

B. Chromatography/modern methods

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❖ Classical methods

- ❖ There is no complete separation, and

- ❖ Require large amounts of material

❖ Chromatography used when:

- ❖ Small amount of material is available &

- ❖ The components closely resemble each other

- ✓ E.g alkaloids, anthraquinones.

Chromatography....

- Chromatography:
 - Greek word
 - Chroma- *color*
 - Graphein- *to write*, chromatography means *to write in color*
 - First employed by Russian scientist Mikhail Tsyet
 - Used for separation of pigments such as chlorophyll.

Chromatography

- A technique of separating the components of a mixture based on their relative partitioning characteristics between a
 - **Mobile phase (MP):** the solvent moving through the column, and
 - **Stationary phase(Sp):** a substance fixed inside the column.
- Components move through the column based on the difference in their interaction towards MP & Sp.
- Small differences in a compounds' **partition coefficient** result in differential retention on the Sp
 - Leads to separation of components of the mixture.

Chromatography...

- Chromatography may be **analytical** or **preparative**
- **Analytical chromatography**
 - For **smaller amounts of material**,
 - For **measuring the relative proportions** of analytes in a mixture.
 - Provide the qualitative profile or **finger print** of the compound.
- **Preparative chromatography**
 - To purify sufficient quantities of a substance for further use.
 - Provide the quantitative profile
 - For isolation and purification of the components

Classification

◉ Shape of Stationary phase

- Column chromatography
- Planar chromatography
 - Paper chromatography
 - Thin layer chromatography

◉ Physical state of mobile phase

- Gas chromatography
- Liquid chromatography
- Supercritical fluid chromatography

◉ Separation mechanism

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

Chromatography...

- Solute molecules move through the column only when they are in the MP.
 - The more the solute stays on the MP the faster it elutes.
 - As the Mp velocity increases the velocity of the solute particles also increase and vice versa.
- However, the longer the molecules of solute stay on Sp the longer the retention time
 - **Elute from the column later**

Chromatography...

Chromatographic terms

- **Analyte:** substance to be separated during chromatography.
- **Solute:** the sample components in partition chromatography.
- **Solvent:** any substance capable of solubilizing another substance/the mobile phase in liquid chromatography.
- **Stationary phase:** substance fixed in place for the chromatography procedure. Example the [silica](#) layer in TLC
- **Bonded phase:** Sp covalently bonded to the support particles or to the inside wall of the column tubing.
- **Immobilized phase:** Sp that is immobilized on the support particles, or on the inner wall of the column tubing.
- The **mobile phase** is the phase that moves in a definite direction. It may be a liquid, a gas, or a supercritical fluid.
- **Retention time:** characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions.

Chromatography...

- A **chromatograph**: equipment that enables a sophisticated separation.
- **Chromatography**: physical method of separation that distributes components to separate between stationary and mobile phases.
- A **chromatogram**: visual output of the chromatograph. peaks or patterns on the chromatogram correspond to different components of the separated mixture.
- **Eluate**: is what is coming out of the column.
- **Eluent**: portion of the MP which carries the sample components with in it. It is the solvent used as MP in LC & the carrier gas in GC.
- An **eluotropic series** is a list of solvents ranked according to their eluting power.

1. Adsorption Chromatography

- Separation based on the interaction of adsorbate with adsorbent.
 - **Hydrogen bonding** occurs between the compound functional groups and free hydroxyls groups (silanols) on the sorbent.
- Separation occurs when one compound is more strongly adsorbed by the sorbent than the other components.
 - Least strongly adsorbed compounds are carried down the column by the passage of solvent, & elute 1st.

