flow rates which are largely independent of column back pressure and solvent viscosity.
- Continuous use (no refill time)

• Reciprocating pumps usually consist of a small chamber in which the solvent is pumped by the back and forth motion of a motor-driven piston
• have the disadvantage of producing a pulsed flow, which must be damped because the pulses appear as baseline noise on the chromatogram. Modern LC instruments use dual pump heads to minimize such pulsations. One, two, or three pump heads (- more heads, less pulse)

Displacement (Syringe) Pumps

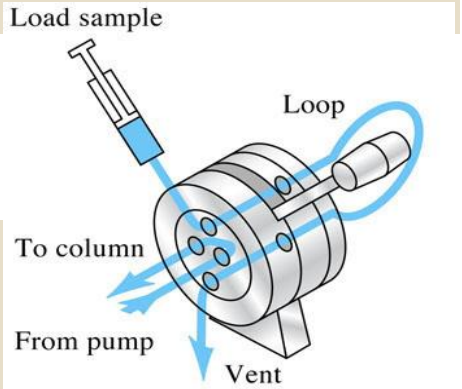
Displacement pumps usually consist of large, syringelike chambers equipped with a plunger activated by a screw-driven mechanism powered by a stepping motor.

- Constant flow rate pump
- Non-pulsating flow (pulse free)
- Low flow rates (1 to 100 mL/min)
- Isocratic flow only
- Low sample capacity (Refill required)

Sample Injection Systems

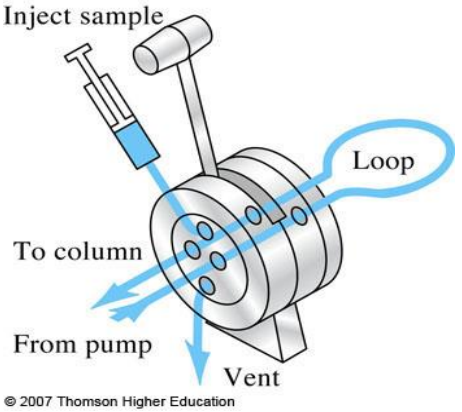
• The limiting factor in the precision of liquid chromatographic measurements is the *reproducibility with which samples can be introduced* onto the column
• The problem is exacerbated by band broadening, which accompanies a lengthy sample injection plug.
• Thus, sample volumes must be very small- a few tenths of a microliter to perhaps 500 μL. Furthermore, it is convenient to be able to introduce the sample without depressurizing the system.

• The most widely used method of sample introduction in LC is based on sampling loops.



- Valve-type injectors-
  - fixed volume reproducible injection volumes
  - variable loop size (1 μL to 100μL
  - easy to use, reliable

• Most chromatographs today are sold with autoinjector
• Such units are capable of injecting samples into the LC from vials on a sample carousel or from microtiter plates.
• Some have controlled temperature environments that allow for sample storage and for carrying out derivatization reactions prior to injection.



Auto Injectors

- Continuous injections
- operator free
- Comparable precision and accuracy to manual
- Much more expensive initially
- Much more convenient Up 100 samples and standards with microprocessor control

Liquid-Chromatographic Columns

Liquid-chromatographic columns are ordinarily constructed from

- smooth-bore stainless steel tubing,
- heavy-walled glass tubing (occasionally encountered and is restricted to pressures that are lower than about 600 psi.)
- polymer tubing, such as polyetheretherketone (PEEK).
- In addition, stainless steel columns lined with glass or PEEK are also available
- Hundreds of packed columns differing in size and packing are available. The cost of standard-sized nonspeciality columns ranges from $200 to more than $500. Specialized columns, such as chiral columns can cost more than $1000.

- Analytical Columns

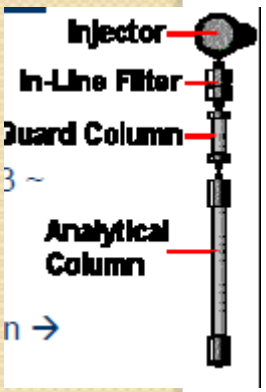
- length from 5 to 25 cm

- when needed, coupling two or more columns together.

- 3 to 5 mm inside diameter

- 3 or 5 μm is the most common particle size of the packings.

- The column of this type contain 40,000 to 70,000 plates/meter.



- Guard Columns

- before the analytical column to protect more expensive analytical column

- particulate matter, contaminants from the solvents and sample components that bind irreversibly to the stationary phase are removed

- the lifetime of the analytical column increases

- serves to saturate the mobile phase with the stationary phase so that losses of this solvent from the analytical column are minimized.

- Packing composition is similar to that of the analytical column; the particle size is usually larger. When the guard column has become contaminated, it is repacked or discarded and replaced with a new one.

Column Temperature Control

- For some applications, close control of column temperature is not necessary and columns are operated at room temperature.
- Often, however, better, more reproducible chromatograms are obtained by maintaining constant column temperature.
- Most modern commercial instruments are now equipped with heaters that control column temperatures to a few tenths of a degree from near ambient to 150 C.
- Columns may also be fitted with water jackets fed from a constant temperature bath to give precise temperature control.
- Many chromatographers consider temperature control to be essential for reproducible separations.

28C-5 Types of Column Packings

- Two basic types of packings have been used in LC, *pellicular and porous particle.*
- *The original pellicular* particles were spherical, nonporous, glass or polymer beads with typical diameters of 30 to 40 μm.
- A thin, *porous* layer of silica, alumina, a polystyrene-divinylbenzene synthetic resin, or an ion-exchange resin was deposited on the surface of these beads. Small porous microparticles have completely replaced these large pellicular particles.
- In recent years, small (~5 μm) pellicular packings have been reintroduced for separation of proteins and large biomolecules.
- The typical porous particle packing for LC consists of particles having diameters ranging from 3 to 10 μm; for a given size particle, a very narrow particle size distribution is desirable.
- The particles are composed of silica, alumina, the synthetic resin polystyrene-divinylbenzene, or an ion-exchange resin.
- Silica is by far the most common packing in LC. Silica particles are prepared by agglomerating submicron silica particles under conditions that lead to larger particles having highly uniform diameters

Detectors:

Unlike gas chromatography, liquid chromatography has *no detectors* that are as *universally applicable* and as reliable as the flame ionization and thermal conductivity detectors. A major challenge in the development of liquid chromatography has been in detector improvement.

- LC detector need not be responsive over as great a temperature range. In addition, an HPLC detector should have minimal internal volume to reduce zone broadening and should be compatible with liquid flow.

Types of Detectors:

Bulk property detectors respond to a mobile-phase bulk property, such as refractive index, dielectric constant, or density.

Solute property detectors respond to some property of solutes, such as UV absorbance, fluorescence, or diffusion current, that is not possessed by the mobile phase.

• The most widely used detectors for LC are based on absorption of ultraviolet or visible radiation.
• Fluorescence, refractive-index, and electrochemical detectors are also widely used.
• Mass spectrometry (MS) detectors are currently quite popular. Such LC/MS systems can greatly aid in identifying the analytes exiting from the HPLC column as discussed later in this section.

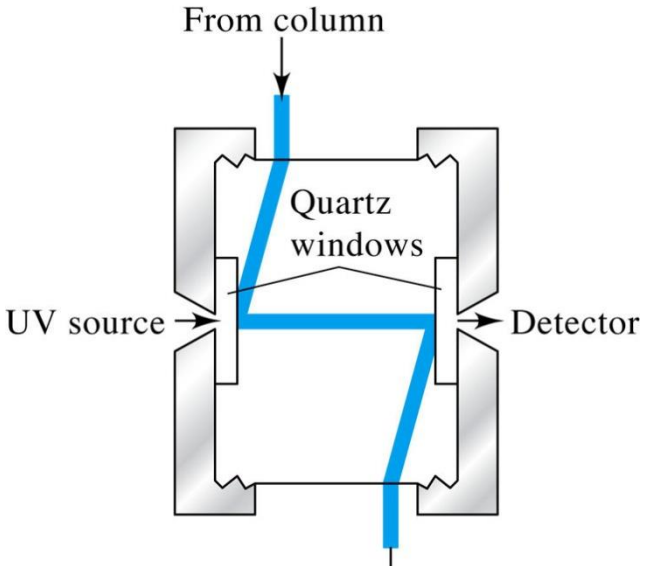
TABLE 28-1 Performance of HPLC Detectors

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

Absorbance Detectors:

- Z-shaped, flow-through cell for absorbance measurements on eluents from a chromatographic column.

- double-beam, in which one beam passes through the eluent cell and the other is a referance beam.



UV-Absorbance Detectors with Filters:

-The simplest UV absorption detectors are filter photometers with a mercury lamp as the source.

- Most commonly the intense line at 254 nm is isolated by filters.
- Deuterium or tungsten filament sources with interference filters also provide a simple means of detecting absorbing species.

UV Absorbance Detector with Monochromator:

- scanning spectrophotometer with grating optics.
- limited to uv or both uv and visible radiation.
- diode-array, the most powerful uv spectrophotometric detector

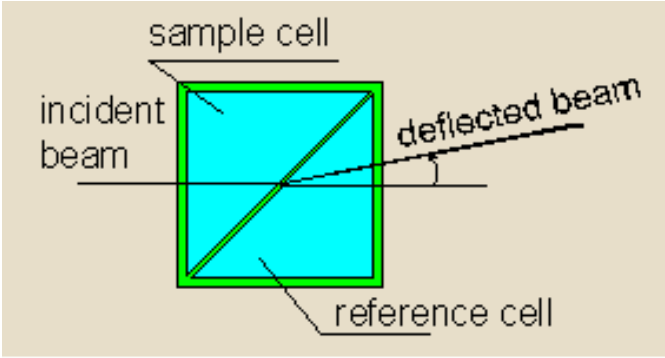
Infrared Absorbance Detectors:

- Two types of infrared detectors are offered commercially.
- The range of the first instrument is from 2.5 to 14.5 μm or 4000 to 690 cm^{-1}.
- The second type of infrared detector is based upon FT instruments.
- A major limitation to the use of IR detectors lies in the low transparency of many useful solvents. For example, the broad infrared absorption bands for water and the alcohols largely preclude the use of this detector for many applications.
- Also the use of aqueous mobile phases can lead to rapid deterioration of cell materials unless special windows are used

Fluorescence Detectors:

- Fluorescence is observed by a photoelectric detector located at 90 deg to the excitation beam.
- The simplest detectors employ a mercury excitation source and one or more filters to isolate a band of emitted radiation.
- More sophisticated instruments are based upon a Xenon source and employ a grating monochromator to isolate the fluorescent radiation.
- An inherent advantage of fluorescence methods is their high sensitivity, which is typically greater by more than an order of magnitude than most absorbance procedures.

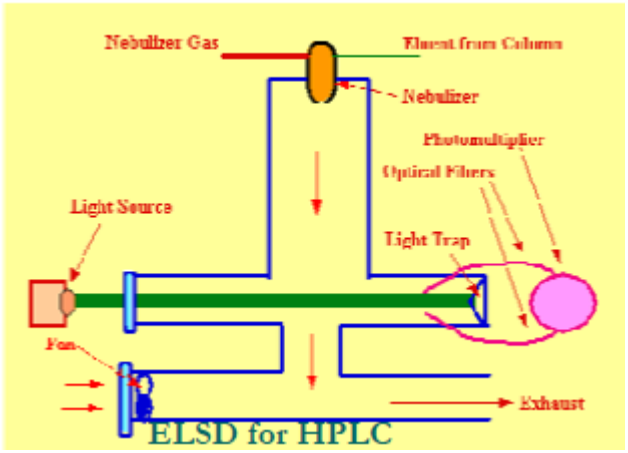
Refractive-Index Detectors:



- It is the only universal detector in HPLC , having a significant advantage of responding to nearly all solutes.
- They are general detectors analogous to flame or thermal conductivity detectors in gas chromatography.
- The detection principle involves measuring of the change in refractive index of the column effluent passing through the flow-cell.
- HPLC Refractive Index Detectors are used with substances with limited or no UV absorption.
- These chemical components included alcohols, sugars, fatty acids, polymers and carbohydrates. Applications where HPLC RI detectors are used include polymer analysis.
- In addition, they are reliable and unaffected by flow rate.
- They are, however, highly temperature sensitive and must be maintained at a constant temperature to a few thousandths of a degree centigrade.
- Furthermore, they are not as sensitive as most other types of detectors, and are not compatible with gradient elution methods..

