Liquid Chromatography

Liquid Chromatography (LC) is a chromatographic technique in which the mobile phase is a liquid.

LC is a much older technique than GC, but was overshadowed by the rapid development of GC in the 1950's and 1960's.

LC is currently the dominate type of chromatography and is even replacing GC in its more traditional applications.

Advantages of LC compared to GC:

LC can be applied to the separation of any compound that is soluble in a liquid phase.

- LC more useful in the separation of biological compounds, synthetic or natural polymers, and inorganic compounds

Liquid mobile phase allows LC to be used at lower temperatures than required by GC

- LC better suited than GC for separating compounds that may be thermally labile

Advantages of LC compared to GC (continued):

Retention of solutes in LC depend on their interaction with both the mobile phase and stationary phase.

GC retention based on volatility and interaction with stationary phase
LC is more flexible in optimizing separations → change either stationary or mobile phase

Most LC detectors are non-destructive

LC is better suited for preparative or process-scale separations

Disadvantage of LC compared to GC:

LC is subject to greater peak or band-broadening. RESOLUTION!!!!
much larger diffusion coefficients of solutes in gases vs. liquids

Low- and High-performance Liquid Chromatography:

Many types of liquid chromatography are available, based on different stationary phase and mobile phase combinations.

- each type may be further characterized based on its overall efficiency or *performance*

Low-performance liquid chromatography

– LC methods that use large, non-rigid support material

particles > 40 μm in diameter

– poor system efficiencies and large plate heights

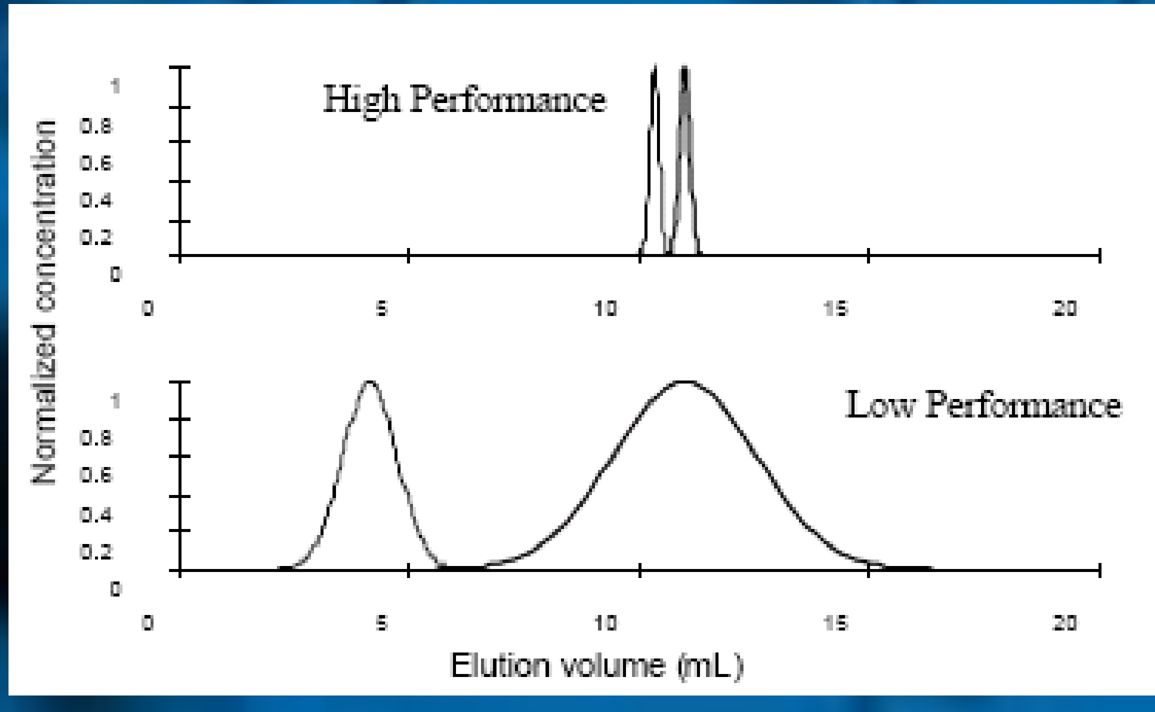
– such systems have the following characteristics:

broad peaks

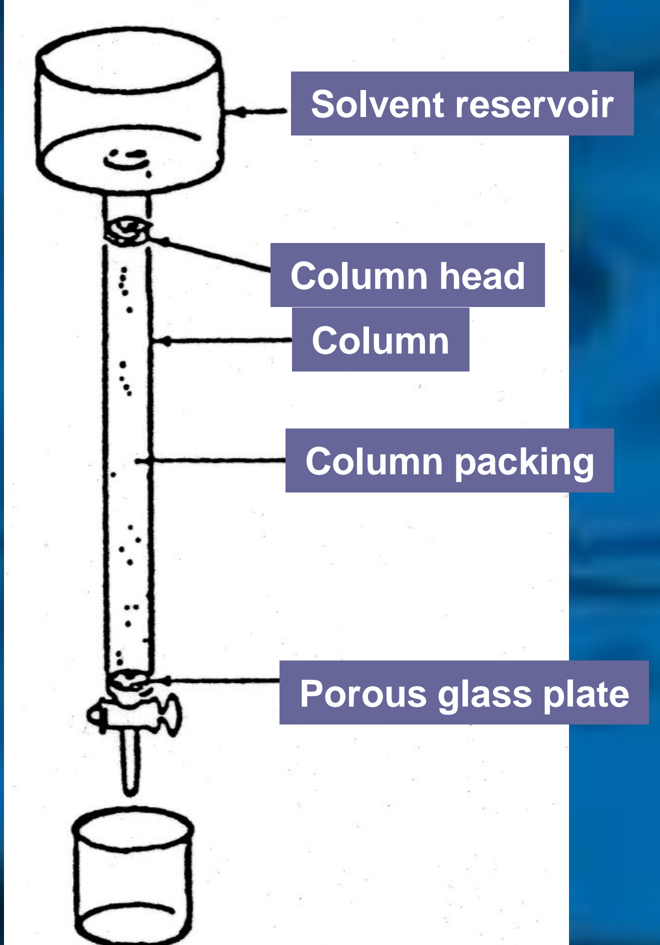
poor limits of detection

long separation times

columns can only tolerate low operating pressures



Column chromatography – an example of the equipment used in low-performance liquid chromatography



Sample is usually applied directly to the top of the column.
Detection is by fraction collection with later analysis of each fraction

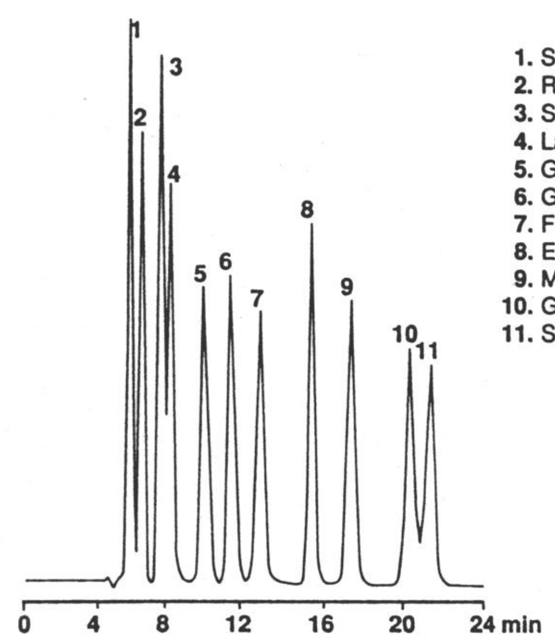
High-performance liquid chromatography (HPLC)

– LC methods that use small, uniform, rigid support material
  - particles < 40 μm in diameter
  - usually 3-10 μm in practice
  - Nano-particles??????????