1. Adsorption Chromatography...

- When the sorbent is silica or alumina, polar natural products move slowly compared to nonpolar ones.
- **Adsorbent:** adsorb substances on its porous surface. It is the Sp. **Silica gel, alumina**, and cellulose microcrystalline are some of the adsorbents.
- Adsorption mechanism of separation is employed in
 - Column chromatography,
 - Thin layer chromatography,
 - Ion exchange chromatography, and
 - Gas – solid chromatography

1. Adsorption Chromatography...

- **Adsorption** depends on
 - Migration of solute
 - Relative strengths molecular interactions of:
 - solute – solute
 - solute – solvent
 - solvent – solvent
 - solute and solvent affinities for active sites
 - effect of molecules in adsorbed state

2. Partition chromatography

- Separation based on the relative solubility of the compound between the sorbent and the Mp.
 - Partitioning between two phases
 - Compounds that are more soluble in the MP migrate up the plate to a greater extent than components that are more soluble in the Sp.
- Both the Sp and MP are liquids or MP is gas
- **Partition coefficient (α) is the ratio** in which the solute distributes itself between the Sp &MP
 - Constant at
 - Constant temperature
 - Over a limited range of concentration

2. Partition chromatography...

$$\alpha = c_A / c_B$$

c_A and c_B are solute concentrations in solvents A and B

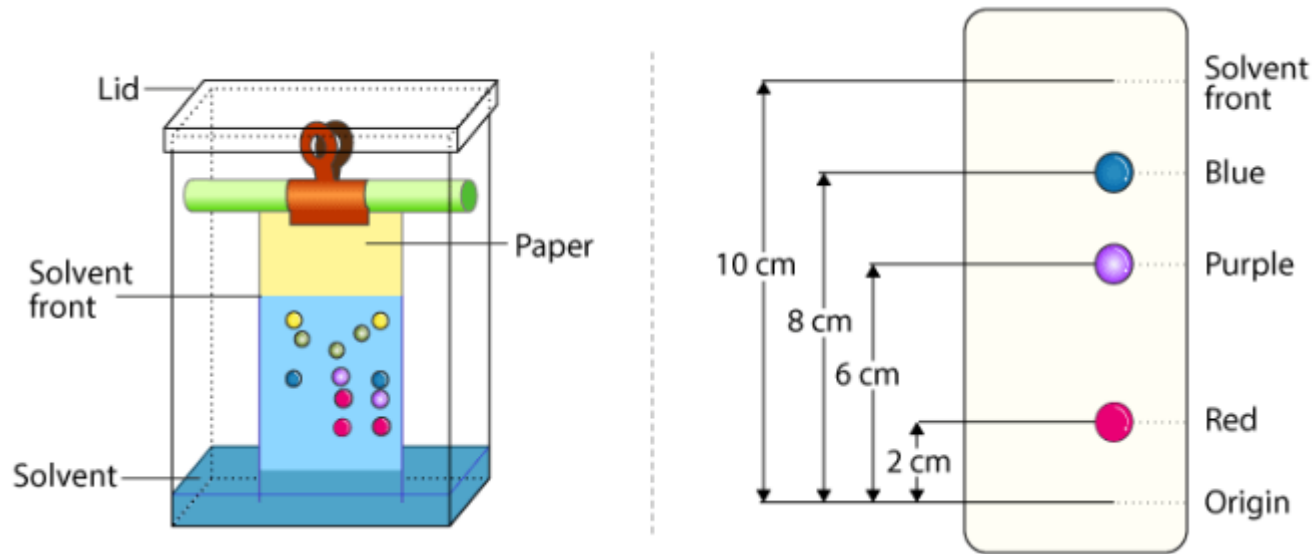
□ Types of partition chromatography

- Liquid-liquid Chromatography: paper chromatography
- Gas-liquid Chromatography

• Application of partition chromatography

- To separate and identify amino acids, tannins, alkaloids, carbohydrates, and glycosides.