Evaporative light scattering detector:

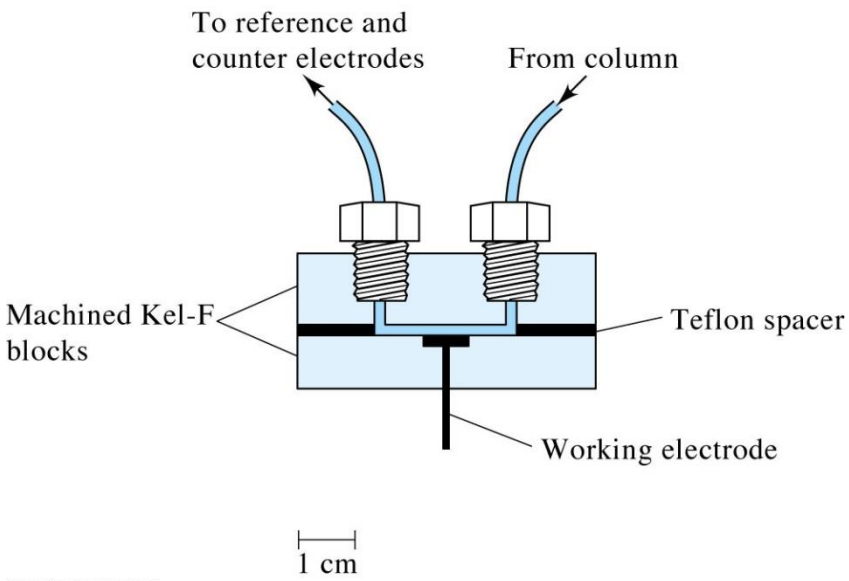
- One of the newer types of detectors for HPLC is the *evaporative light scattering detector (ELSD).*
- *In this detector,* the column effluent is passed into a nebulizer where it is converted into a fine mist by a flow of nitrogen or air.
- The fine droplets are then carried through a controlled-temperature drift tube where evaporation of the mobile phase occurs, leading to formation of fine particles of the analyte.
- The cloud of analyte particles then passes through a laser beam. The scattered radiation is detected at right angles to the flow by a silicon photodiode.
- A major advantage of this type of detector is that its response is approximately the same for all nonvolatile solutes.
- In addition it is significantly more sensitive than the refractive-index detector, with detection limits of 0.2 ng/μL.
- A disadvantage is that mobile-phase compositions are limited to only volatile components.



Electrochemical Detectors:

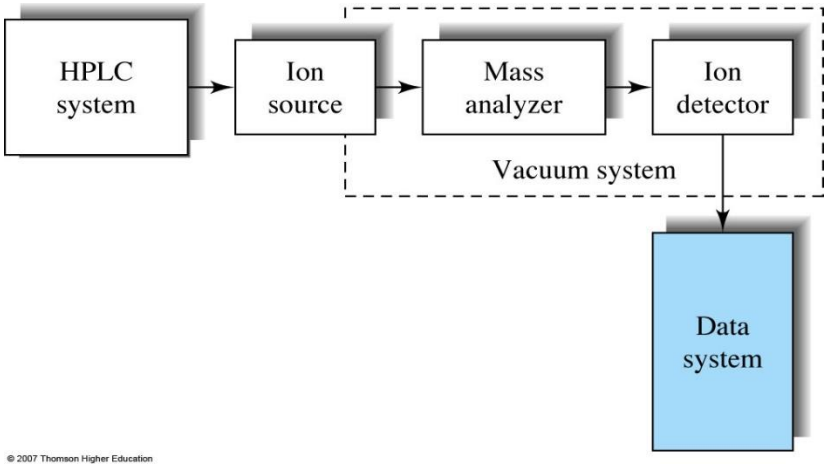
- These devices are based upon amperometry, polarography, coulometry, and conductometry.
- They appear to offer advantages, in many instances, of high sensitivity, simplicity, convenience, and widespread applicability.
- A major limitation of electrochemical detectors, however, is that they are not compatible with gradient elution.
- Thin-layer flow cell of Teflon : 50 μm thick, 1 ~ 5 μL cell volume
  – Indicator Electrode : Pt, Au, C
  – RE and CE: located down stream from the indicater
- – Multi-electrode: simultaneous

detection or sample purity indication



Mass Spectrometric Detectors:

A problem in coupling liquid chromatography with MS is the enormous mismatch between the relatively large solvent volumes and the vacuum requirements. Several interfaces have been developed for solving this problem. Today, the most popular approaches use a low-flow-rate atmospheric pressure ionization techniques.



- Electrospray (ESI)
  - high flow rate (100µL/min – 1mL/min)
  - capillary flow rate (2µL/min - 100µL/min)
  - low flow rate (<2µL/min)
  - – nanospray (200-500nL/min) – ESI is most sensitive at these low flow rates
- Atmospheric Pressure Chemical Ionization (APCI)
- Atmospheric Pressure PhotoIonization (APPI)
- Atmospheric Pressure MALDI

28D PARTITION CHROMATOGRAPHY

Partition chromatography can be subdivided into

(i) **liquid-liquid** chromatography and

(ii) **bonded-phase** chromatography.

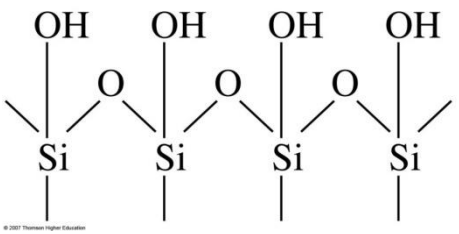
- With liquid-liquid, a liquid stationary phase is retained on the surface of the packing by physical adsorption. This stationary phase is immiscible with the liquid mobile phase.
- With bonded-phase, the stationary phase is bonded chemically to the support surfaces. These highly stable packings insoluble in the mobile phase.
- Bonded-phase columns are also compatible with gradient elution techniques therefore, our discussion focuses exclusively on bonded-phase partition chromatography.

°Early partition chromatography was the liquid-liquid type; now the bonded-phase method has become predominate because of certain disadvantages of liquid-liquid systems.

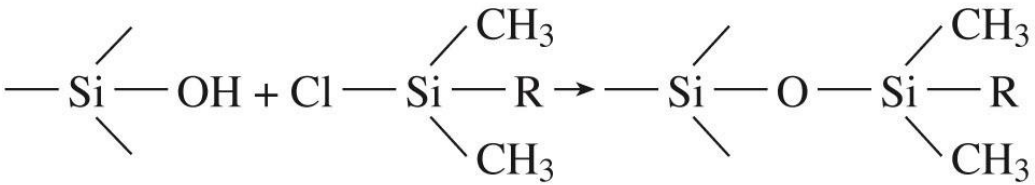
° One of these disadvantages is the loss of stationary phase by dissolution in the mobile phase, which requires periodic recoating of the support particles. Furthermore, stationary-phase solubility problems prohibit the use of liquid-phase packings for gradient elution.

28D-1 Columns for Bonded-Phase Chromatography

The supports for the majority of bonded-phase packings for partition chromatography are prepared from rigid silica, or silica-based compositions. These solids are formed as uniform, porous, mechanically sturdy particles commonly having diameters of 3, 5, or 10 μm. The surface of fully hydrolyzed silica is made up of chemically reactive silanol groups.



The most useful bonded-phase coatings are siloxanes formed by reaction of the hydrolyzed surface with an organochlorosilane.



Surface coverage by silanization is limited to 4 μmol/m^2 or less because of steric effects. The unreacted SiOH groups, unfortunately, impart an undesirable polarity to the surface, which may lead to tailing of chromatographic peaks, particularly for basic solutes.

To lessen this effect, siloxane packings are frequently *capped by further reaction* with chlorotrimethylsilane that, because of its smaller size, can bond some of the unreacted silanol groups.

Reversed-Phase and Normal-Phase Packings

- Two types of partition chromatography are distinguishable based upon the relative polarities of the mobile and stationary phases.

1) normal-phase chromatography

- *highly polar stationary phases* such as water or triethyleneglycol supported on silica or alumina particles; and a

- *relatively non-polar solvent (mobile phase)* such as hexane or i-propylether

Compound polarity ↓ or solvent polarity ↑ ∴ tR ↓

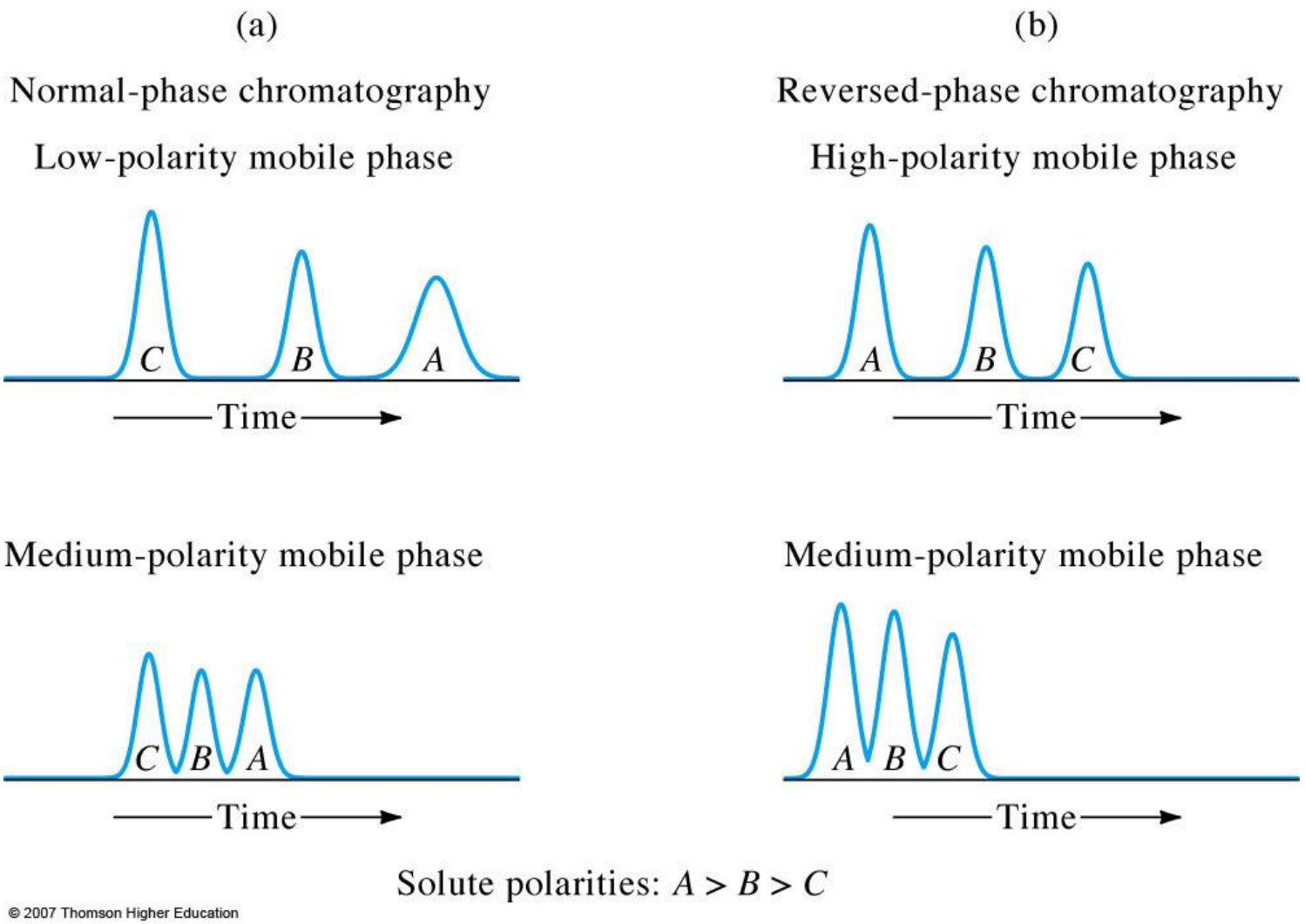
2) reversed-phase chromatography,

- *stationary phase is non-polar*, often a hydrocarbon,

- *mobile phase is relatively polar* (such as water, methanol, acetonitrile, or tetrahydrofuran).