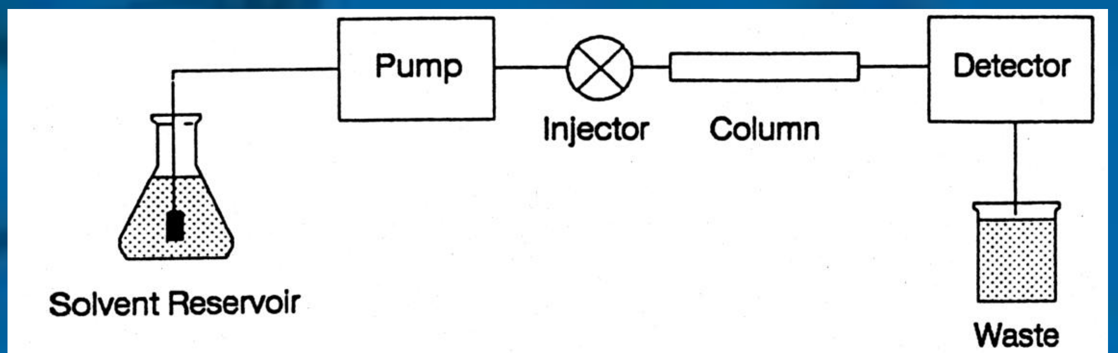
– good system efficiencies and small plate heights

– such systems have the following characteristics:

- narrow peaks
- low limits of detection
- short separation times
- columns can only tolerate high operating pressures and faster flow-rates



A typical HPLC system:

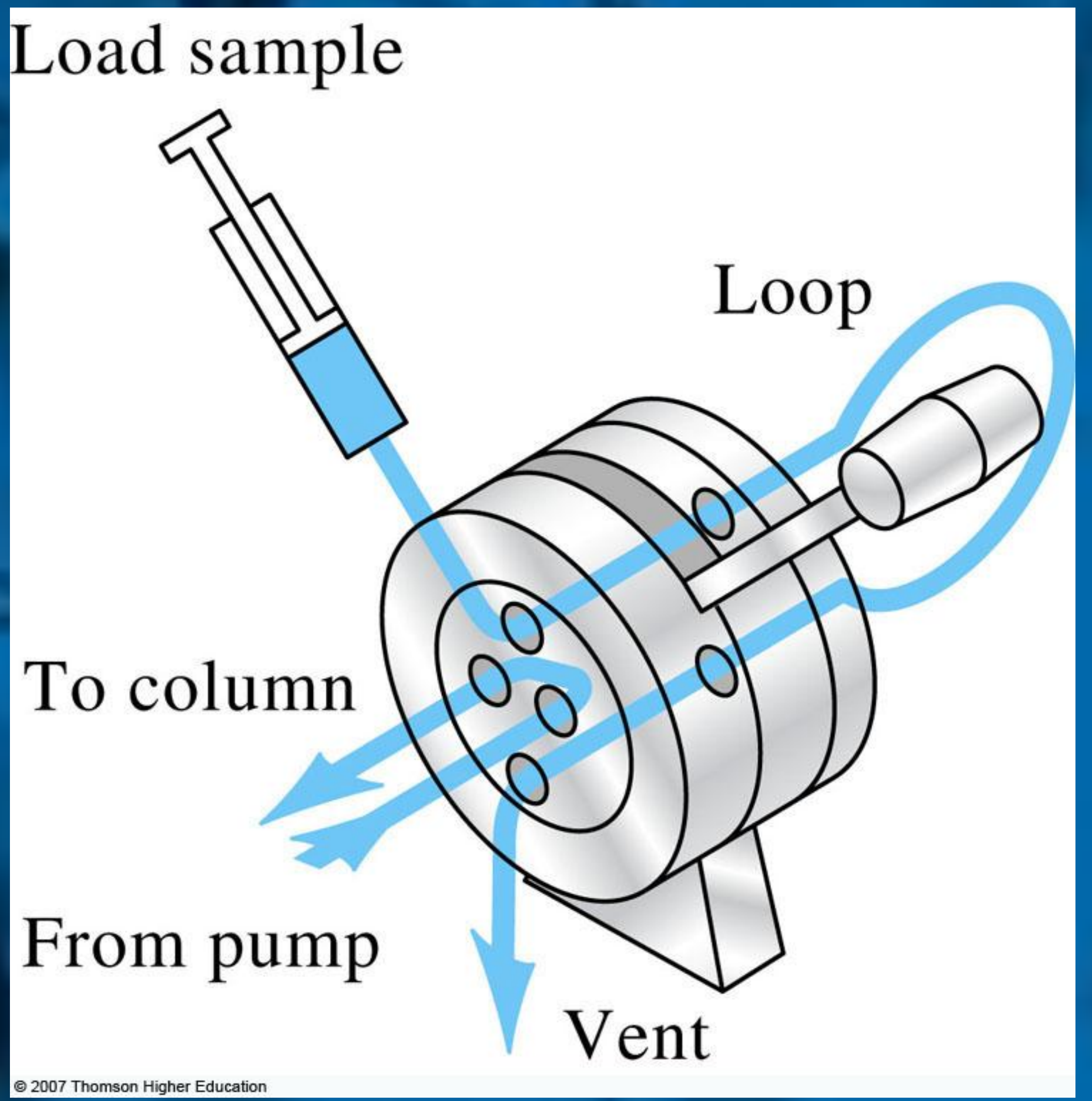


- Higher operating pressures need for mobile phase delivery requires special pumps and other system components

- Sample applied using closed system (i.e., injection valve)

- detection uses a flow-through detector

Regulated helium source
Output check valve
Solvent reservoirs
Sparger
Inlet filter
Pump
Inlet check valve
Pulse damper
To waste
Drain valve
Priming syringe
Solvent proportioning valve
To detector
Column
Pressure transducer
Backpressure regulator
Filter
Injector valve



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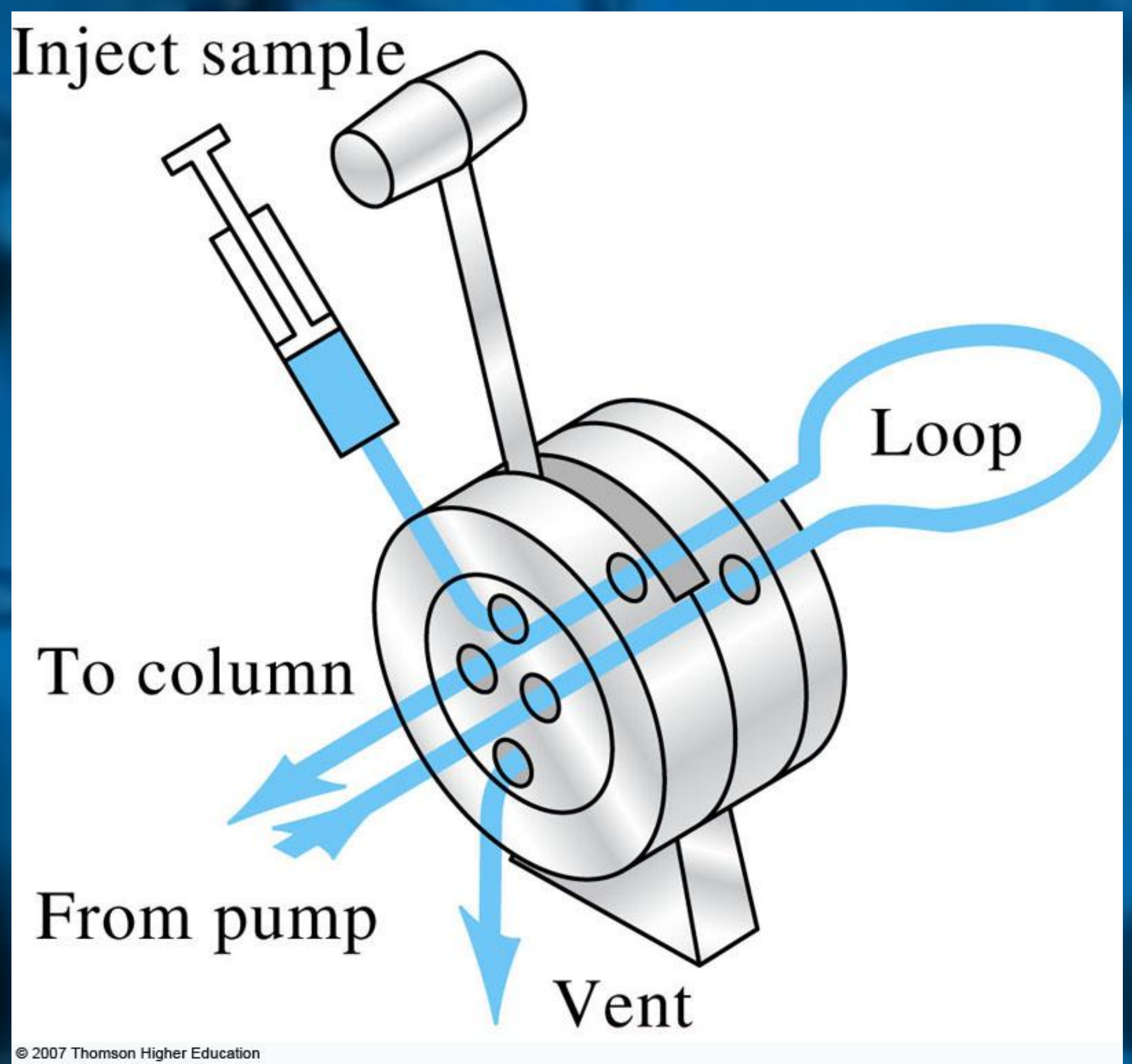