2. Partition chromatography...



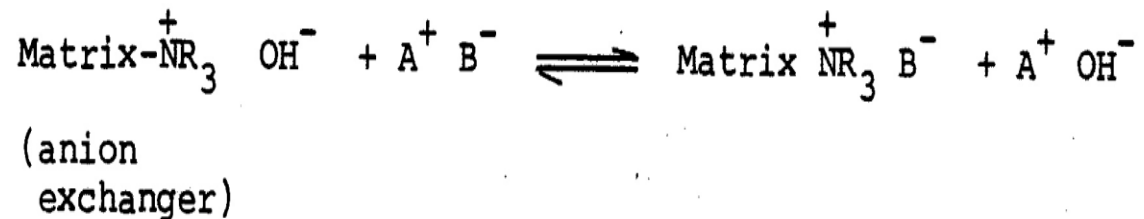
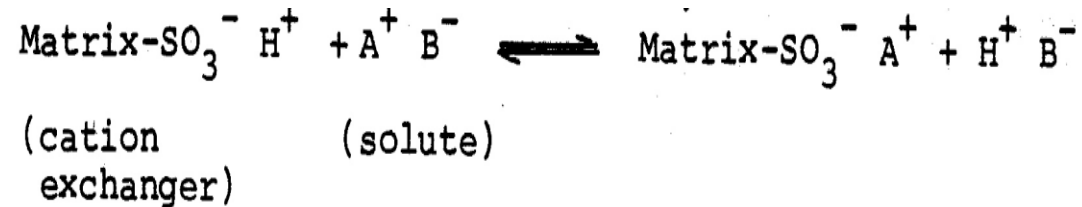
Diagrammatic representation of partition (paper) chromatography

3. Ion exchange chromatography

- Consists of an insoluble matrix with chemically bound charged groups and **mobile counter ions**.
- Involves reversible binding of charged sample molecules to the insoluble matrix in exchange for the counter ions:
- Separation on the basis of differences in their net surface charge
- Eluted through displacement by stronger ionized species in the eluent.

3. Ion exchange chromatography...

- IEC consists of
 - **Mobile phases:** an aqueous buffer system into which the mixture to be resolved is introduced
 - **Stationary phases:** an inert organic matrix bonded to ionizable fg that carry a displaceable oppositely charged counterion.



3. Ion exchange chromatography...

1. Anion Exchangers/AX

- consists of polymeric cation and active anion.
- Used to purify carboxylic acid natural products that ionized and negatively charged at pH>6.0.
- **Strong AX:** Trimethyl benzyl ammonium, Quaternary ammonium, Trimethyl benzyl hydroxyethyl ammonium
- **Weak AX:** Dimethyl amine, Diethyl amine, Diethyl amino ethyl

Anionic exchange



3. Ion exchange chromatography...

2. Cation Exchangers/CX

- CX consists of a polymeric anion and active cation.
- To purify amine groups in natural products that can be protonated and positively charged at pH<7.0.
- **Strong CX:** Sulfonic acid, Sulfopropyl
- **Weak CX:** Carboxylic acid, Carboxymethyl

Cationic exchange



3. Ion exchange chromatography...

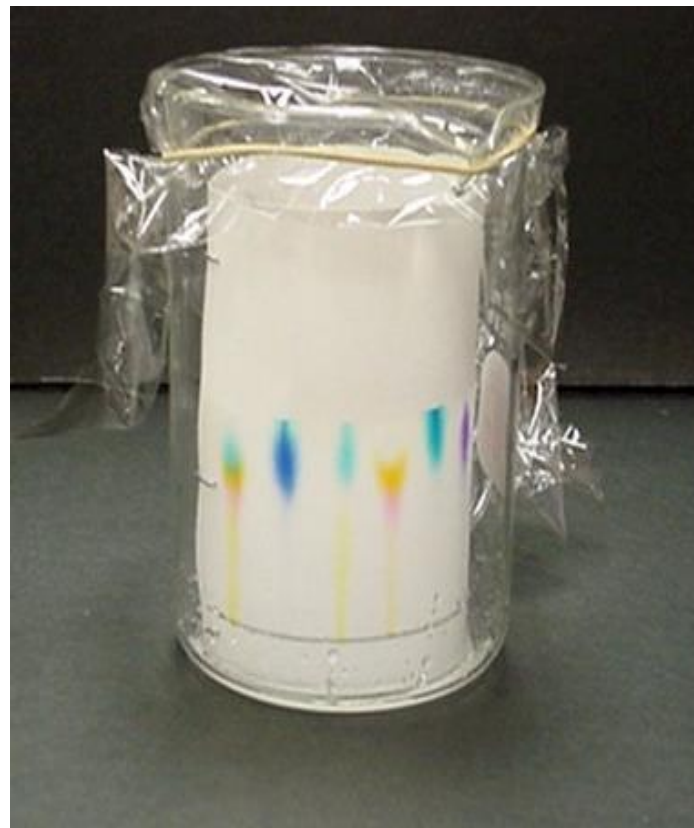
- Effectiveness of ion exchange depend on
 - The affinity of the charged resin group toward ions and
 - Their relative concentrations ($[C]$ and $[M]$).
- Highly polarized ions are strongly attracted to the exchange resin.
- Polyvalent ions have greater affinity for charged resin than monovalent
 - Due to multiple binding at more than one charged group on the resin.