compound polarity ↓ or solvent polarity ↑ ∴ tR ↑

Mobile phase polarity:
– Water > acetonitrile > methanol > ethanol > tetrahydrofuran > propanol > cyclohexane > hexane

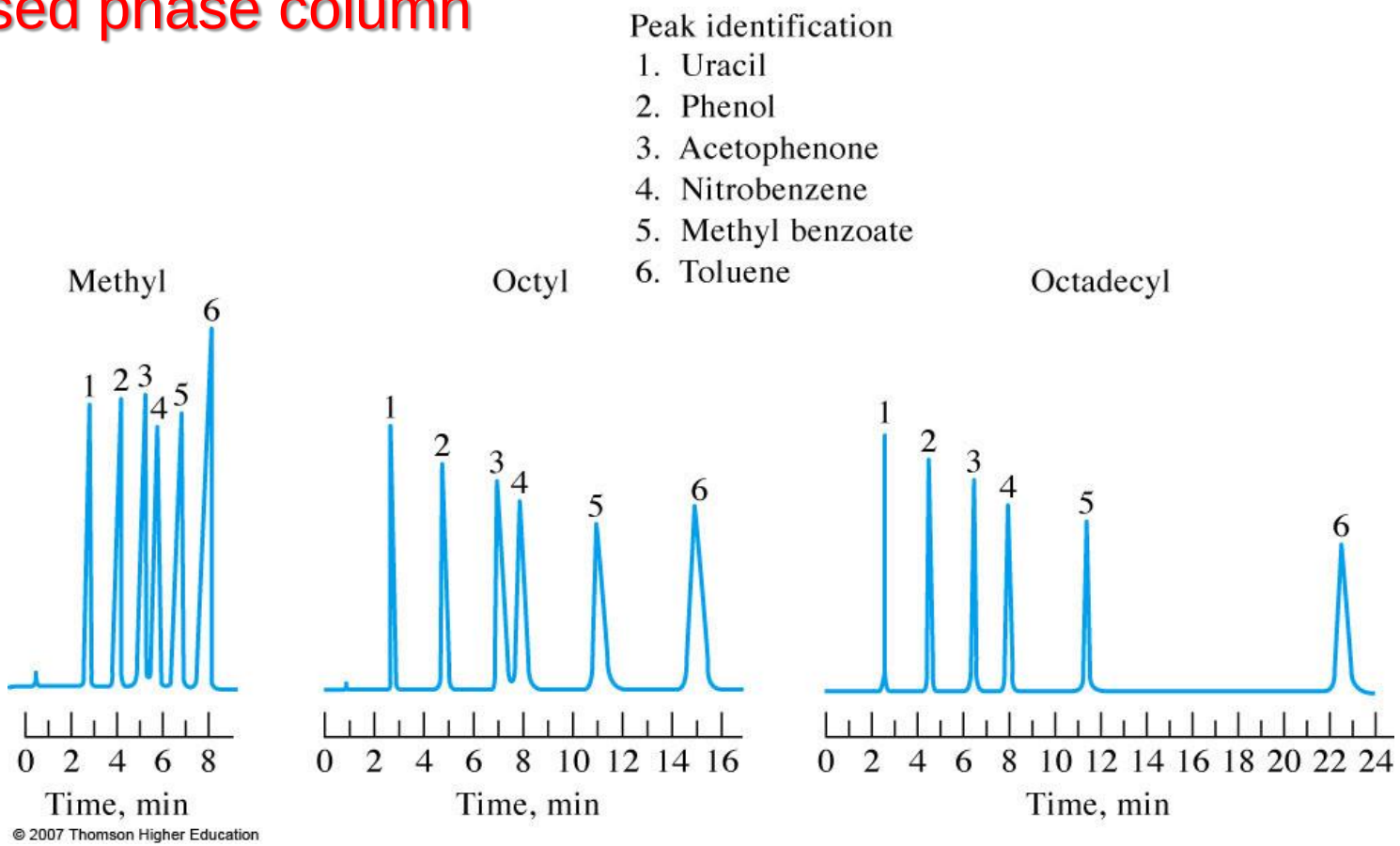


In normal-phase chromatography, the least polar component is eluted first because it is the most soluble in the mobile phase; increasing the polarity of the mobile phase has the effect of decreasing the elution time.

In contrast, in the reversed-phase method, the most polar component appears first, and increasing the mobile phase polarity increases the elution time.

- normal-phase partition chromatography and adsorption chromatography overlap considerably. In fact, retention in most types of normal-phase chromatography appears to be governed by adsorption-displacement processes.
- *Bonded-phase packings* are classified as reversed phase when the bonded coating is nonpolar in character and as normal phase when the coating contains polar functional groups. It has been estimated that more than three quarters of all HPLC separations are currently performed in columns with reversed-phase packings. The major advantage of reversed-phase separations is that water can be used as the mobile phase.
- Water is an inexpensive, nontoxic, UV-transparent solvent compatible with biological solutes. Also, mass transfer is rapid with nonpolar stationary phases, as is solvent equilibration after gradient elution.
- Most commonly, the R group of the siloxane in these coatings is a C_8 chain (n-octyl) or a C_{18}chain (n-octyldecyl).
- The mechanism by which these surfaces retain solute molecules is not entirely clear. The retention mechanism appears to be quite complex and very different from bulk phase partitioning because the nonpolar stationary phase is anchored at one end.
- Regardless of the detailed mechanism of retention, a bonded coating can usually be treated as if it were a conventional, physically retained liquid.

Effect of chain length of alkyl group on performance of reversed phase column



✓ Longer chains produce packings that are more retentive.

✓ In addition, longer chain lengths permit the use of larger samples. For example, the maximum sample size for a C_{18} packing is roughly double that for a C_4 preparation under similar conditions.

• In most applications of reversed-phase chromatography, elution is carried out with a highly polar mobile phase, for example, an aqueous solution containing various concentrations of such solvents as methanol, acetonitrile, or tetrahydrofuran. In this mode, care must be taken to avoid pH values greater than about 7.5 because the silica can form soluble silicate species, causing dissolution of the stationary phase.

• Also, hydrolysis of the siloxane can occur in alkaline solutions, which leads to degradation or destruction of the packing. Acid hydrolysis of the siloxane can limit the pH to about 2.5 in acidic solutions.

• In commercial normal-phase bonded packings, the R in the siloxane structure is a polar functional group such as cyano, $-C_2H_4CN$; diol, $-C_3H_6OCH_2CHOHCH_2OH$; amino, $-C_3H_6NH_2$; and dimethylamino, $C_3H_6N(CH_3)_2$.

• The polarities of these packing materials vary over a considerable range, with the cyano type being the least polar and the amino types the most. Diol packings are intermediate in polarity. With normal-phase packings, elution is carried out with relatively nonpolar solvents, such as ethyl ether, chloroform, and n-hexane.

28D-2 Method Development in Partition Chromatography

Method development tends to be more complex in LC than in GC because in a liquid MP the sample components interact with both the SP and the MP In contrast, in GC, the MP simply carries the sample components through the SP and makes no contribution to the separation process.

That is, in gas chromatography separations are not significantly affected by whether the MP is helium, nitrogen, or hydrogen.

In marked contrast, the success of a partition chromatographic separation is often critically dependent on whether the MP is, say, Acetonitrile, Hexane, or Dioxane.

Systematic approach to the separation of 6 steroids

Column Selection in Partition Chromatographic Separations

- Successful chromatography with interactive MP requires a proper balance of intermolecular forces among the three active participants of the separation process - *the solute, the mobile phase, and the stationary phase*.
- These intermolecular forces are described qualitatively in terms of the relative polarity of each of the three reactants. The polarities of the various analyte functional groups increase in the following order:

hydrocarbons < ethers < esters < ketones <aldehydes < amides < amines < alcohols.

- Water is more polar than compounds containing any of the functional gr.
- Often, in choosing a column for a partition chromatographic separation, the polarity of the stationary phase is matched roughly with that of the analytes; a mobile phase of considerably different polarity is then used for elution.
- This procedure is generally more successful than one in which the polarities of the solute and mobile phase are matched but different from that of the stationary phase. Here, the stationary phase often cannot compete successfully for the sample components; retention times then become too short for practical application.

Column Selection in Partition Chromatographic Separations

- At the other extreme, of course, is the situation where the polarity of the solute and stationary phases are too much alike and totally different from that of the mobile phase. Here, retention times become inordinately long.
- In summary, then, polarities for solute, mobile phase, and stationary phase must be carefully blended if good partition chromatographic separations are to be realized in a reasonable time.
- Unfortunately, theories of mobile-phase and stationary-phase interactions with any given set of sample components are imperfect, and at best, the user can only narrow the choice of stationary phase to a general type. Having made this choice, some trial-and-error experiments must be performed in which chromatograms are obtained with various mobile phases until a satisfactory separation is realized.
- The high degree of correlation between mobile-phase composition and retention factors, however, allows prediction of approximate retention times from only a few trial-and-error experiments. If resolution of all of the components of a mixture proves to be impossible, a different type of column may have to be chosen.

MP Selection in Partition Chromatography

- *N, k, and* α *are the* three parameters for improving the resolution of a chromatographic column;
- *In* LC, *the retention factor, k, is experimentally the most* easily manipulated of the three because of the strong dependence of this constant on the composition of the mobile phase. For optimal performance, *k should be in the ideal range between 2 and* 10; for complex mixtures, however, this range must often be expanded to perhaps 0.5 to 20 to provide time for peaks for all of the components to appear.
- Sometimes, adjustment of *k alone is not sufficient* to produce individual peaks with no overlap. In this case we must resort to variations in the *selectivity factor,* α *. Here again, the simplest way of bringing about* changes in α *is by altering the mobile-phase composition,* taking care, however, to keep *k within a reasonable* range. Alternatively, α *can be changed by choosing* a different column packing.

Effect of Solvent Strength on Retention Factors

➢ Solvents that interact strongly with solutes are termed *"strong" solvents*. Strong solvents are often, but not always, polar solvents. Solvent strength depends on the nature of the analyte and stationary phase. Several indexes have been developed for quantitatively describing the polarity of solvents.

➢ The most useful of these for partition chromatography is the polarity index *P', which was developed by Snyder.*

This parameter is based on solubility measurements for the substance in question in three solvents:

- *dioxane (a low dipole proton acceptor),*
- *nitromethane (a high dipole proton acceptor), and*
- *ethyl alcohol (a high dipole proton donor).*

The polarity index is a numerical measure of the relative polarity of various solvents. Table 28-2 lists polarity indexes (and other properties) for a number of solvents used in partition chromatography.

➢ Note that the polarity index varies from 10.2 for highly polar water to - 2 for the highly nonpolar fluoroalkanes.

Any desired polarity index between these limits can be achieved by mixing two appropriate solvents. Thus the polarity index P'_{AB} of a mixture of solvents A and B is given by

$$P'_{AB} = \phi_A P'_A + \phi_B P'_B$$

Where P'_A *and* P'_B are the polarity indexes of the two solvents A and B

ϕ_A *and* ϕ_B are the volume fractions of solvents A and B.

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

the easiest way to Improve the chromatographic resolution of two species is by manipulating the *retention factor k. which* can in turn be varied by changing the polarity index of the solvent.