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Similar to GC, solutes can be eluted from a column by using either a constant column conditions or gradient elution

Isocratic elution: use of a constant mobile phase composition to elute solutes

simple, inexpensive

difficult to elute all solutes with good resolution in a reasonable amount of time → general elution problem

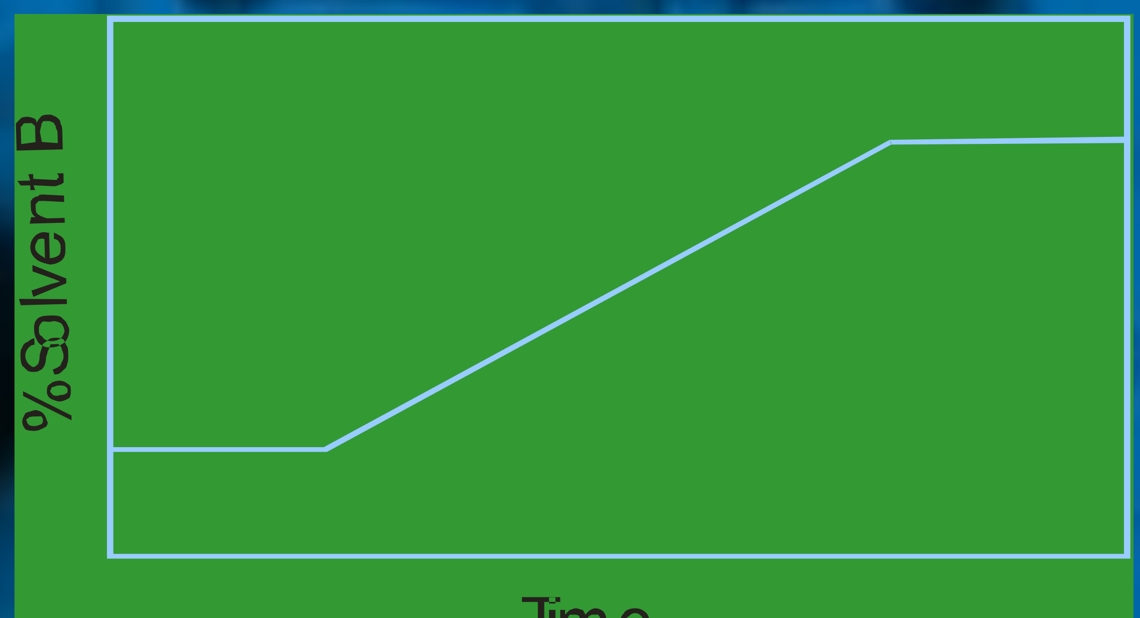
Gradient elution: changing the composition of the mobile phase with time → *solvent programming*

going from a weak mobile phase to a strong one.

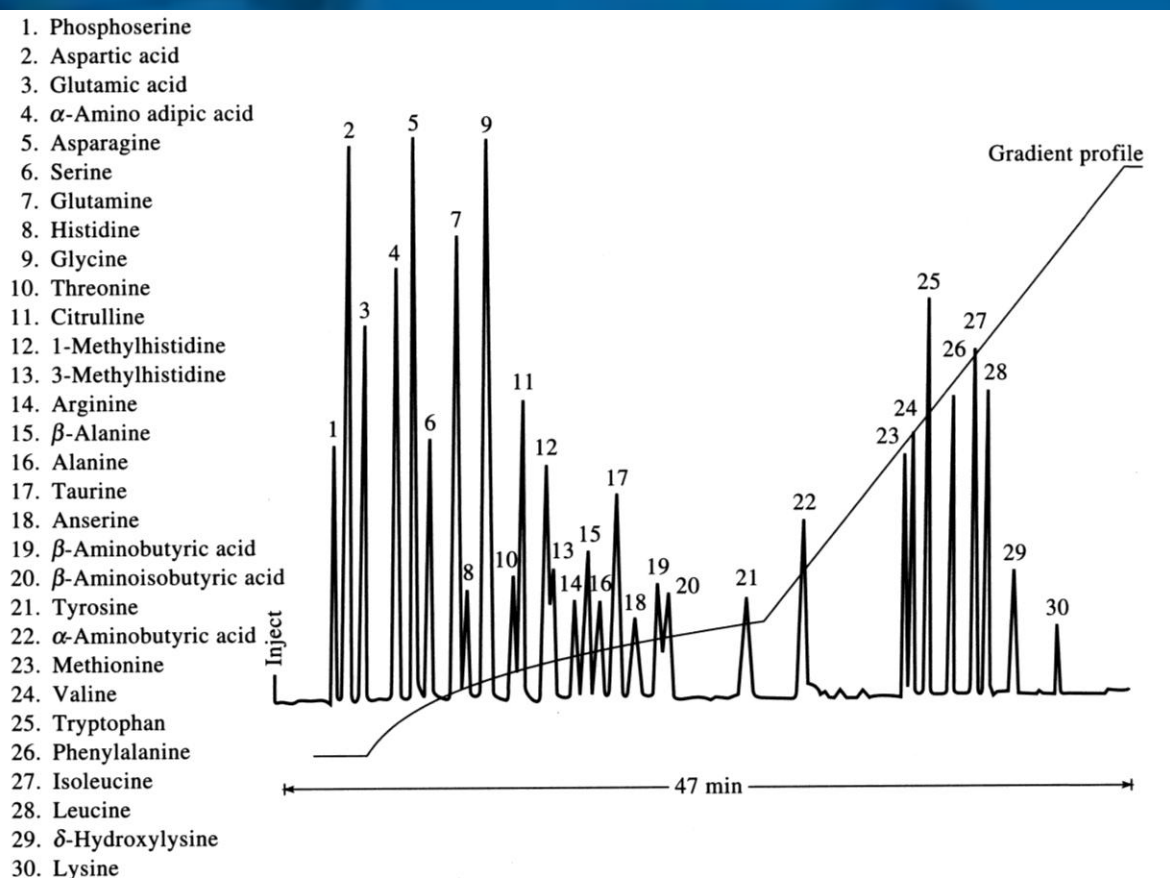
weak mobile phase → *solvent A*

strong mobile phase → *solvent B*

solvent change can be stepwise, linear or non-linear



Gradient elution of mixture of 30 amino-acids



In choosing a mobile phase for LC, several factors need to be considered

– type of stationary phase used

determines what will be a strong or weak mobile phase

– solubility of the solutes

– viscosity of the mobile phase

– type of detector used and solvent's background signal

– purity of the solvents

– miscibility of the solvents (for gradient elution)

Selection of a mobile phase for a particular LC application can be done by using various tables that summarize properties for common LC solvents:

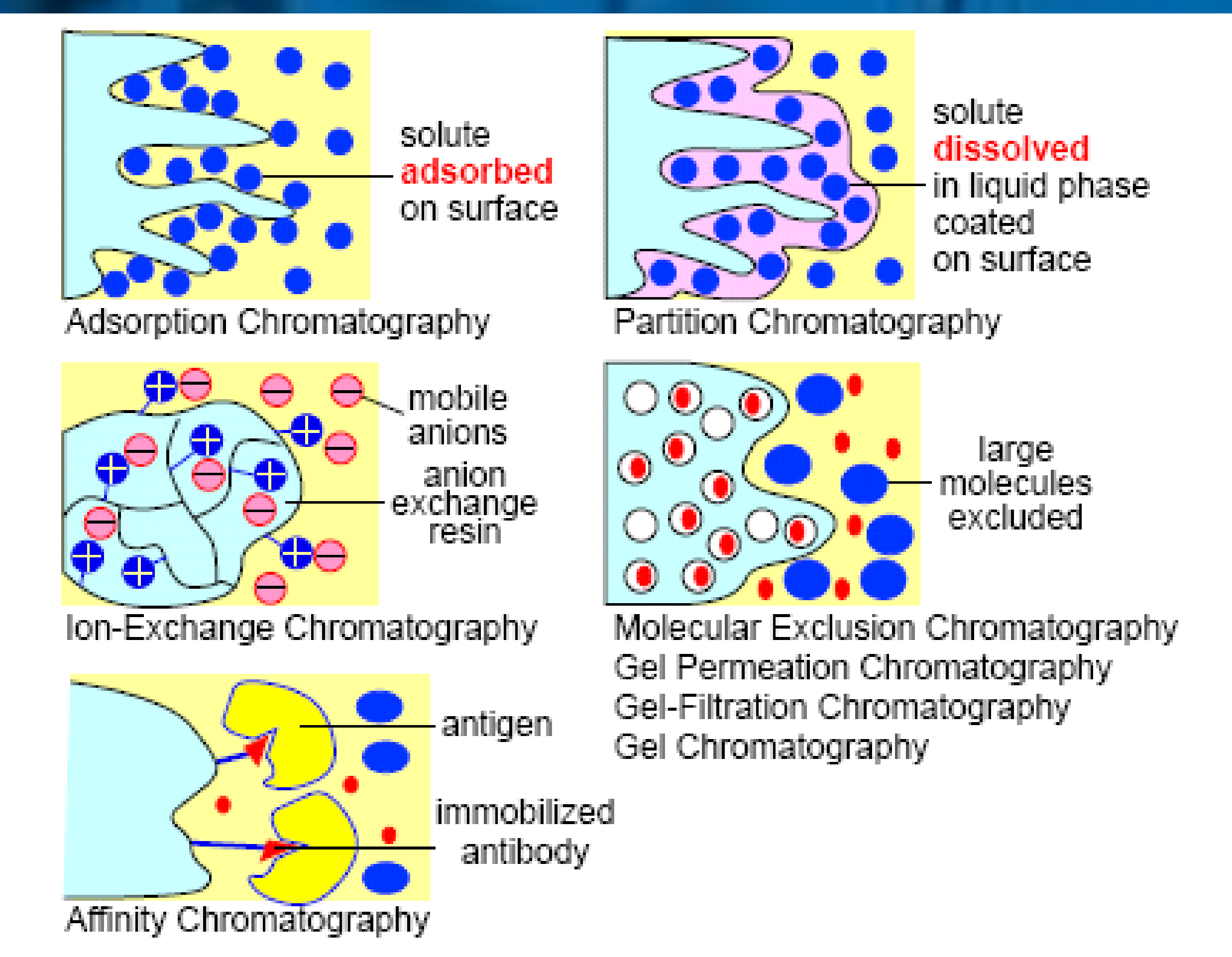
LIKE DISSOLVES LIKE!

Solvent	Refractive Index	Viscosity (cP)	Boiling Point (°C)	Polarity Index (P)	Eluent Strength (ε^o)
Fluoroalkanes	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	1.327	0.30	69	0.1	0.01
1-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

Types of Liquid Chromatography:

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

- Adsorption chromatography
- Partition chromatography
- Ion-exchange chromatography
- Affinity chromatography
- Size-exclusion chromatography



Adsorption chromatography stationary phase (or solid support) may be either polar or non-polar

Adsorbent	Surface Type	Application
Silica	Slightly acidic	General Purpose – Basic compounds
Alumina	Slightly basic	General Purpose – Acidic Compounds
Charcoal	Non-polar	Non-polar Compounds
Florisil	Strongly acidic	General purpose – Basic Compounds
Polyamides	Basic	Phenols and Aromatic Nitro Compounds
Others (clay, Kieselguhr, diatomaceous earth, celite, etc.)	Relatively Non-polar	Polar Compounds

For polar supports (silica/alumina), the weak mobile phase is a non-polar solvent (hexane, benzene, etc.) and the strong mobile phase is a polar solvent (water, methanol, etc.)

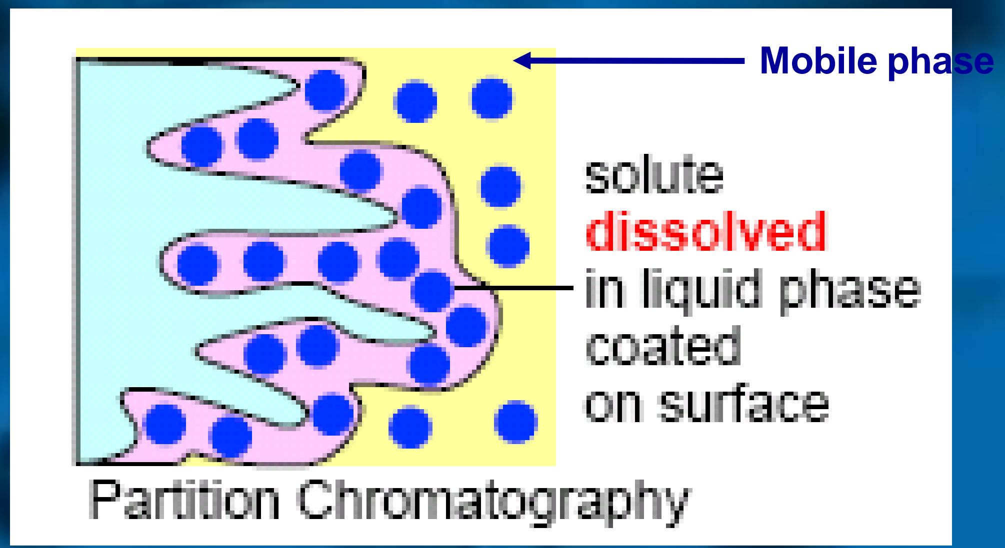
For non-polar supports (charcoal), the weak mobile phase is a polar solvent and the strong mobile phase is a non-polar solvent.

Common applications of Adsorption LC:

- purification of synthetic organic compounds from reaction mixtures
- separation of geometrical isomers (ortho/meta/para, cis/trans, etc)

Partition Chromatography

Separates solutes based on their partitioning between a liquid mobile phase and a liquid stationary phase coated on a solid support.



Support Material – is usually silica, originally involved coating this support with some liquid stationary phase that was not readily soluble in the mobile phase

Two main types of partition chromatography based on the type of stationary phase:

normal-phase liquid chromatography (NPLC)

reversed-phase liquid chromatography (RPLC)

Normal Phase liquid Chromatography (NPLC).

partition chromatography where the stationary phase is polar

NPLC column strongly retains polar compounds

- weak mobile phase is a non-polar liquid: organic solvent
- strong mobile phase is a polar liquid: water or methanol

Use stationary phases chemically attached to the support

CN	Cyanopropyl	$-Si-CH_2CH_2CH_2CN$
NH2	Aminopropyl	$-Si-CH_2CH_2CH_2NH_2$
PSA	N-propylethylenediamine	$-Si-CH_2CH_2CH_2NHCH_2CH_2NH_2$

Reverse Phase liquid Chromatography (RPLC).