4. Paper Chromatography

- The Sp is thin film of liquid supported by the paper
- **Cellulose** serve as binder due to interaction of its hydroxyl group with water molecule
- The solution to be separated is applied as a spot near one end of a prepared filter-paper strip.
- As the solvent rises through the paper, it meets the sample mixture, which starts to travel up the paper with the solvent.

4. Paper Chromatography...

- The paper is supported in an airtight chamber which has an atmosphere saturated with solvent.
- Separation based on partitioning of solute between two **immiscible** phases



4. Paper Chromatography...

Application of paper chromatography

- Separating Colored Pigments
- Obtaining Pure Compounds
- Qualitative Analysis
- Pathology and Forensic Science

5. Thin Layer Chromatography (TLC)

- The Sp is a thin layer of adsorbent (usually silica gel or alumina) coated on a glass/ aluminum plate.
- Binder such as CaSO_4 is spread on a glass plate & allowed to dry.
- The plates are activated at 110°C for 30 minutes and used.
- Compared to paper, it has the advantage of **faster runs**, better separations, and the choice between different adsorbents.
 - TLC plates can withstand strong solvent and colour forming reagents

5. Thin Layer Chromatography (TLC)...

- The mixture to be separated is applied as a spot near the base of the plate, which is then placed in a closed glass tank containing a layer of developing solvent.
- The solvent moves up the plate by capillary action
 - carrying the less strongly adsorbed components of the mixture with it,
 - The more strongly adsorbed compounds remain near the base of the plate.

5. Thin Layer Chromatography (TLC)...



Visualization

- ❖ If invisible, the spots may be made visible by
 - ▶ Heating for a specific period
 - ▶ Examining under U.V light (if substances are florescent).
 - ▶ Spraying the finished chromatogram with a suitable reagent
 - ▶ e.g. Dragendrof's reagent are used as sprays for the general detection of alkaloids (although they are not specific for alkaloids).

5. Thin Layer Chromatography (TLC)...

- Each spot on TLC plate can be distinguished by its **Retention factor** (R_f) value on the surface of the stationary phase,

$$R_f \text{ value} = \frac{\text{Distance travelled by compound}}{\text{Distance travelled by solvent front}}$$

- R_f value is a constant for a compound under constant chromatographic conditions
 - Used for identification of unknown substance.

5. Thin Layer Chromatography (TLC)...

Advantages of TLC

- Simple, inexpensive
- Quick – results can be achieved from between 30 minutes to a few hours
- Good separation of spots (compounds)
- Very sensitive
- The chromatogram is resistant to the action of chemicals used for the visualization of compounds.