For a normal phase separation

$$\frac{k_2}{k_1} = 10^{(P_1' - P_2')/2}$$

for a reversed-phase column

$$\frac{k_2}{k_1} = 10^{(P_2' - P_1')/2}$$

EXAMPLE 28-1 In a reversed-phase column, a solute was found to have a retention time of 31.3 min, and an unretained species required 0.48 min for elution when the mobile phase was 30% (by volume) methanol and 70% water. Calculate (a) *k and (b) a water-methanol composition that* should bring *k to a value of about 5.*

Solution

(a) Application of Equation 26-12 for retention factor yields

$$k = (31.3 - 0.48)/0.48 = 64$$

(b) To obtain *P' for the mobile phase we substitute* polarity indexes for methanol and water from Table 28-2 into Equation 28-2

$$P'_{AB} = \phi_A P'_A + \phi_B P'_B$$

$$P' = (0.30 \times 5.1) + (0.70 \times 10.2) = 8.7$$

Substitution of this result into Equation

$$\frac{k_2}{k_1} = 10^{(P'_2 - P')/2}$$

$$5/64 = 10^{(P'_2 - 8.7)/2}$$

If we take the logarithm of both sides, we can solve for P'_2 $P'_2 = 6.5$

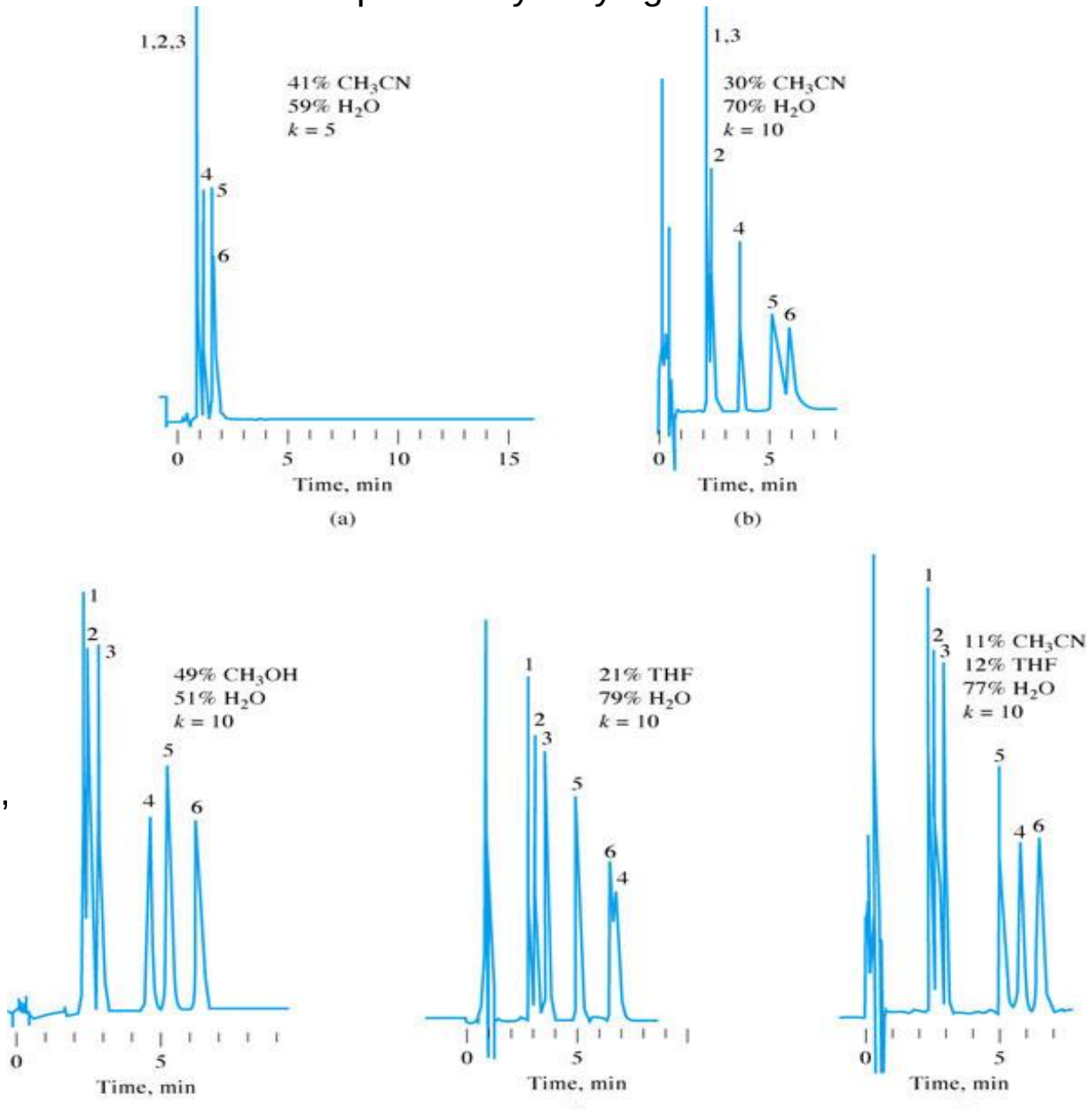
Letting *x be the volume fraction of methanol in* the new solvent mixture and substituting again into Equation 28-2, we find

$$6.5 = (x \times 5.1) + (1 - x) \times 10.2$$

$$x = 0.73, \text{ or } 73\%$$

➢ Often, in reversed-phase separations, we use a solvent mixture containing water and a polar organic solvent. The retention factor can then be manipulated by varying the water concentration as shown by Example 28-1.

➢ The effect of such manipulations is shown by the chromatograms in Figure 28-16a and b where the sample was a mixture of six steroids. With a mixture of 41% acetonitrile and 59% water, *k had a value of 5* and all of the analytes were eluted in such a short time (2 min) that the separation was quite incomplete. By increasing the concentration of water to 70%, elution took place over 7 min, which doubled the value of *k.* Now the total elution time was sufficient to achieve a separation, but the α *value for compounds 1 and 3 was* not great enough to resolve them.



Effect of Mobile Phase on Selectivities

Solvent Optimization

➢The most widely accepted approach for this type of optimization is called the *solvent selectivity triangle."*

➢ *Here, the mobile-phase effects contributing* to selectivity are considered to be the result of proton donor, proton acceptor, and dipolar interactions.

➢ Solvents incorporating these interactions are then chosen for statistically designed experiments to find the optimal solvent mixture.

• For reversed-phase chromatography, the three solvent modifiers are methanol, acetonitrile, and tetrahydrofuran.

• Water is then used to adjust the solvent strength of the mixtures and yield a suitable value of *k.*

• Three binary solvent compositions (water plus modifier) define the three vertices of the solvent triangle Figure 28-17.

• Usually, seven to ten experiments are enough to define a solvent composition that will produce the best selectivity for a suitable *k range.*

• Software is also available for solvent optimization.

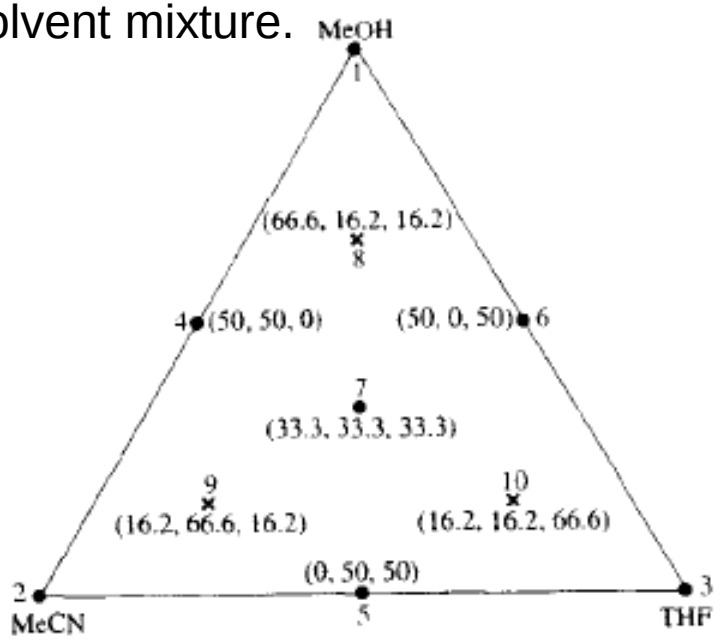


FIGURE 28-17 Solvent selectivity triangle for optimization of reversed-phase separations. Ten mixtures of the three organic solvents (methanol, acetonitrile, and tetrahydrofuran) are shown with the relative proportions indicated in parentheses (MeOH, MeCN, and THF). Water is used to maintain solvent strength and keep the *k* value within an appropriate range.

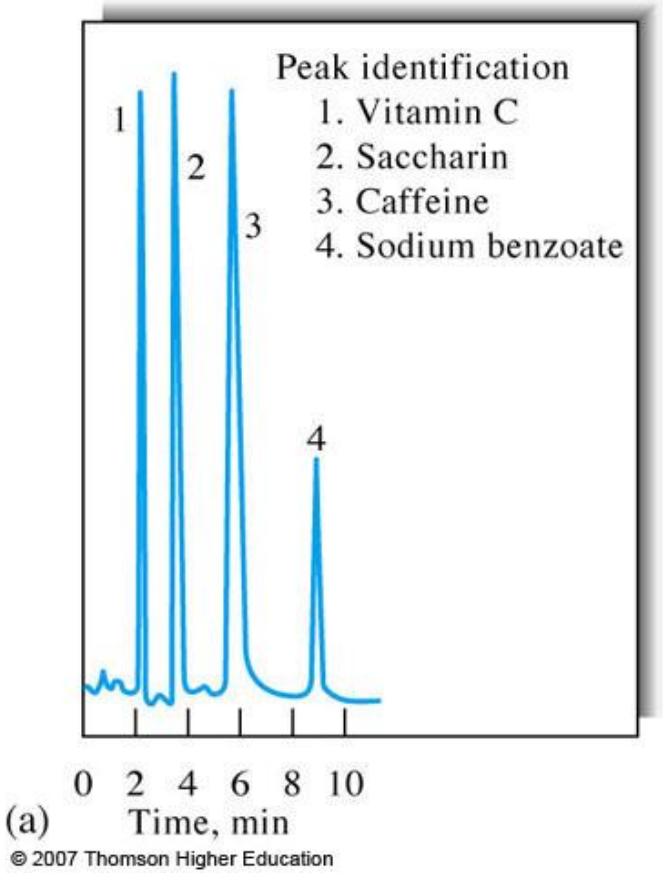
- For normal-phase separations, a similar solvent triangle is used in which the selectivity solvents are
  - ethyl ether,
  - methylene chloride, and
  - chloroform.
- The solvent strength adjustment is then made with n-hexane.
- With these solvent systems, optimization can be accomplished with a minimal number of experiments.

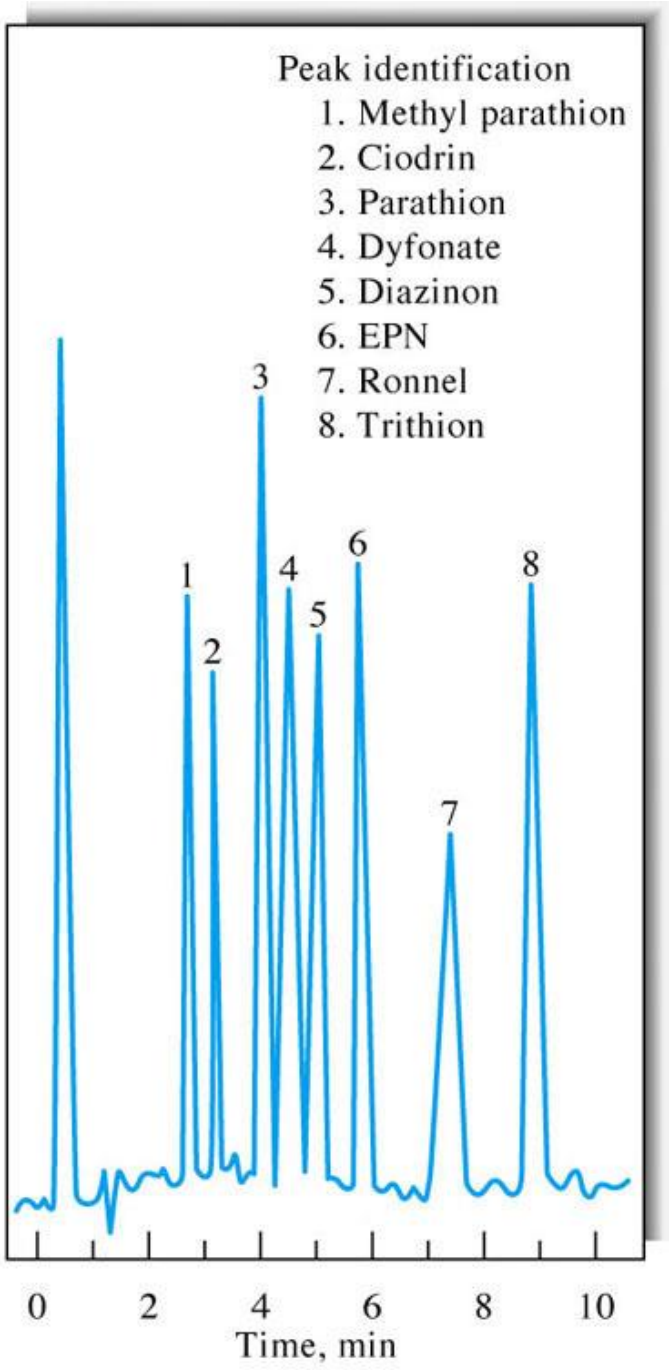
TABLE 28-3 Typical Applications of Partition Chromatography

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, polychlorinated biphenyls
Forensic science	Drugs, poisons, blood alcohol, narcotics
Clinical chemistry	Bile acids, drug metabolites, urine extracts, estrogens

Typical applications of bonded-phase chromatography

Soft drink additives Column: 4.6 x 250 mm packed with polar (nitrile)bonded-phase packing. Isocratic elution with 6% HOAc to
94% H20. Flow rate 1.0 ml/min. (Courtesy of BTRSeparations, a DuPont ConAgra affiliate.)
b)Organophosphate insecticides. Column 4.5 x 250 mm packed with 5 μm C8 bonded-phase particles. Gradient elution: 67% CH30H to 33% H20, to 80% CH30H to 20% H20. Flow rate : 2 mL/min. Both used 254-nm-UVdetectors.