partition chromatography where the stationary phase is non-polar

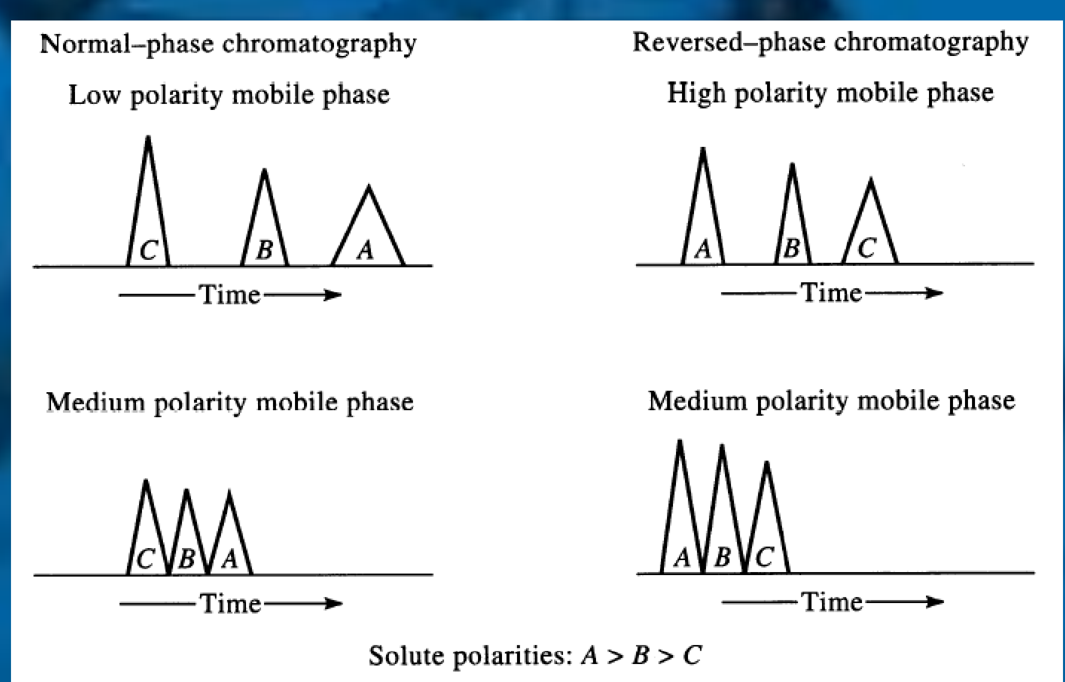
reverse polarity of normal phase LC

retains non-polar compounds most strongly

- weak molar phase is a polar liquid: water
- strong mobile phase is more non-polar liquid: methanol or acetonitrile

- stationary phase must have a low miscibility with the mobile phase so the stationary phase is not dissolved from the column

Like NPLC, these liquid stationary phases slowly bleed from the column, changing the properties and solute retention time .



Use stationary phases chemically attached to the support, C_8 and C_{18} are most common

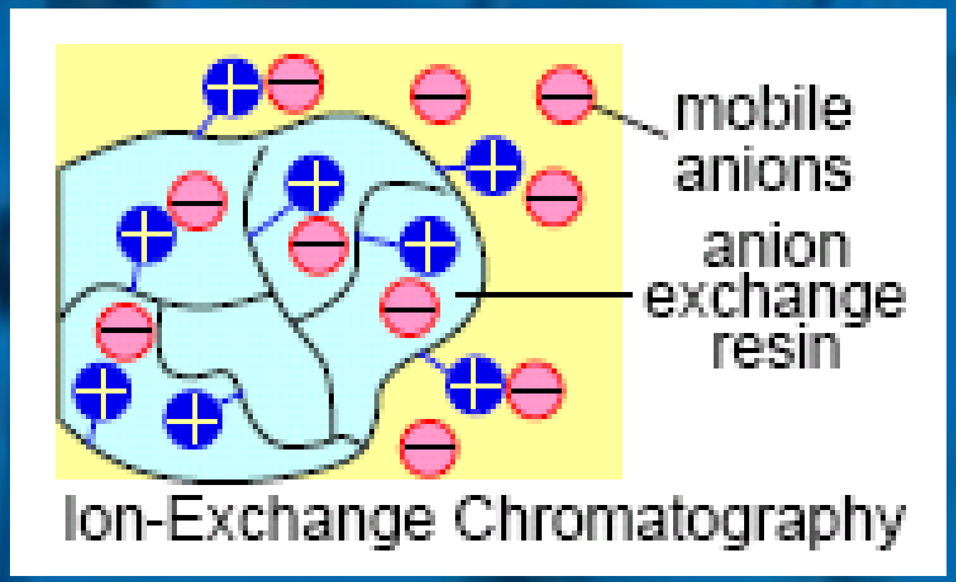
C18	Octadecyl	$-Si-C_{18}H_{37}$
C8	Octyl	$-Si-C_8H_{17}$
C2	Ethyl	$-Si-C_2H_5$
CH	Cyclohexyl	–Si–(cyclohexyl ring)
PH	Phenyl	–Si–(phenyl ring)

Common applications of RPLC:

- most popular type of liquid chromatography
  separation of a wide variety of non-polar **and** polar solutes
- popularity → weak mobile phase is a polar solvent (e.g., water)
  ideal for the separation of solutes in aqueous-based samples, such as biological compounds

Ion-exchange Chromatography (IEC)

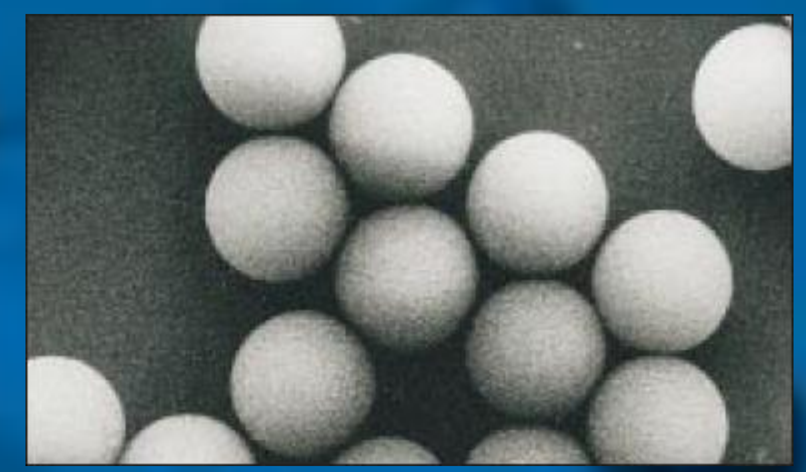
Separates solutes by their adsorption onto a support containing fixed charges on its surface. A high concentration of a competing ion is often added to the mobile phase to elute the analytes from the column



$$xRSO_3^-H^+ + M^{x+} \rightarrow (RSO_3^-)_xM^{x+} + xH^+$$

$$xRN(CH_3)_3OH^- + A^{x-} \rightarrow [RH(CH_3)_3^+]_xA^{x-} + xOH^-$$

Cross-linked polystyrene resins: for use with the separation of inorganic ions and small organic ions
Carbohydrate-based resins: for low-performance separations of biological molecules (dextran, agarose, cellulose)
Silica-based supports: for high-performance separations of biological molecules



rigid polystyrene/divinyl benzene beads

A strong mobile phase in IEC:

- contains a high concentration of a competing ion for displacement of the sample ion from the stationary phase

cation exchange resin (K_{ex}):

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

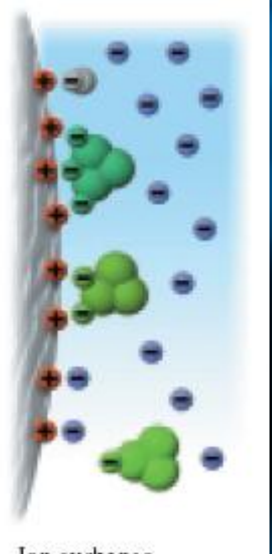
$Ba^{2+} > Pb^{2+} > Sr^{2+} > Ca^{2+} > Ni^{2+} > Cd^{2+} > Cu^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} > UO_2^{2+}$

anion exchange resin (Kex):