5. Thin Layer Chromatography (TLC)...

Application of TLC

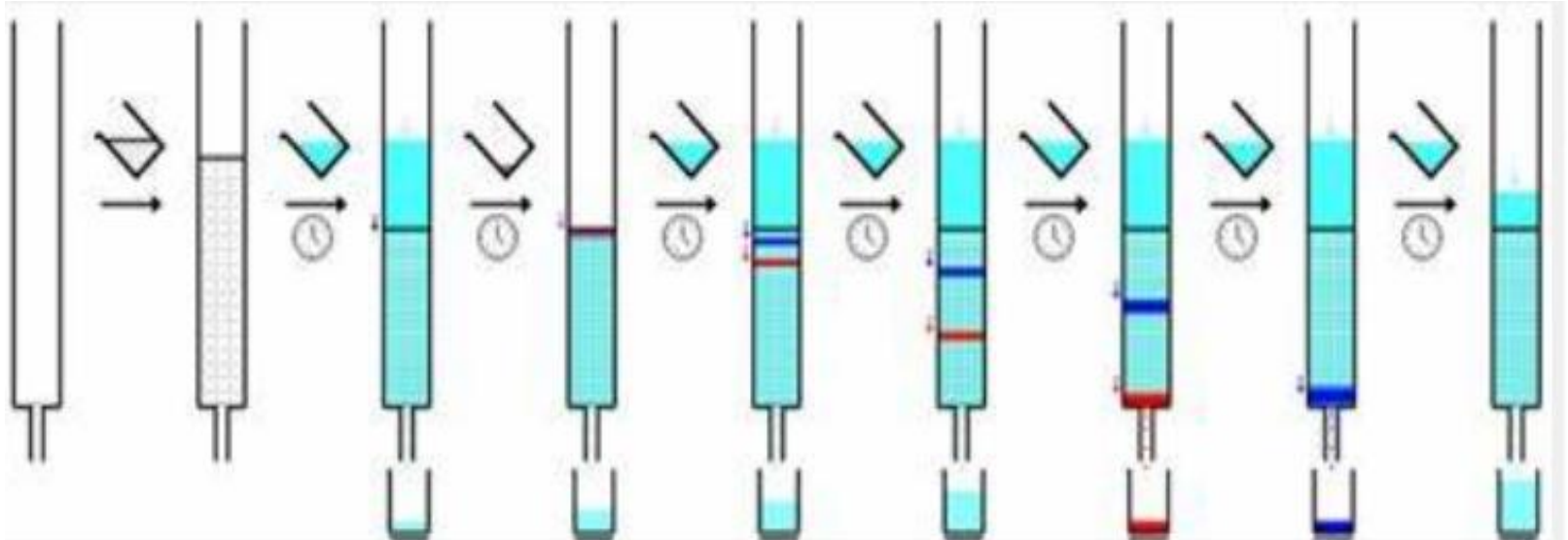
1. To determine the number of components in a mixture.
2. To determine the identity of two substances.
3. To monitor the progress of a reaction.
4. To determine the effectiveness of a purification.
5. To determine the appropriate conditions for a column chromatographic separation.
6. To monitor column chromatography.

6. Column chromatography

- The liquid MP passes over the Sp packed in a column tube.
- The column is either a glass or metal.
- Adsorbents– Starch, Calcium carbonate, Magnesia, Lime, **Silica gel, Alumina** etc.
- **Mobile phases**—either singly or in combination of Pet. Ether, dichloromethane, Cyclohexane, Chloroform, Acetone, Water, and Organic acids

6. Column chromatography...

Column packaging



6. Column chromatography...

Application of column chromatography

- Isolation of active ingredients
- Separating components of a mixture
- Drug estimation from drug formulation
- Purification of a sample from impurities
- Isolation of metabolites from biological fluids e.g proteins

7. High Performance Thin Layer Chromatography (HPTLC)

- The plates are similar to conventional TLC plates.
- Silica gel of **very fine particle size** is widely used as a sorbent in HPTLC.
- The use of smaller particle size helps in **greater resolution and sensitivity**.
- HPTLC is applied for
 - “Finger- print” patterns of herbal formulations,
 - Quantification of active ingredients, and
 - Detection of adulteration.

Parameter	TLC	HPTLC
Chromatographic plate used	Hand made /pre-coated	Pre-coated
Sorbent layer thickness	250 mm	100-200mm
Development distance	10-15 cm	3-5 cm
Particle size range	5-20 µm	4-8 µm
Pre-washing of the plate	Not followed	Must
Application of sample	Manual/Semi automatic	Semi automatic/Automatic
Shape	Spot	Spot/Band
Spot size	2-4mm	0.5-1mm
Sample volume	1-10 µl	0.2-5 µl
Application of larger volume	Spotting leads to over loading	applied as bands
No. of samples/plate (20X20)	15-20	40-50
Development time	Depends on mobile phase	40% Less than TLC
Development chamber	Require large quantity solvent	Require lower quantity of solvent
Reproducibility of results	Difficult	Reproducible
Resolution	poor resolution	High resolution.
Scanning	Not possible	Uses UV/vis/ fluorescence scanner to scan the entire chromatogram quantitatively and qualitatively.