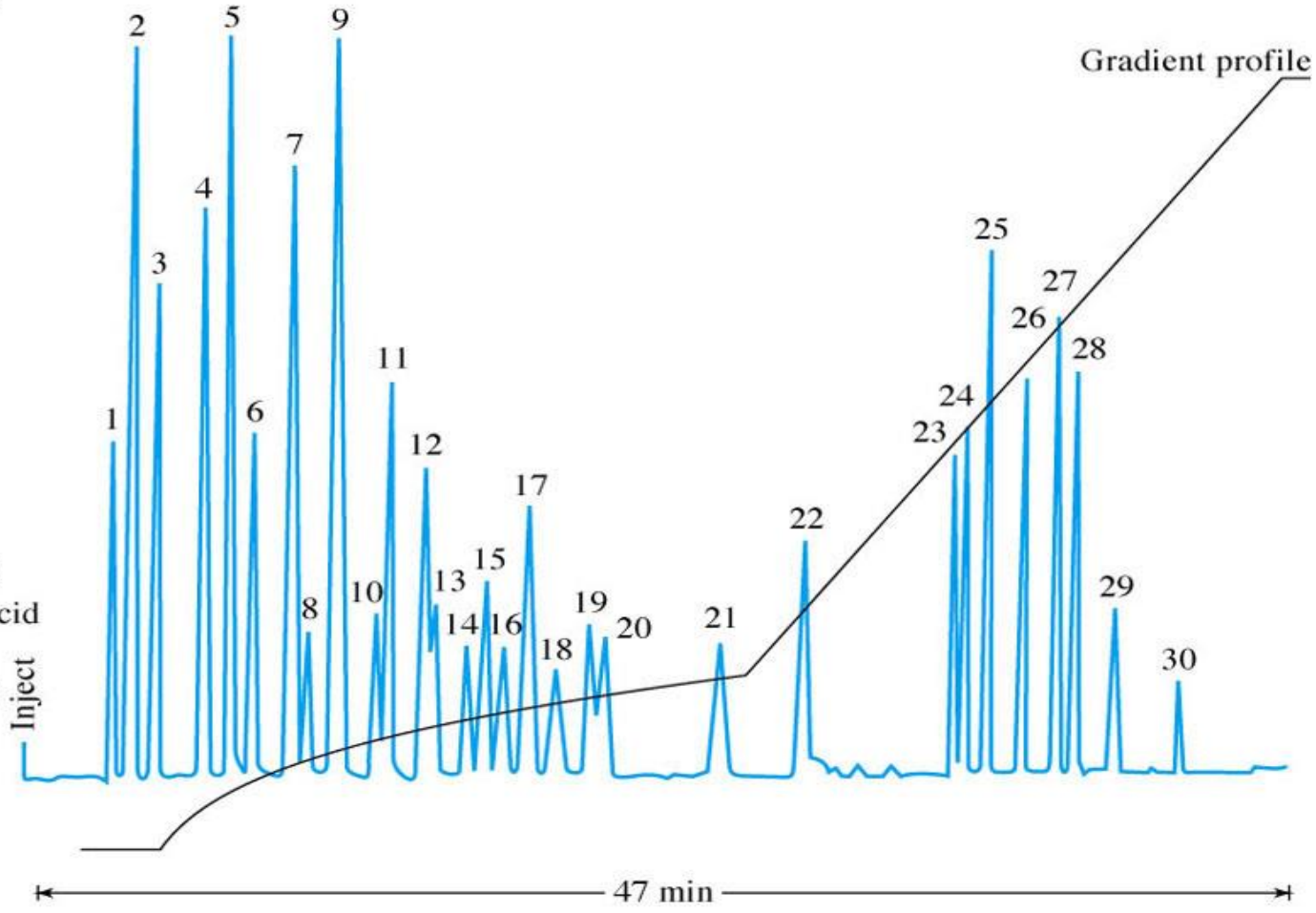
HPLC Derivatization Methods

- To convert the components of a sample to a different chemical form (derivative) before or after the column
- Why derivatize?
  - to reduce the polarity of the species so that partition rather than adsorption or ionexchange columns can be used;
  - to increase the detector response, and thus sensitivity, for all of the sample components; and
  - to selectively enhance the detector response to certain components of the sample.

Thirty amino acids of physiological importance.

precolumn derivative formation with orthophthalaldehyde.

1. Phosphoserine
2. Aspartic acid
3. Glutamic acid
4. α-Amino adipic acid
5. Asparagine
6. Serine
7. Glutamine
8. Histidine
9. Glycine
10. Threonine
11. Citrulline
12. 1-Methylhistidine
13. 3-Methylhistidine
14. Arginine
15. β-Alanine
16. Alanine
17. Taurine
18. Anserine
19. β-Aminobutyric acid
20. β-Aminoisobutyric acid
21. Tyrosine
22. α-Aminobutyric acid
23. Methionine
24. Valine
25. Tryptophan
26. Phenylalanine
27. Isoleucine
28. Leucine
29. δ-Hydroxylysine
30. Lysine



Column: 5 µm C18. reversed-phase.
Solvent A: 0.05 MNa2HPO4 pH 7.4,96:2:2 CH30H, THF,H20.
Fluorescence detector: excitation 334 nm: emission 425 nm. (Reprinted with permission from R. Pfiefer et al.,*Amer. Lab., 1983, 15 (3),86.*

Ion-Pair Chromatography

Ion-pair chromatography, sometimes called paired-ion chromatography is a subset of reversed-phase chromatography in which easily ionizable species are separated on reversed-phase columns.

In this type of chromatography an organic salt containing a large organic counterion, such as a quaternary ammonium ion or alkyl sulfonate, is added to the mobile phase as an ion-pairing reagent. Two mechanisms for separation are postulated.

In the first, the counterion forms an uncharged ion pair with a solute ion of opposite charge in the mobile phase. This ion pair then partitions into
the nonpolar stationary phase, giving differential retention of solutes based on the affinity of the ion pair for the two phases.

Alternatively, the counter ion is retained strongly by the normally neutral stationary phase and imparts a charge to this phase. Separation of organic solute ions of the opposite charge then occurs by formation of reversible ion-pair complexes with the more strongly retained solutes forming the strongest complexes with the stationary phase.
Some unique separations of both ionic and nonionic compounds in the same sample can be accomplished by this form of partition chromatography.

ion-pair chromatography frequently overlap those of ion-exchange chromatography. For the separalion of small inorganic and organic ions, ion exchange is usually preferred.

- ✓ Organic counter-ion (ion-pairing reagent) in mobile phase: NR_4^+, RSO_3^-
- ✓ Partition of neutral species or retention by charges

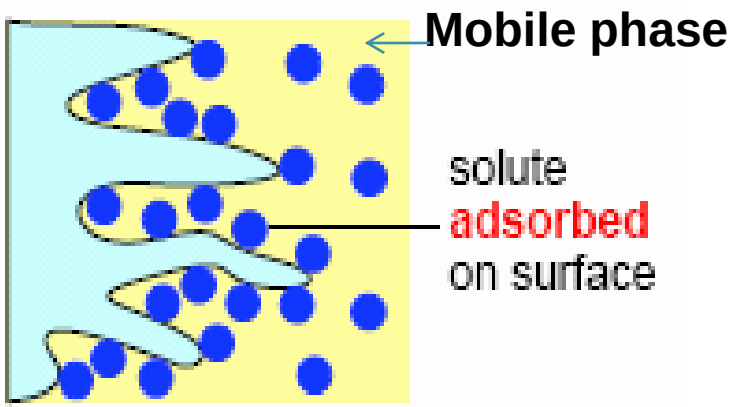
Chiral Chromatography

chiral compounds, Nonsuperimposable mirror images of each other, e*nantiomers.*

- Either chiral MP additives or chiral SP are required for these separations.
- Separation based on the use of chiral stationary phases which interact to varing degrees with optical isomers of the solute
- Systems differ little from conventional HPLC systems
- Chiral stationary phases have received the most attention. Here. a chiral agent is immobilized on the surface of a solid support.
- Several different modes of interaction can occur between the chiral resolving agent and the solute. In one type. the interactions are due to attractive forces such as those between π *bonds, hydrogen bonds, or dipoles. In another* type, the solute can fit into chiral cavities in the stationary phase to form inclusion complexes.
- No matter what the mode, the ability to separate these very closely related compounds is of extreme importance in many fields. Figure 28-21 shows the separation of a racemic
- mixture of an ester on achiral stationary phase. Note

Adsorption Chromatography

- Liquid-solid chromatography: adsorption of analyte on a solid surface
- Stationary phase: polar solid, finely divided silica SiO_2(higher sample capacity ∴ more common) or alumina Al_2O_3
- Analyte polarity ↑, t_R↑
- Mobile phase: the variable
- Application: relatively nonpolar, water-insoluble organic compounds
- Decreased application due to versatility and ready availability of bonded stationary phases of normal phase LC



Adsorption Chromatography

Advantages:

- retain and separate special compounds like geometrical isomers

Disadvantages:

– very strong retention of some solutes

– may cause catalytic changes in solutes

– solid support may have a range of chemical and physical environments

- non-symmetrical peaks
- variable retention times

Ion Chromatography

- Separation of inorganic cations and anions and low molecular weight water-soluble organic acids and bases
- Ion-exchange processes are based on exchange equilibria between ions in solution and ions of like sign on the surface of an essentially insoluble, high-molecular mass solid.
- clays and zeolites, natural ion-exchangers
- Synthetic ion-exchange resins (mid-1930s) for water softening, water deionization, and solution purification.
- Cation exchange resin: sulfonic acid – $SO_3^- H^+$ *(strong acid)*

  carboxylic acid –$COO^- H^+$ *(weak acid)*
  - When a sulronic acid ion-exchanger is brought in contact with an aqueous solvent containing a cation M*x+, an exchange equilibrium is set up that can be* described by

$$xRSO_3^- H^+ + M^{x+} \leftrightarrow RSO_3^- M^{x+} + xH^+$$

- Anion exchange resin: tertiary amine – $N(CH_3)_3^+ OH^-$ (strong base)

  primary amine – $NH_3^+ OH^-$ *(weak base)*

a strong-base exchanger interacts with the anion A^{x-} as shown by the reaction

$$xRN(CH_3)_3OH^- + A^{x-} \leftrightarrow [RN(CH_3)_3^+]_x A^{x-} + xOH^-$$

Concentrations, ppm	
F^-	3
Formate	8
BrO_3^-	10
Cl^-	4
NO_2^-	10
HPO_4^{2-}	30
Br^-	30
NO_3^-	30
SO_4^{2-}	25

