Chromatography

Part-II: Coloumn Chromatographic Techniques

Column Chromatography (CC)

This includes chromatographic methods in which:

- **The stationary phase is packed into a column.**
- **The mobile phase is a moving liquid or gas.**

- **According to the mechanism of separation of solutes, five major types of CC are distinguished. Usually, one mechanism predominates but does not exclude the others**

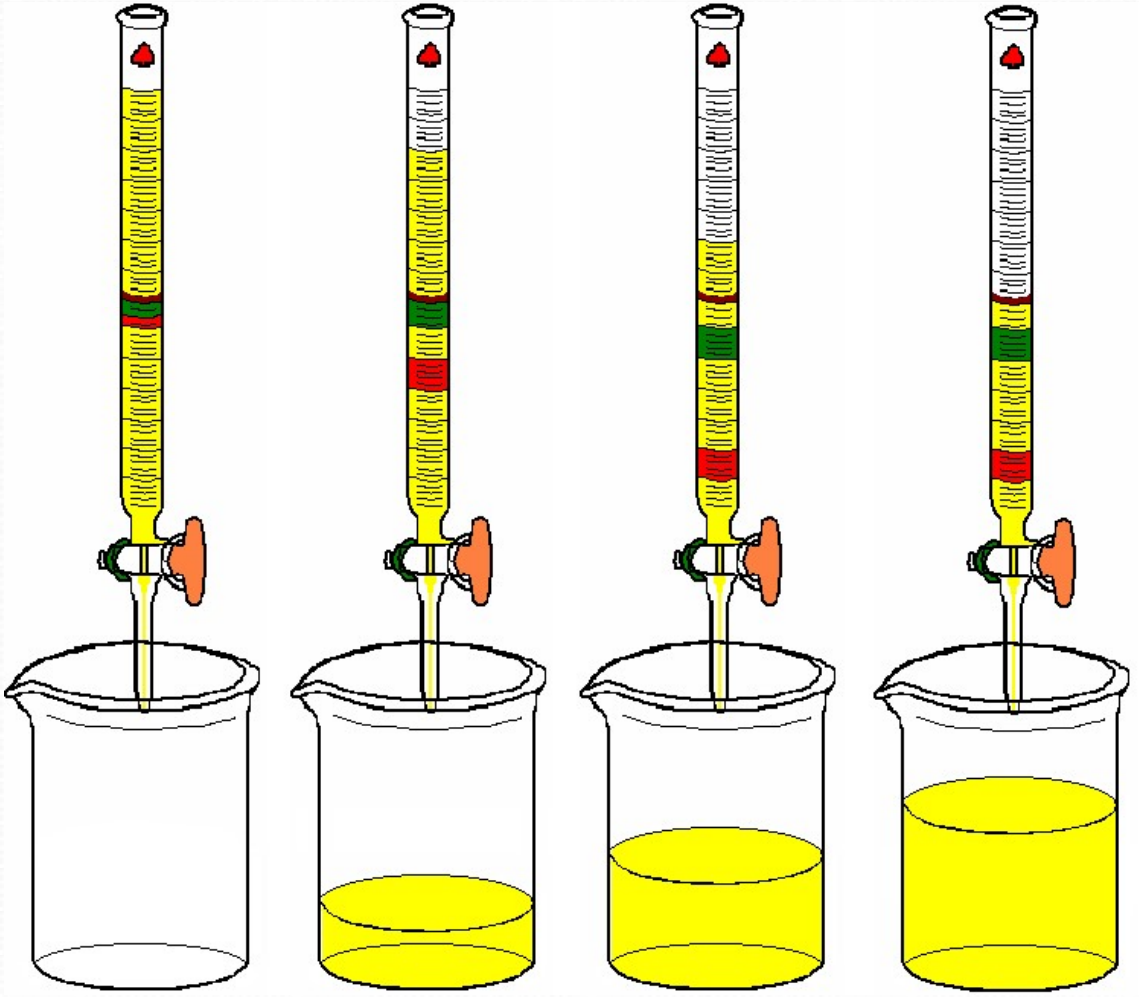
Different Types of chromatography

Mode or type	Stationary phase	Mobile phase	Mechanism
Adsorption Chromatography	Solid that attracts the solutes	Liquid or gas	Solutes move at different rates according to the forces of attraction to the stationary phase.
Partition Chromatography	Thin film of liquid formed on the surface of a solid inert support	Liquid or gas	Solutes equilibrate between the 2 phases according to their partition coefficients
Ion Exchange Chromatography	Solid resin that carries fixed ions & mobile couterions of opposite charge attached by covalent bonds	Liquid containing electrolytes	Solute ions of charge opposite to the fixed ions are attracted to the resin by electrostatic forces & replace the mobile counter ions.
Molecular Exclusion Chromatography	Porous gel with no attractive action on solute molecules	Liquid	Molecules separate according to their size: 1.Smaller molecules enter the pores of the gel, and need a larger volume of eluent. 2.Larger molecules pass through the column at a faster rate.
Affinity Chromatography	Solid on which specific molecules are immobilized	Liquid or gas	Special kind of solute molecules interact with those immobilized on the stationary phase

Column Chromatography

Column chromatography

Stationary phase is held in a narrow tube through which the mobile phase is forced under pressure or under the effect of gravity



Term	Definition
Solvent	Mobile liquid phase with no affinity to the stationary phase (i.e. inert towards it) & no effect on solutes.
Developer	Any liquid with more affinity to the stationary phase than the solvent but less than solutes and just capable to move them through the column.
Effluent	Any liquid that passes out of the column.
Eluent	Any liquid that has lesser affinity to the stationary phase than solutes but is capable to move them out of the column.
Eluate	Fraction of eluent containing a required specific substance.
Retention volume (V_R)	(or retardation volume): Volume of mobile phase that passes out of the column, before elution of a specific substance.

Open Column Chromatography (Traditional column chromatography)