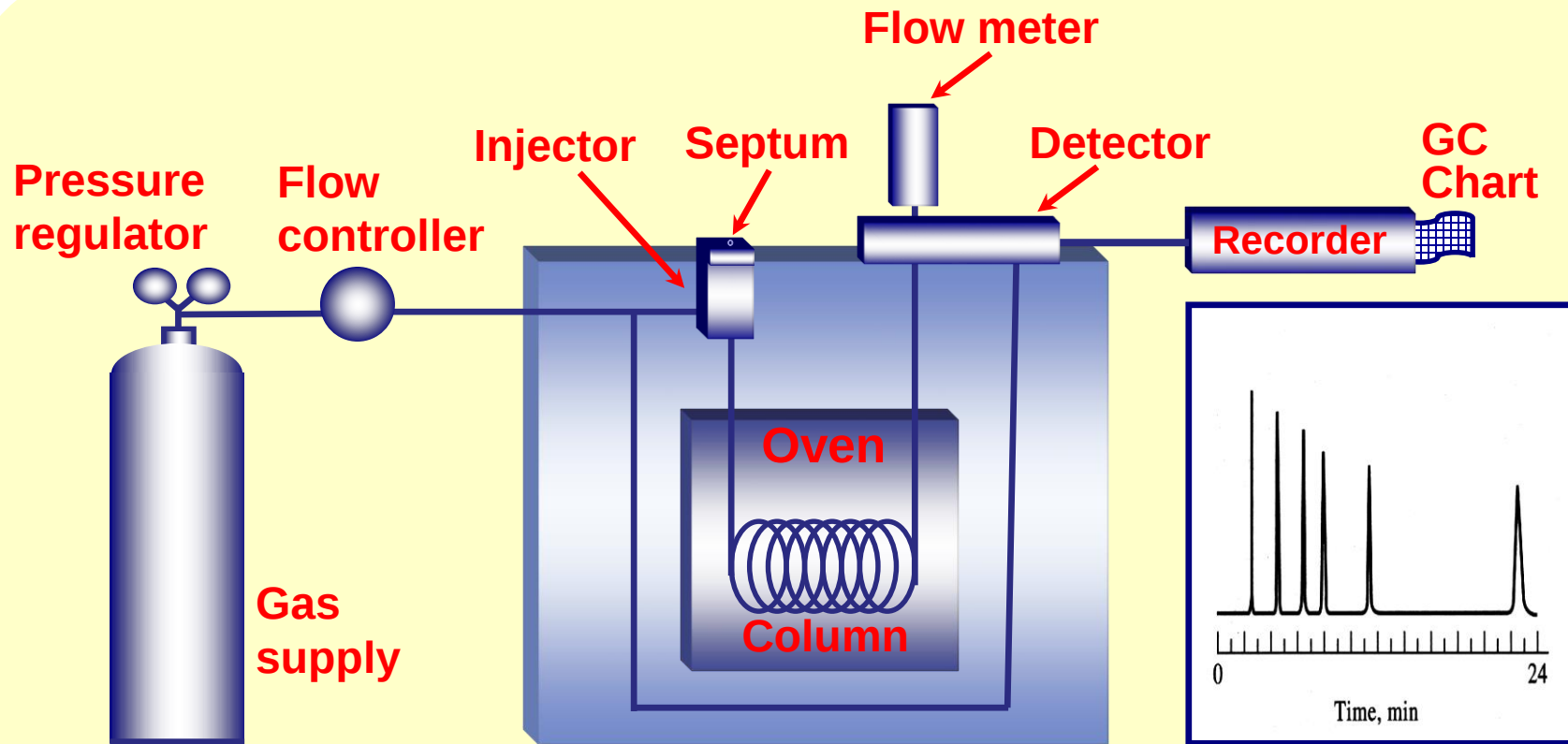
8. Gas chromatography (GC)