Concentrations, ppm	
Ca^{2+}	3
Mg^{2+}	3
Sr^{2+}	10
Ba^{2+}	25

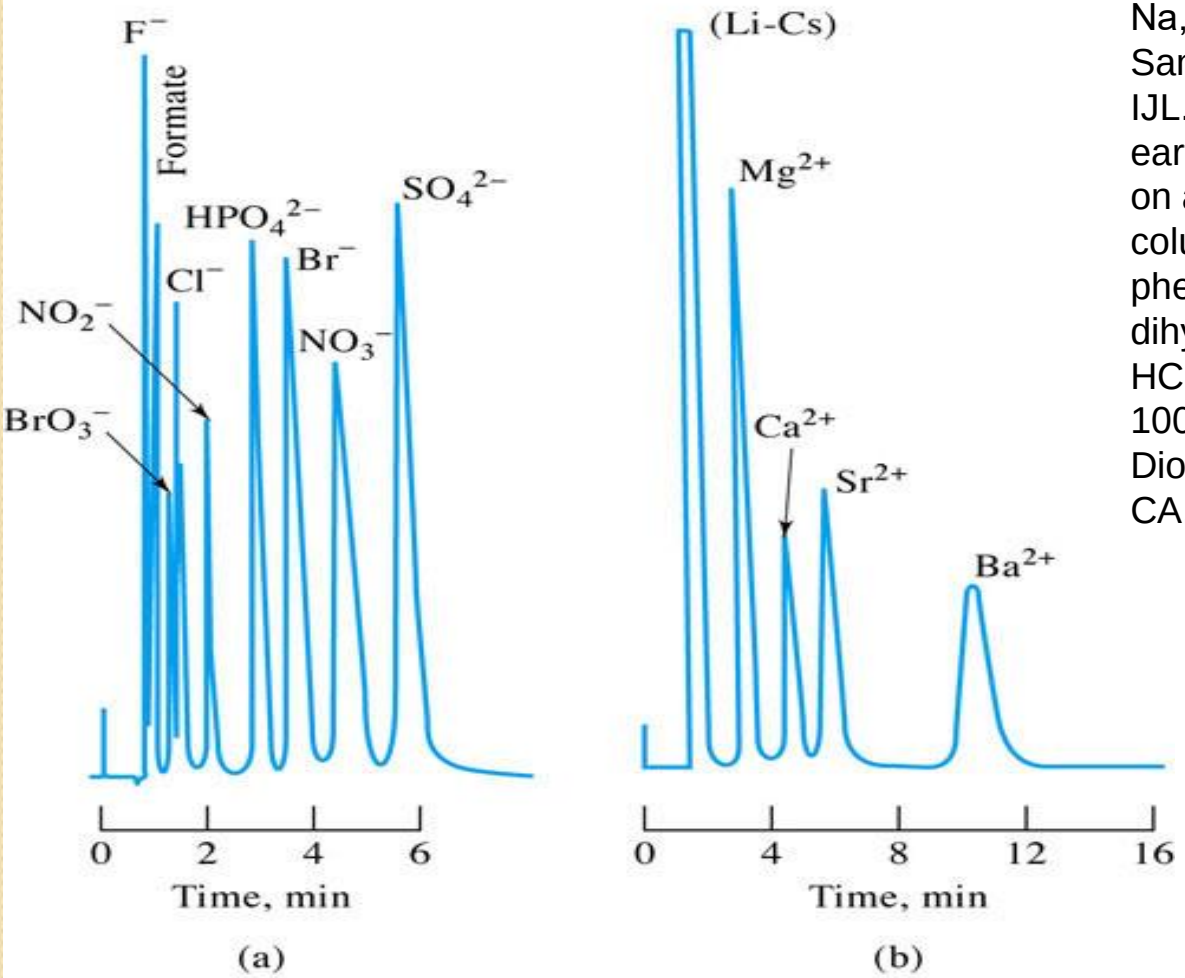


FIGURE28-24 Typical applications of ion chromatography. (a)Separation of anions on an anion-exchange column. Eluent: 0.0028 M NaHC03 to 0.0023 M Na,C03• Sample size: 50 IJL.(b)Separation of alkaline earth ions on a cation-exchange column. Eluent: 0.025 M phenylenediamine dihydrochloride to 0.0025 M HCI.Sample size: 100 IJL.(Courtesy of DionexCorp., Sunnyvale, CA.)

Ion Exchange Chromatography

- *Keq represents the* affinity of the resin for the ion B^+ relative to another ion (here, H^+).
- Where *Keq is large, a strong tendency* exists for the solid phase to retain B^+; where *Keq is* small, the reverse is true.
- $K_{eq} \uparrow \; t_R \uparrow$:
- Such experiments reveal that polyvalent ions are much more strongly held than singly charged species.
- Within a given charge group, however, differences appear that are related to the size of the hydrated ion as well as to other properties. Thus, for a typical sulfonated cation-exchange resin, values for K_{eq} *decrease in the order*

$$Tl^+ > Ag^+ > Cs^+ > Rb^+ > K^+ > NH_4^+ > Na^+ > H^+ > Li^+$$

For divalent cations,

$$Ba^{2+} > Pb^{2+} > Sr^{2+} > Ca^{2+} > Ni^{2+} > Cd^{2+} > Cu^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} > UO_2^{2+}$$

For anions, *Keq for a strong-base resin decreases in* the order

$$SO_4^{2-} > C_2O_4^{2-} > I^- > NO_3^- > Br^- > Cl^- > HCO_2^- > CH_3CO_2^- > OH^- > F^-$$

Ion Exchange Packings

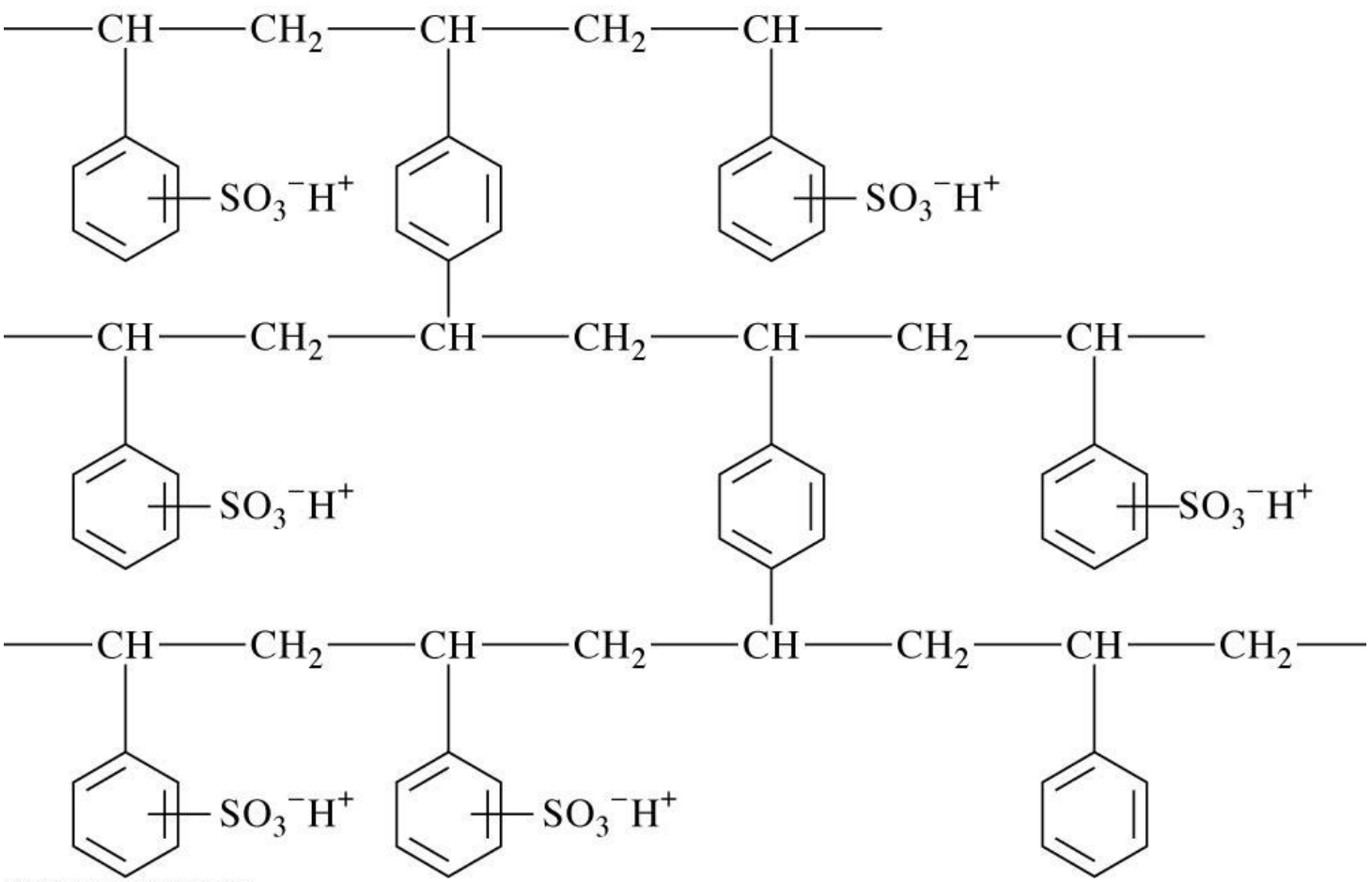


Figure 28-22 The structure of a strong acid resin. Note the cross-linking that holds the linear polystyrene molecules together. The other types of resins have similar structures except for the active functional group.

IEC Stationary Phases

- Silica based materials
  - Pellicular particles
- Organic materials
  - porous beads
  - styrene/divinylbenzene crosslinked co-polymers
  - methacrylic acid/divinylbenzene crosslinked co-polymers
- Inorganic materials

Ion Exchange Chromatography

•Suppressor-based IEC:

Eluent suppressor column: eliminates the high conductivity of eluent ions by the introduction of an *eluent suppressor column immediately following the ion exchange* column.

•The suppressor column is packed with a second ion-exchange resin that effectively converts the ions of the eluting solvent to a molecular species of limited ionization without affecting the conductivity due to analyte ions.

– Cation separation: HCl (eluting reagent), suppresor column (anion-exchange resin)

$$H^+(aq) + Cl^-(aq) + resin^+ OH^-(s) \rightarrow resin^+ Cl^-(s) + H_2O$$

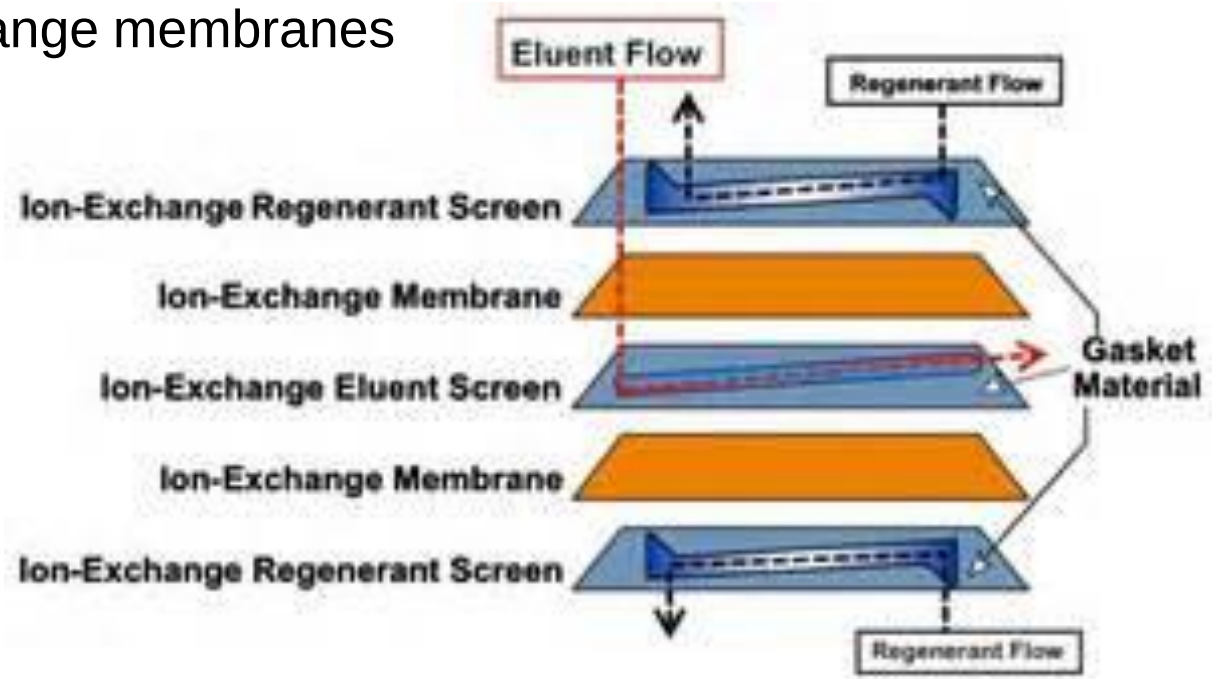
– Anion separation: $NaHCO_3$ or Na_2CO_3 (eluting reagent), cation-exch. resin

$$Na^+(aq) + HCO_3^-(aq) + resin^- H^+(s) \rightarrow resin^- Na^+(s) + H_2CO_3(aq)$$