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

or

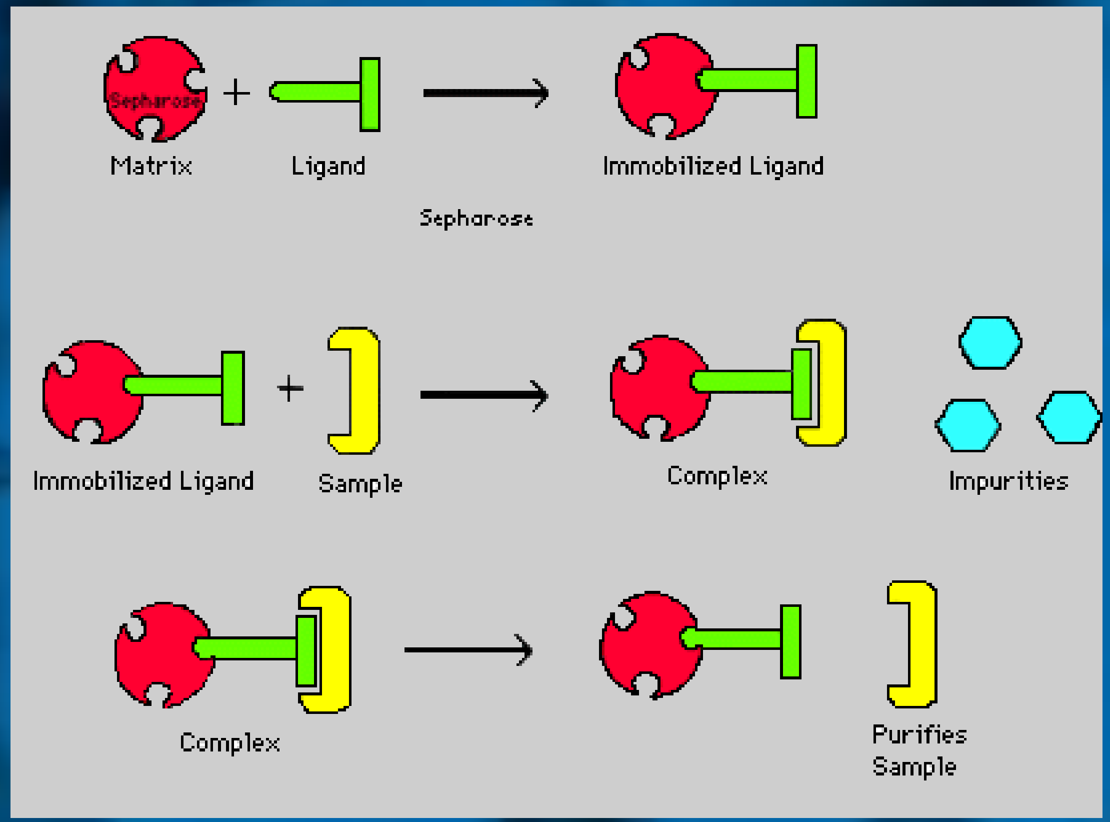
- a solvent that has a pH which decreases ionization of the analyte or stationary phase



Ion exchange

Affinity Chromatography (AC)

Separates based on the use of immobilized biological molecules (and related compounds) as the stationary phase



Based on the selective, reversible interactions that characterize most biological systems

- binding of an enzyme with its substrate or a hormone with its receptor
- immobilize one of a pair of interacting molecules onto a solid support
- immobilized molecule on column is referred to as the *affinity ligand*

Two Main Types of Affinity Ligands Used in AC:

High-specificity ligands – compounds which bind to only one or a few very closely related molecules

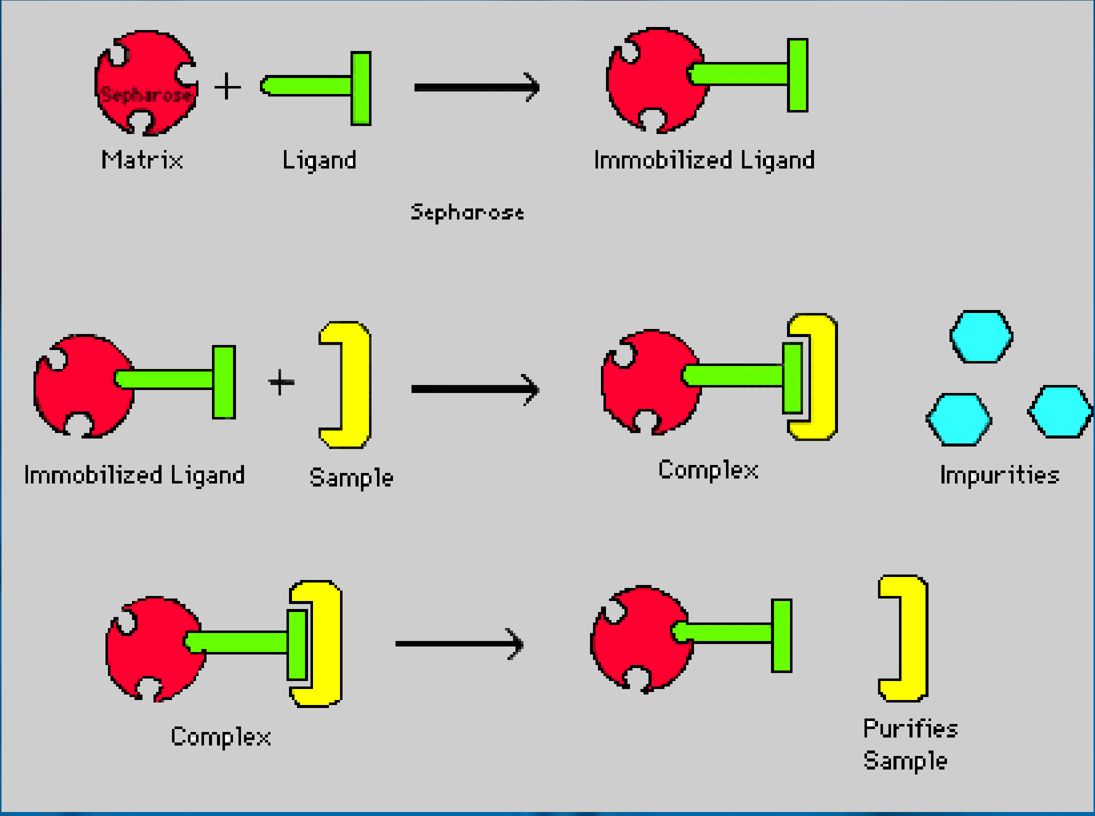
Affinity Ligand	Retained Compounds
Antibodies	Antigens
Antigens	Antibodies
Inhibitors/Substrates	Enzymes
Nucleic Acids	Complimentary Nucleic acids

General or group specific ligands – molecules which bind to a family or class of related molecules

Affinity Ligand	Retained Compounds
Lectins	Glycoproteins, carbohydrates, membrane proteins
Triazine dyes	NADH- or NADPH Dependent Enzymes
Phenylboronic acid	*Cis*-Diol Containing Compounds
Protein A/Protein G	Antibodies
Metal Chelates	Metal-Binding Proteins & Peptides

***Note:* the affinity ligand does not necessarily have to be of biological origin**

Due to the very selective nature of most biological interactions, the solute of interest is often retained with little interference from other components of the sample.



A weak mobile phase is usually a solvent that mimics the pH, ionic strength and polarity of the solute and ligand in their natural binding environment.

A strong mobile phase is a solvent that produces low retention for the solute-ligand interaction:

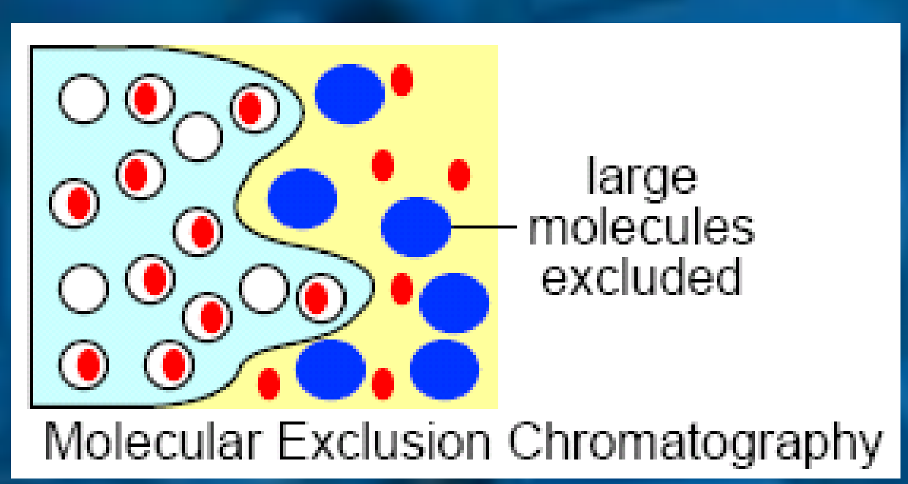
- by decreasing its binding constant

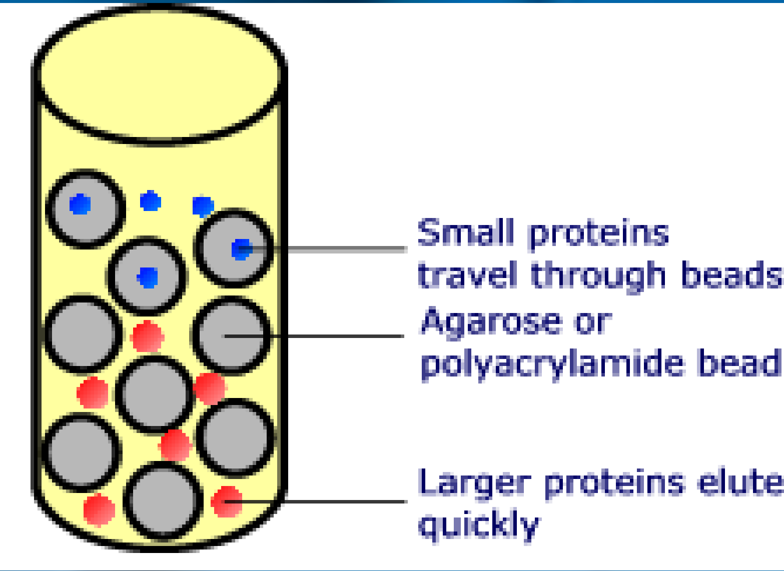
or

- displaces solute by the addition of an agent with competes for solute sites on the column