- Gas-liquid chromatography, (GLC) have.
 - **Mobile phase:** inert gas (helium, nitrogen)
 - **Stationary phase:** supported liquid (SiO₂ coated with polymer)

GC:

- ✓ Involves a sample being vaporized and injected onto the head of the chromatographic column.
- ✓ The sample is transported through the column by the flow of inert, gaseous mobile phase.
- ✓ The column itself contains a liquid Sp which is adsorbed onto the surface of an inert solid

Instrumentation of GC



8. Gas chromatography (GC)...

- For GC analysis the sample
 - Must be volatile, or
 - Be able to be converted to volatile derivative.
 - Form volatile metal chelate with trifluoroacetylacetone (TFA) and hexafluoroacetylacetone (HFA) if it is inorganic metals (aluminum, beryllium, and chromium).

8. Gas chromatography (GC)...

Application of GC

- Food Analysis
- Drug Analysis
- Environmental Analysis
- Forensic Analysis

9. High Performance Liquid Chromatography (HPLC)

- Utilizes very small packing particles and a relatively high pressure.
- Works by forcing the Mp through the column at much higher rate increasing the resolution rate.
- It can be
 - Normal phase HPLC
 - Reverse phase HPLC

9. High Performance Liquid Chromatography (HPLC)...

1. Normal phase HPLC

- Polar Sp: SiO_2 , Al_2O_3
- Non polar Mp: Hexane, heptane, cyclohexane...
- Polar compounds travel slower and eluted slowly due to higher affinity to the Sp

1. Reverse phase HPLC

- Non polar Sp: n-octadecyl, n-octyl, phenyl diol,
- Polar Mp: methanol or acetonitrile/water
- Polar compounds elute faster than non polar compounds

HPLC system

- Solvent Reservoir
- Degasser
- Solvent Delivery System (Pump)
- Injector
- Column & oven
- Detectors
- Recorder (Data Collection)



10. Supercritical fluid chromatography/SFC

- It is a normal phase chromatography using SCF as Mp
- Polar Sp: silica/Alumina
- Non polar Mp: commonly CO₂
- SFC is not suitable for elution of polar compounds.
 - Overcomed by addition of modifiers (alcohols, acetonitrile, chloroform)
 - Modifiers increase the solvating power of CO₂.

10. Size Exclusion Chromatography(SEC)

- Is non-adsorption and non-destructive method
- Applied to large molecules or macromolecular complexes such as proteins and industrial polymers.
- Unlike IEC, SEC does not involve chemical interaction with sample

Mechanism of separation

- Separate molecules in solution by their hydrodynamic volumes
- Smallest molecules enter deep into the pores and elute last.
- Medium sized components penetrate some depth and elute separately according to their size.
- Macromolecules, bigger than the pore size will not be retained.
- **Exclusion limit** is maximum molecular weight that can enter the pore