Largely undissociated carbonic acid does not contribute significantly to the conductivity

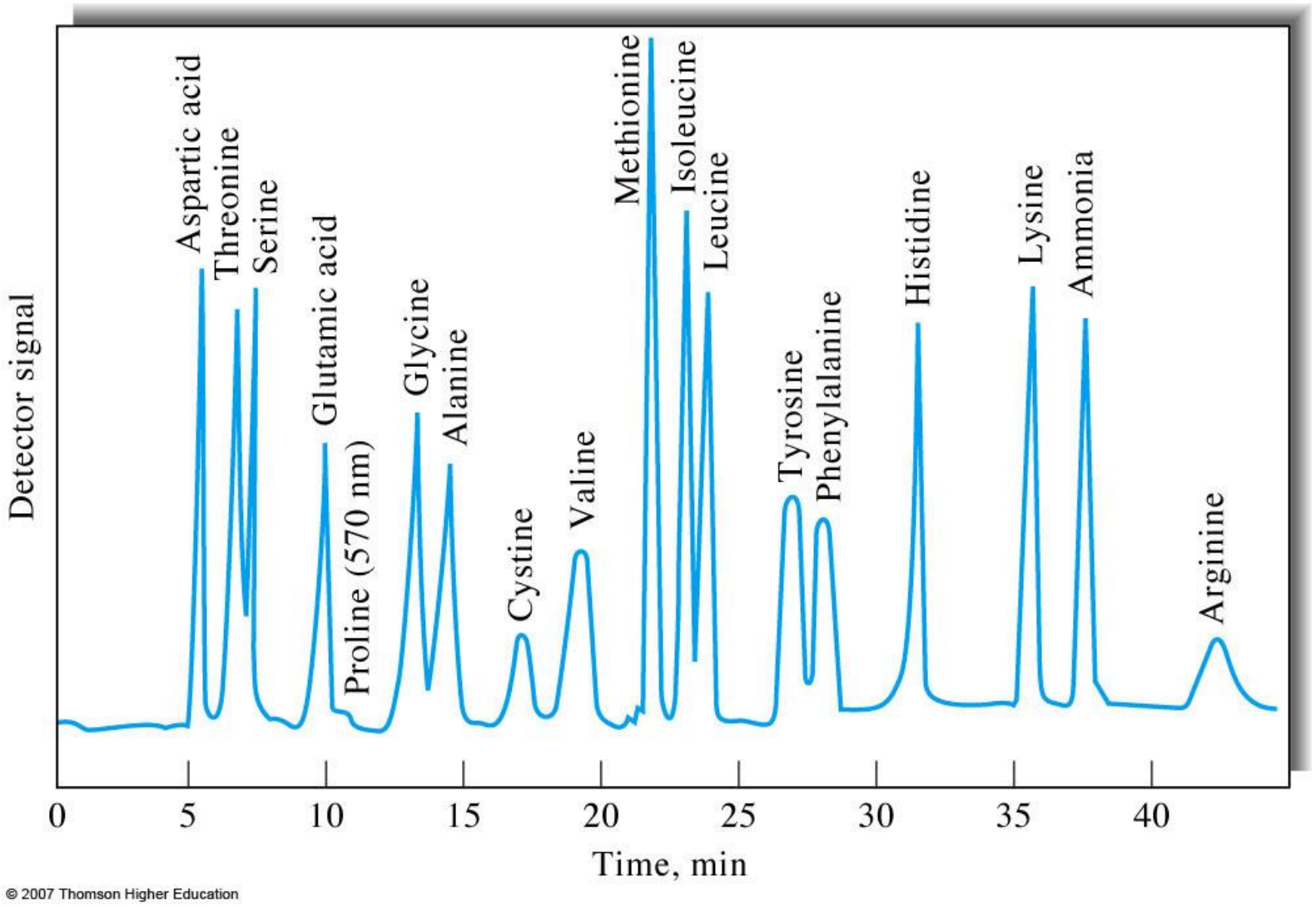
- Suppresor columns need to regenerate periodically (every 8 to 10 h), inconvenience
  - Micromembrane suppressor: no need for regeneration
  - Ultra-thin ion-exchange membranes

-Analyte elution in one direction
- Regeneration in opposite direction



Single Column Ion Chromatography

- Commercial ion chromatography instrumentation that requires no suppressor column is also available
- Less sensitive and limited range comp. To suppressor column IC.

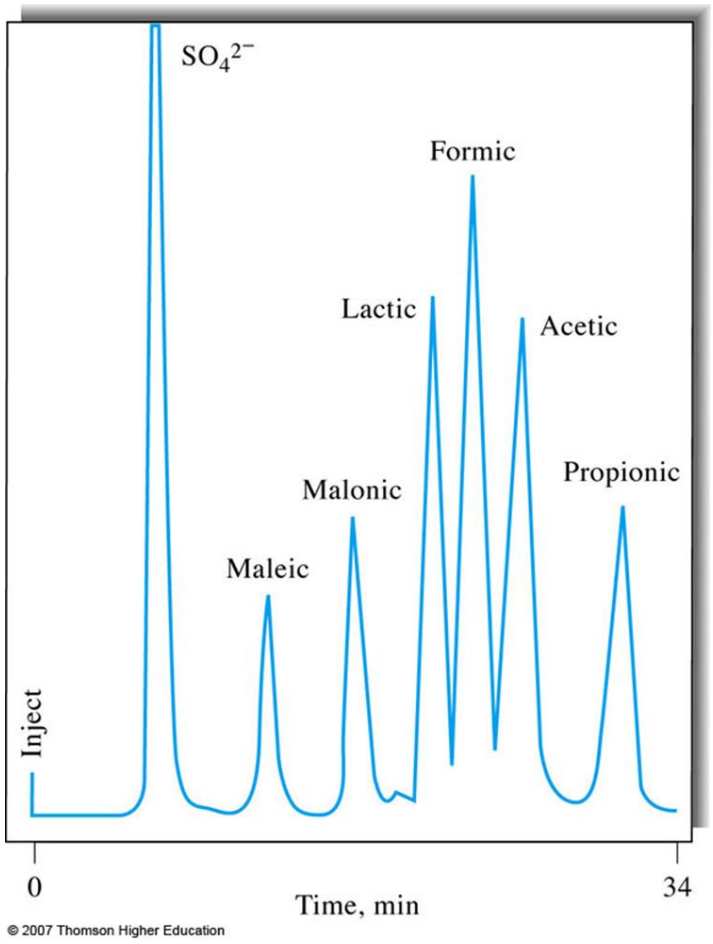


Separation of amino acids on a cation-exchange column

Ion Exclusion Chromatography

- is not a form of ion chromatography because neutral species are separated using strong cation and anion exchange columns

Here, the stationary phase was a cation-exchange resin in its acidic form, and elution was accomplished with a dilute solution of hydrochloric acid. The analytical column was followed by a suppressor column packed with a cation-exchange resin in the silver form. The hydrogen ions of the eluent were exchanged for silver ions, which then precipitated the chloride ions, thus removing the ions contributed by the eluent. The undissociated form of the analyte acids was distributed between the mobile phase in the column and the immobilized liquid held in the pores of the packing. The distribution constants for the various acids are primarily related to the inverse of their dissociation constants, although other factors also play a part in the extent to which various species are distributed between the two phases.



Size Exclusion Chromatography

- Also known as gel permeation or gel filtration chromatography
- used for large MW compounds - proteins and polymers
- Separation mechanism is sieving not partitioning
- Stationary phase porous silica or polymer particles (polystyrene, polyacrylamide) (5-10 µm) with well-defined pore sizes (40-2500 Å) into which solute and solvent molecules can diffuse
- The average residence time in the pores depends upon molecular size and shape of the analyte molecules
  - 1. Large molecules excluded from pores - not retained, first eluted
  - 2. Intermediate molecules - retained, intermediate elution times
  - 3. Small molecules permeate into pores - strongly retained, last eluted

➢ *Note that size-exclusion separations differ from the other procedures we have been considering in that no chemical or physical interaction between analytes and the stationary phase are involved. Indeed, such interactions are avoided because they lead to lower column efficiencies.*

➢ *Also note that, unlike other forms of chromatography, there is an upper limit to retention time because no analyte species is retained longer than those that totally permeate the stationary phase.*

TABLE 28-4 Properties of Typical Packings for Size-Exclusion Chromatography

Type	Particle Size, μm	Average Pore Size, Å	Molecular Mass Exclusion Limit*
Polystyrene-divinylbenzene	10	100	700
		1000	$(0.1 \text{ to } 20) \times 10^4$
		10^4	$(1 \text{ to } 20) \times 10^4$
		10^5	$(1 \text{ to } 20) \times 10^5$
		10^6	$(5 \text{ to } >10) \times 10^6$
Silica	10	125	$(0.2 \text{ to } 5) \times 10^4$
		300	$(0.03 \text{ to } 1) \times 10^5$
		500	$(0.05 \text{ to } 5) \times 10^5$
		1000	$(5 \text{ to } 20) \times 10^5$

*Molecular mass above which no retention occurs.

Originally, styrene-divinylbenzene gels were hydrophobic and thus could be used only with non-aqueous mobile phases. Now, however, hydrophilic gels are available, making possible the use of aqueous solvents for the separation of large, water-soluble molecules such as sugars.

These hydrophilic gels are sulfonated divinylbenzenes or polyacrylamides. Chromatography based on the hydrophilic packings was once called *gel filtration,* and techniques based on hydrophobic packings were termed *gel permeation. Today, both techniques* are described as size-exclusion methods.

Affinity Chromatography

- Affinity chromatography involves covalently bonding a reagent called an *affinity ligand, to a solid support.*
- Typical affinity ligands are antibodies, enzyme inhibitors, or other molecules that reversibly and selectively bind to analyte molecules in the sample.
- When the sample passes through the column, only the molecules

that selectively bind to the affinity ligand are retained.

- Molecules that do not bind pass through the column with the mobile phase. After the undesired molecules are removed, the retained analytes can be eluted by changing the mobile-phase conditions.
- The stationary phase for affinity chromatography is a solid such as agarose or a porous glass bead to which the affinity ligand is immobilized.
- The mobile phase in affinity chromatography has two distinct roles to play.

First, it must support the strong binding of the analyte molecules to the ligand. Second, once the undesired species are removed, the mobile phase must weaken or eliminate the analyte-ligand interaction so that the analyte can be eluted.

- Often, changes in pH or ionic strength are used to change the elution conditions during the two stages of the process.
- Affinity chromatography has the major advantage of extraordinary specificity. The primary use is in the rapid isolation of biomolecules during preparative work.