Size Exclusion Chromatography (SEC)

separates molecules according to differences in their size





SEC is based on the use of a support material that has a certain range of pore sizes

- as solute travels through the support, small molecules can enter the pores while large molecules can not
- since the larger molecules sample a smaller volume of the column, they elute before the smaller molecules.
- separation based on size or molecular weight

SEC is based on the different interactions of solutes with the <u>flowing mobile phase</u> and the <u>stagnant mobile phase</u>.

- no true stationary phase is present in this system
- stagnant mobile phase acts as the "stationary phase"

LC DETECTORS

Common types of LC Detectors

- Refractive Index Detector
- UV/Vis Absorbance Detector
- Fluorescence Detector
- Conductivity Detector
- Electrochemical Detector

As in GC, the choice of detector will depend on the analyte and how the LC method is being used (i.e., analytical or preparative scale)

Refractive Index Detector (RI)

Measures the overall ability of the mobile phase and its solutes to refract or bend light.

One of the few universal detectors available for LC

Advantages:

– non-destructive and universal detector

applicable to the detection of any solute in LC

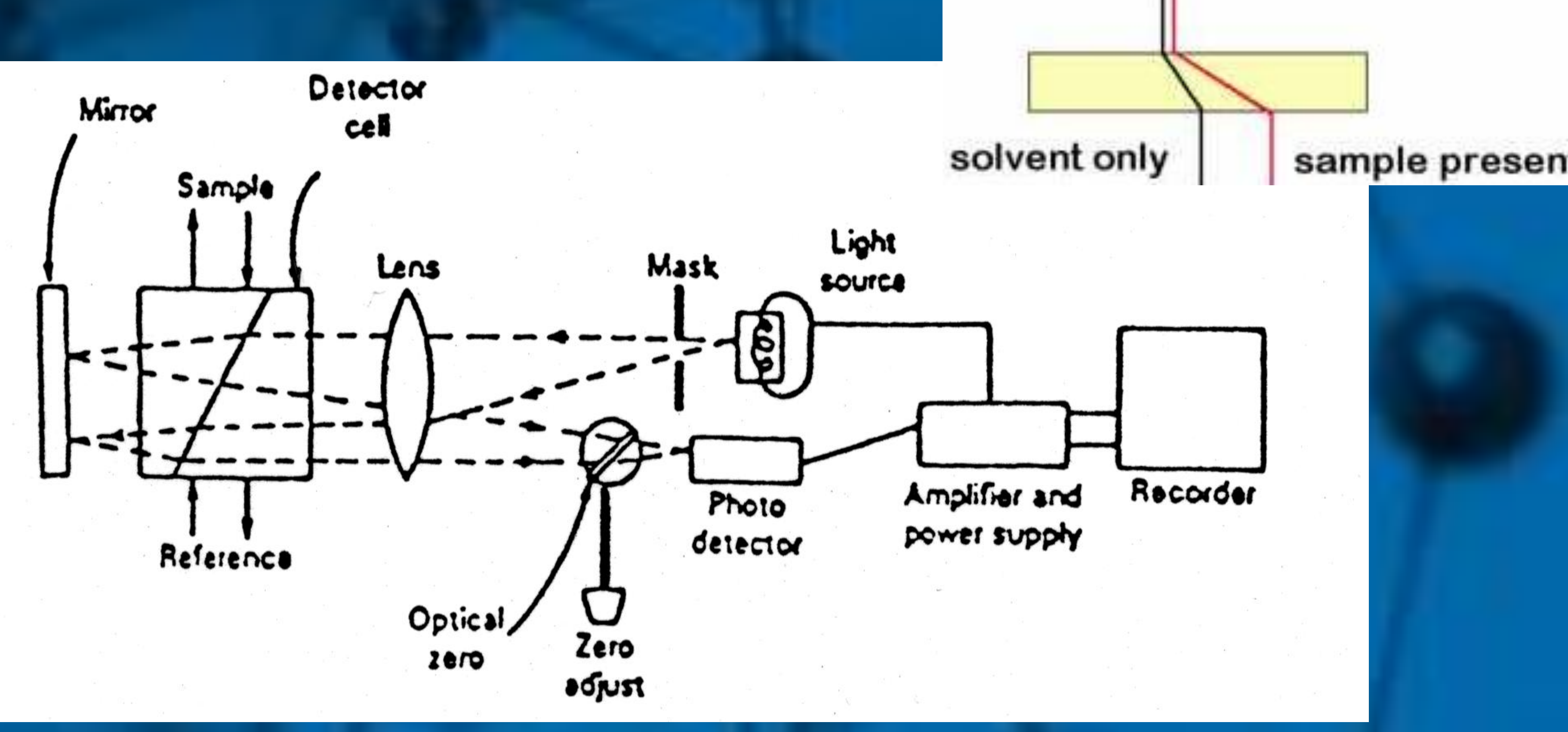
– applicable to preliminary LC work where the nature and properties of the solute are unknown

provided concentration is high enough for detection

Disadvantages:

– high limits of detection (10^{-6} to 10^{-5} M)

– difficult to use with gradient elution



Operating Principle:

- light from source passes through flow-cells containing either sample stream or a reference stream
- when refractive index is the same between the two cells, no bending of light occurs at the interface between the flow-cells
  - maximum amount of light reaches the detector
- as solute elutes, refractive index changes between reference and sample cell
  - light is bent as it passes through flow cell interface
  - amount of light reaching detector is decreased

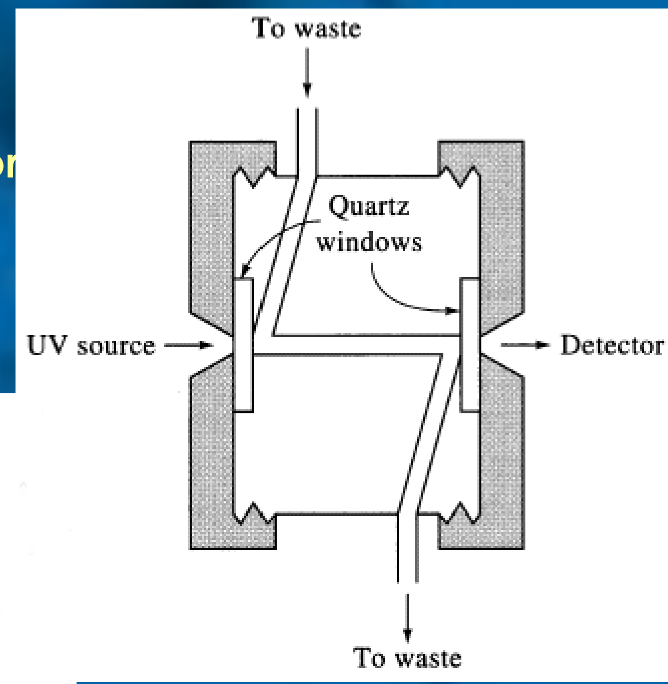
UV/Vis Absorbance Detector

Measures the ability of solutes to absorb light at a particular wavelength(s) in the ultraviolet (UV) or visible (Vis) wavelength range.