- Traditional column chromatography is characterized by addition of **mobile phase** under **atmospheric pressure** and the **stationary phase** is **packed in a glass column**.
- Following slides contain the stepwise procedure of experimental techniques of Traditional coloumn chromatography..

Packing & operating the column

1- Packing

- The selection of the method of packing **depends** mainly **on the density of the solid**. Techniques used are the **wet**, **dry** & **slurry** methods.
- In all cases **avoid inclusion of air bubbles**

2- Sample Application

- Apply **evenly & in a concentrated solution** to the top of the column which is protected from disturbance (e.g. add glass wool or filter paper).

3- Elution

1.Elution techniques

Technique	Procedure
Isocratic elution	**Addition of solvent mixture of fixed composition during the whole process.**
Gradient elution	**<u>Continuous or linear elution</u>: in which there is continuous change in the composition of the mobile phase over a period of time (e.g. polarity, pH or ionic strength).**
	<u>Step wise or fractional elution</u>: in which the change is not continuous i.e. a sudden change in the composition of the mobile phase is followed by a period where the mobile phase is held constant.

4- Detection

- **On-column detection** for **colored** or **fluorescent** compounds directly after developing the chromatogram.

- **Monitoring of eluted fractions** (PC or TLC).

- **Using special detectors** connected to the column such as refractive index, UV detectors, etc…

Factors affecting solutes separation in CC
(Factors affecting column efficiency)

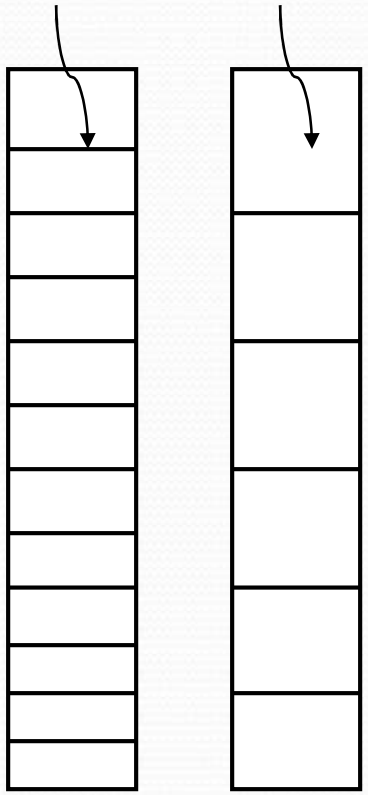
Factor	Effect
Particle size of solid stationary phase (or of support)	Decrease of size improves separation (but very small particles need high pressure).
Column dimensions	Efficiency increases as ratio length / width increases.
Uniformity of packing	Non uniform packing results in irregular movement of solutes through column & less uniform zone formation, (i.e. band broadning or tailing).
Column temperature	Increase in column temperature results in speed of elution but does not improve separation (tailing).
Eluting solvent	Solvents should be of low viscosity (to give efficient resolution) & h igh volatility (to get rapid recovery of the substances).
Solvent flow rate	Uniform & low flow rate gives better resolution.
Continuity of flow	Discontinuous flow disturbs resolution
Condition of adsorbent	Deactivation of adsorbent decreases separation.
Concentration of solutes	Substances of high concentration move slowly.