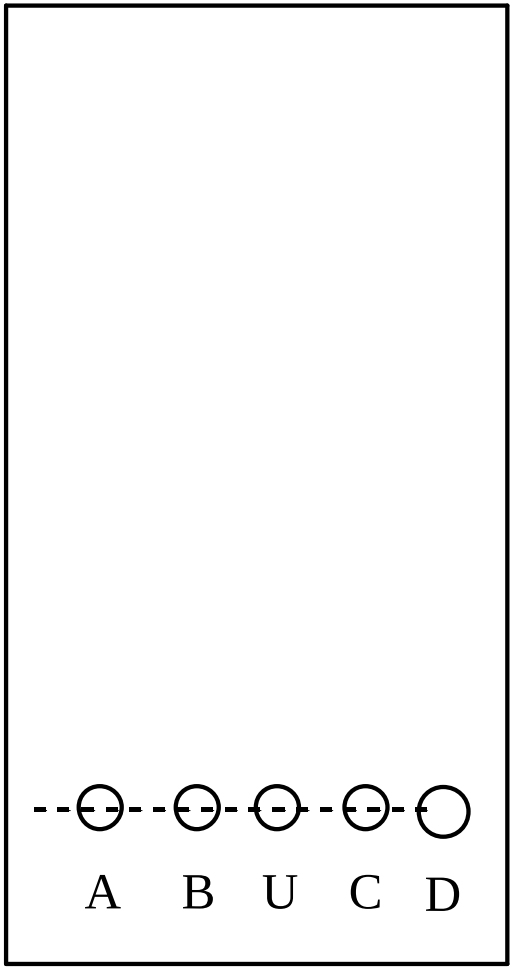
Planar Chromatography

Paper Chromatography
Thin Layer Chromatography

- Stationary Phase: flat, relatively thin layer of material that is either self supporting or is coated on a glass, plastic or metal surface, usually **silica gel, SiO_2**
- Mobile Phase moves through the SP by ***capillary action*** sometimes assisted by gravity or an electrical potential.
- Planar Chromatography is also called 2-D chromatography.
- TLC is more widely used than Paper Chromatography due to
  - faster
  - better resolution
  - more sensitive
  - than Paper Chromatography

Scope of TLC

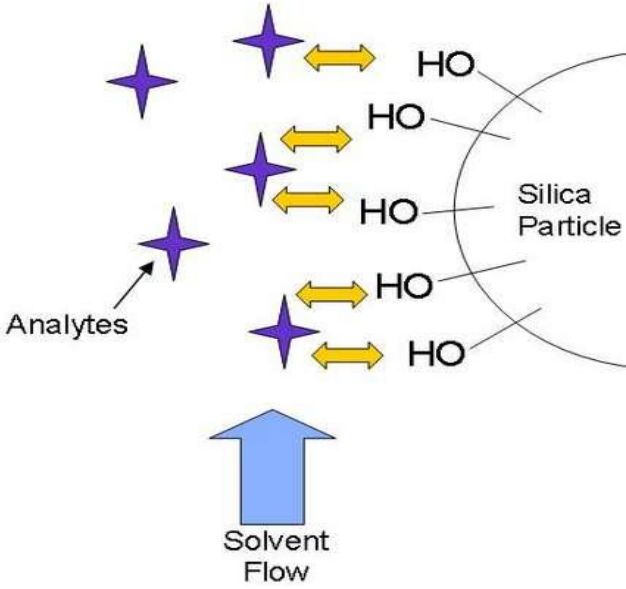
- In TLC, a plastic, glass or aluminum sheet is coated with a thin layer of silica gel.
- A very small amount of a solution of the substance to be analyzed is applied in a small spot with a capillary tube, ~1cm from the bottom of the TLC plate
- The TLC is *developed* in a chamber which contains the developing solvent (the mobile phase). A truncated filter paper placed in the chamber serves to saturate the chamber with mobile phase.



THIN LAYER CHROMATOGRAPHY

As the mobile phase rises up the TLC plate by capillary action, the components dissolve in the solvent and move up the TLC plate.

Individual components move up at different rates, depending on *intermolecular forces* between the component and the silica gel stationary phase and the component and the mobile phase.



http://www.instructables.com/id/EW1YDCYF4REC0IU/

The stationary phase is SiO_2 and is very "*polar*".

It is capable of strong *dipole-dipole* and *H-bond donating and accepting* interactions with the "*analytes*". More *polar* analytes interact more strongly with the stationary phase and move very slowly up the TLC plate.

By comparison, the mobile phase is relatively *nonpolar* and is capable of interacting with analytes by stronger *London forces*, as well as by dipole-dipole and H-bonding.

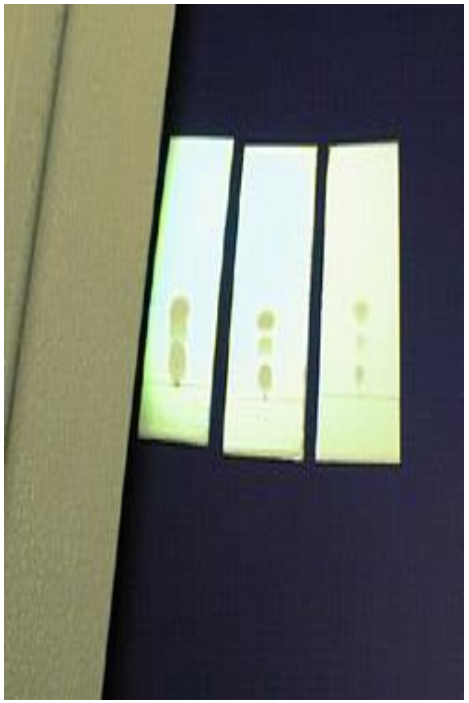
Nonpolar analytes interact less strongly with the polar silica gel and more strongly with the less polar mobile phase and move higher up the TLC plate.

THIN LAYER CHROMATOGRAPHY

✓ Once the solvent is within ~1-2 cm of the top of the TLC sheet, the TLC is removed from the developing chamber and the farthest extent of the solvent (the *solvent front*) is marked with a pencil.

✓ The solvent is allowed to evaporate from the TLC sheet in the hood.

✓ The spots are visualized using a UV lamp.



✓ A fluorescent compound, usually Manganese-activated Zinc Silicate, is added to the adsorbent that allows the visualization of spots under a black light (UV254). The adsorbent layer will fluoresce light green by itself, but spots of analyte quench this fluorescence and appear as a dark spot.

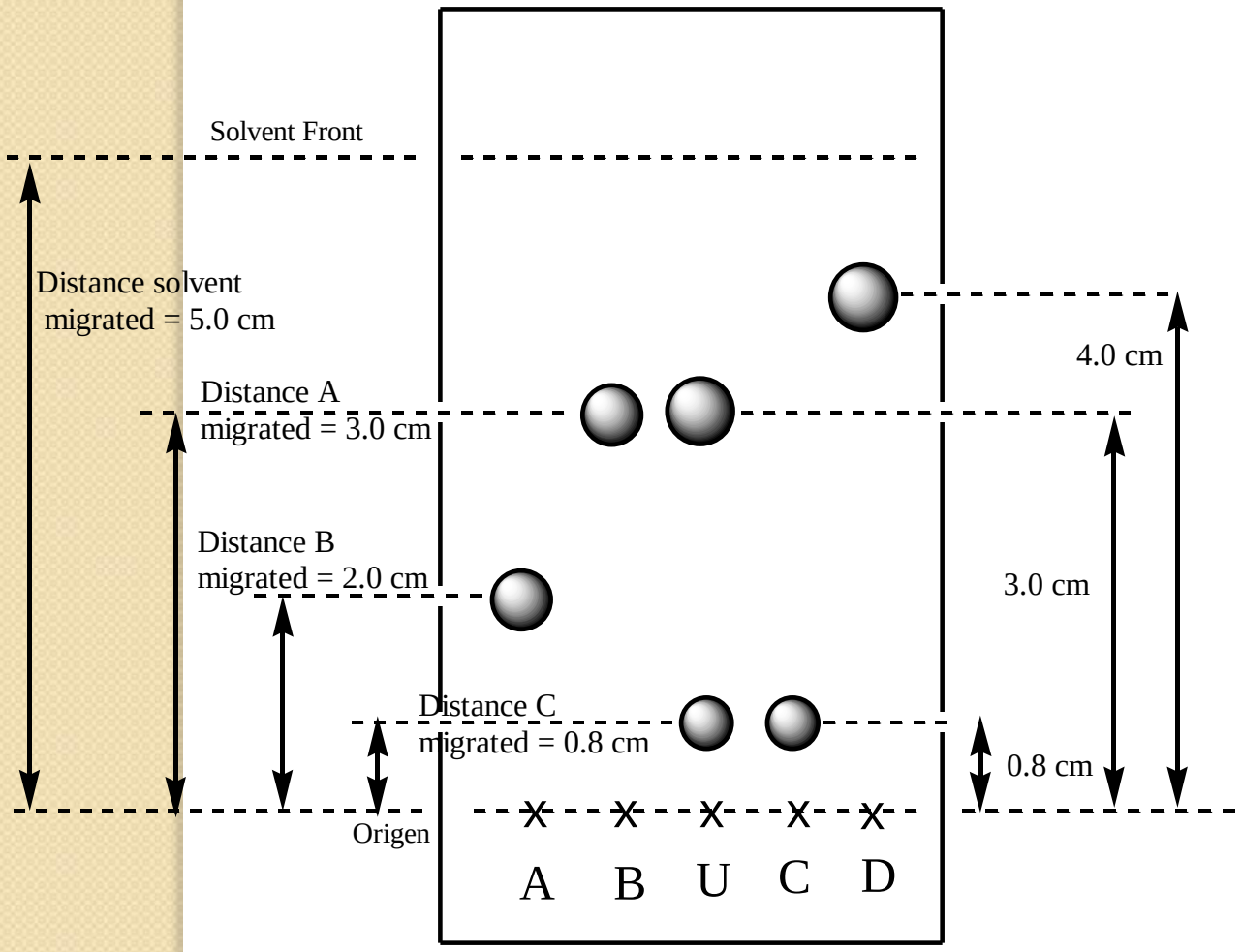
✓ Specific color reagents exist into which the TLC plate is dipped or which are sprayed onto the plate(ninhydrin).

http://orgchem.colorado.edu/hndbksupport/TLC/TLCprocedure.html

Performance Characteristics of a Thin Layer Plates

Retardation Factor, Rf

$$R_F = d_R / d_M$$



$$R_f\,(A) = \frac{2.0\text{ cm}}{5.0\text{ cm}} = 0.40$$

$$R_f\,(B) = \frac{3.0\text{ cm}}{5.0\text{ cm}} = 0.60$$

$$R_f\,(C) = \frac{0.8\text{ cm}}{5.0\text{ cm}} = 0.16$$

$$R_f\,(D) = \frac{4.0\text{ cm}}{5.0\text{ cm}} = 0.80$$

$$R_f\,(U_1) = \frac{3.0\text{ cm}}{5.0\text{ cm}} = 0.60$$

$$R_f\,(U_2) = \frac{0.8\text{ cm}}{5.0\text{ cm}} = 0.16$$

The R_f is defined as the distance the center of the spot moved divided by the distance the solvent front moved (both measured from the origin)

Retardation Factor, Rf

R_f values can be used to aid in the identification of a substance by comparison to standards.

The R_f value is not a physical constant, and comparison should be made only between spots on the same sheet, run at the same time.

Two substances that have the same R_f value may be identical; those with different R_f values are not identical.

Value for Rf can vary from 1 for solutes that are not retarded to a value that approaches 0.

Retention Factor

d_R and d_M in TLC are related to t_R and t_M in eqn's given in Ch.26

$t_M = d_R / u$ $\quad$ $t_R = d_M / u$

Retention factor

$$k' = \frac{t_R - t_M}{t_M} \qquad k' = \frac{d_M - d_R}{d_R}$$

Retention factor in terms of retardation factor

$R_F = d_R / d_M$

$$k' = \frac{1 - R_F}{R_F}$$

Plate Heights

$$N = 16 \left(\frac{d_R}{W} \right)^2$$

$$H = d_R / N$$

Resolution

The separation between two analytes on a chromatogram can be expressed as the resolution, Rs and can be determined using the following equation:

$$Rs = \frac{\text{(distance between center of spots)}}{\text{(average diameter of spots)}}$$

In TLC, if the Rs value is greater than 1.0, the analytes are considered to be resolved.