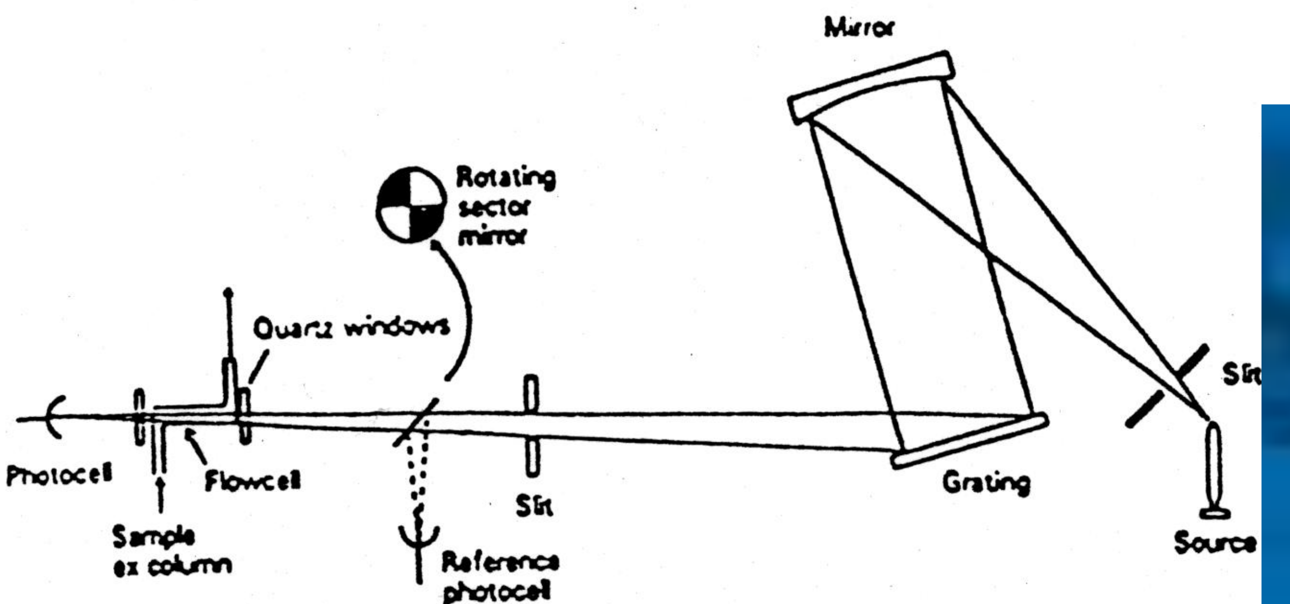
most common type of LC detector

Three Common types of UV/Vis Absorbance Detector

- Fixed wavelength detectors
- Variable wavelength detectors
- Photodiode array detectors

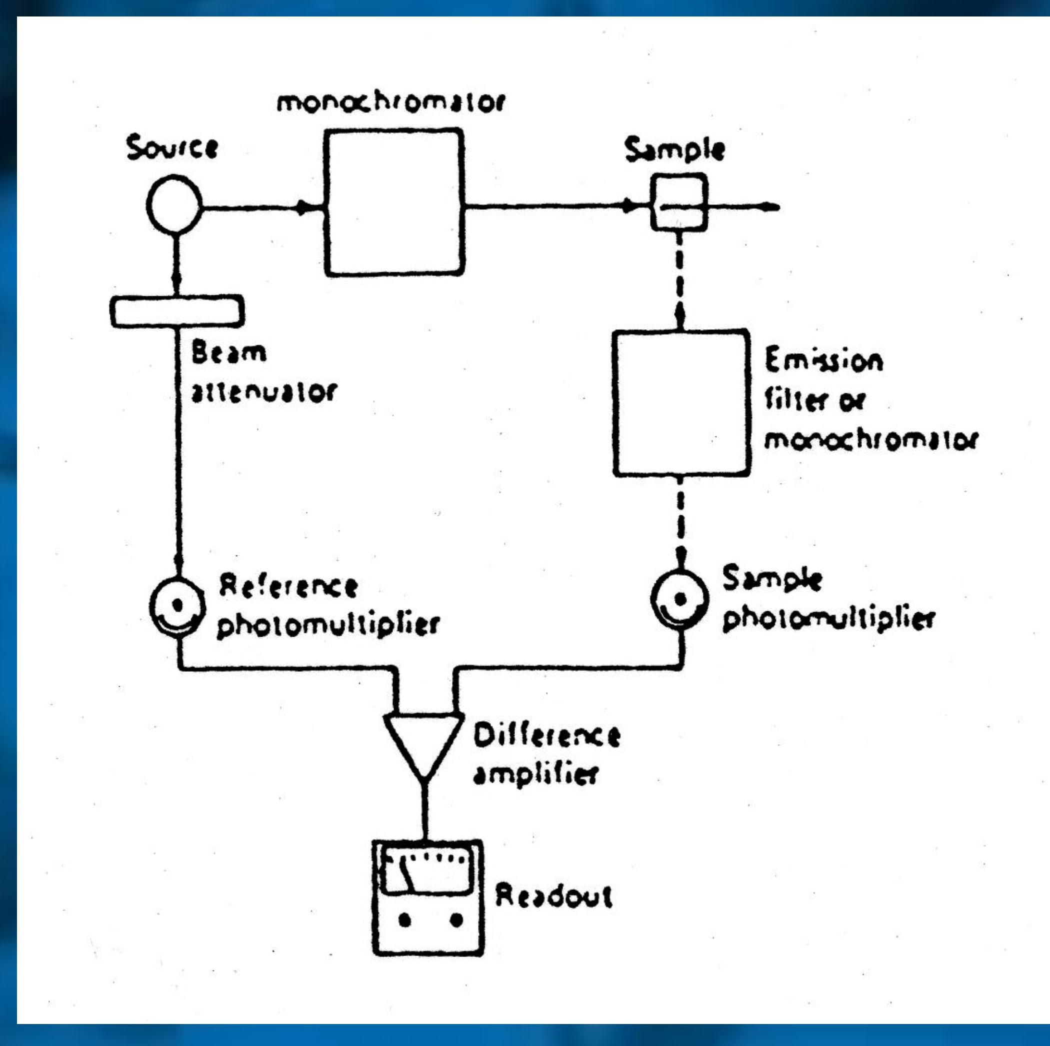


Schematic diagram of a variable wavelength UV detector for HPLC



Fluorescence Detector

A selective LC detector that measures the ability of eluting solutes to fluoresce at a given set of excitation and emission wavelengths

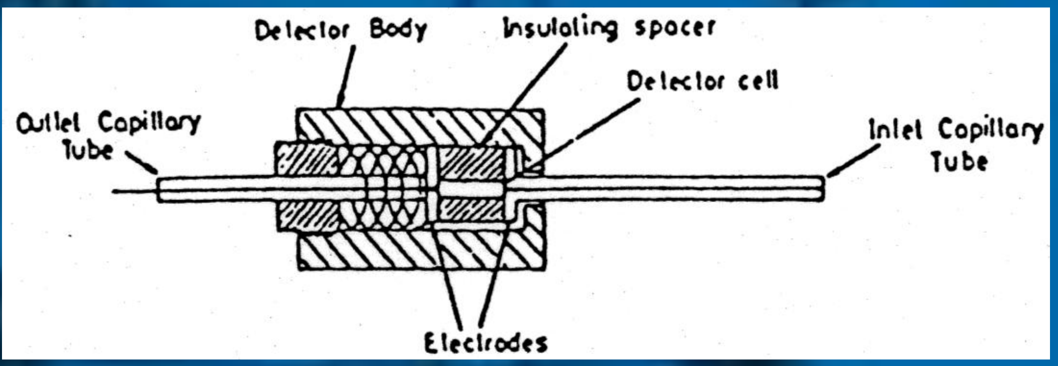


Conductivity Detector

Used in analytical applications of ion-exchange chromatography for the detection of ionic compounds

detector measures the ability of the mobile phase to conduct a current when placed in a flow-cell between two electrodes

current conducted within the cell will depend on the number and types of ions present in the mobile phase



Applications:

- can be used to detect any compound that is ionic or weakly ionic
  - Relatively few compounds undergo fluorescence
  - High selectivity, low background signal
- limits of detection for a conductivity detector are ~ 10^{-6} M

- Can be used with gradient elution
  - Constant ionic strength and pH of mobile phase
  - Background conductance of the mobile phase is sufficiently low