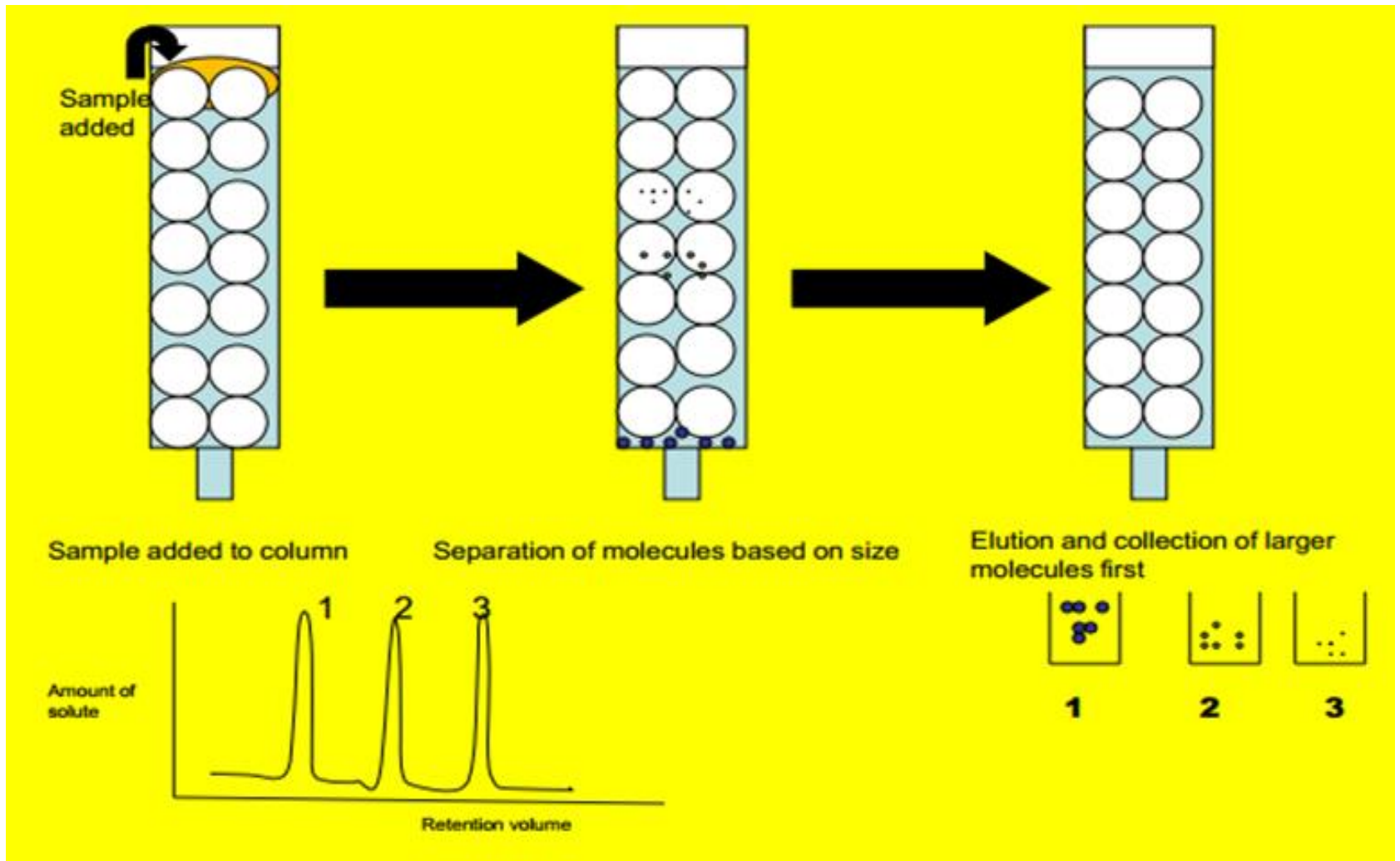


Figure 2: schematic representation of the operation of SEC column

Types of size exclusion chromatography.

- **Gel permeation chromatography (GPC),**
 - The column is packed with porous beads (Sephadex, agarose).
 - Organic solvents like hexane & toluene used as MP
 - To measure the molecular weight distribution of organic polymers.
 - For polymer analysis.
- **Gel filtration chromatography (GFC),**
 - The column is packed with porous beads (Sephadex, agarose).
 - Aqueous solution used as Mp
 - To separate, fractionate, or measure M.weight of molecules soluble in water, such as polysaccharides and proteins.

.....cont.

- Highly specialized gel filtration media
 - composed of macroscopic beads synthetically
 - derived from the polysaccharide, dextran or silica gel.

Merits and demerits of SEC

- **Merits**

- Short analysis time
- Well defined separation
- Good sensitivity
- No sample loss, since no interaction with Sp
- Small MP required

Merits and demerits of SEC...

- **Demerits**

- Prior filtration to prevent dust and other particulate running in the column and interfering with the detector
- Not applicable to similar-sized molecules, like isomers
- Limited number of bands accommodated since short time scale

Applications of SEC:

- Purification.
- Desalting.
- Protein-ligand binding studies.
- Protein folding studies.
- Concentration of sample.
- Relative molecular mass determination

The end!!!