Number of Theoretical Plates (N)

H = Theoretical Plate Height
L = Length of the Column.

$$N = L / H$$

As HETP decreases efficiency of the column increases.



Types of Column Chromatography

A. Adsorption Column Chromatography

- **Adsorbents:** The most common are **Alumina** & **Silica gel** in which the **interactions** with solute molecules is **due to OH groups present on their surface**.
- **More polar molecules** are **adsorbed more strongly** & thus, will **elute more slowly**
- Strength of adsorption of polar groups (solutes) on polar support is in the following order:
- **-C=C- < O-CH_3 < -COOR < >C = O < -CHO < -NH_2 < -OH < -COOH**
- **Olefins < Ethers < Esters < Lactones < Aldehydes < Amines < Phenols < Acids.**

Applications in separation of natural products

- **Alumina**: sterols, dyestuffs, vitamins, esters, alkaloids & inorganic compounds: Not used for compounds containing phenolic or carboxylic groups
- **Silica gel:** sterols & amino acids.
- **Carbon:** peptides, carbohydrates & amino acids.
- **Calcium carbonate:** carotenoids & xanthophylls.

B. Partition Column Chromatography

- In this type, the **packing consists of a theoretically inert support material coated with a film of the liquid stationary phase**.

- The **division into adsorption & partition** is only of **theoretical** significance as **in partition chromatography the adsorption effects of the support can be felt.**

Selection of the solid support

The support material should:

- adsorb & retain the mobile stationary phase.
- expose as large surface as possible to the mobile phase
- be mechanically stable.
- be easy to pack.
- not retard the solvent flow

Examples of solid supports:

Silica gel, diatomaceous earth (as kieselguhr, celite etc.) & cellulose.

Selection of the mobile phase

- The **liquid stationary & mobile phases** should have a considerable difference between their solvent strength parameters.
- **Pure water > Methanol > Ethanol > Propanol > Acetone > Ethyl acetate> Ether > Chloroform > Dichloromethane >Benzene > Toluene > Carbon tetrachloride > Cyclohexane > Hexane > Pentane.**
- e.g. if the **stationary phase** is **water**, **pentane** would be the **eluent** of choice.
- The **mobile phase** is usually **saturated** with the **stationary phase** to **overcome** "**stripping**" (washing of the stationary phase from the column by the mobile phase).

Gel Permeation Chromatography (GPC)

This type is also known as:

Size Exclusion Chromatography (SEC)

Molecular Exclusion Chromatography (MEC)

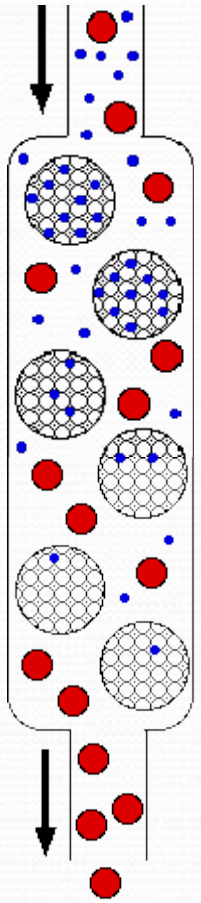
Molecular Sieve Chromatography (MSC)

Gel Filtration Chromatography (GFC)

Gel Chromatography.

Stationary phase

- **Porous polymeric matrix:** formed of spongy particles, with pores completely filled with the liquid mobile phase **(gel)**.
- The gels (polymers) consist of **open**, **three-dimensional networks** formed by cross-linking of long polymeric chains.
- The **pore size** varies with the degree of cross-linking.
- The **diameter of the pores is critical as** separation is based on that **molecules above certain size are totally excluded from the pores because they can not enter the gel**.
- The **interior of the pores is reached**, partially or wholly, **by smaller molecules**.



- **Mobile phase**
- Mobile phase is a liquid: **water** or **dilute acohol**

- **Separation mechanism:** Based on difference between the solutes molecular weights.

- Molecules will distribute themselves outside & inside the pores according to their size.

- **Larger** are excluded, **medium sized** enter half-way & **smallest** permeate all the way.

The **retention volume Vo** of a substance is inversely proportional to the molecular weight (M. Wt) of the solute.

$$Vo \simeq 1 / \text{M.wt}$$

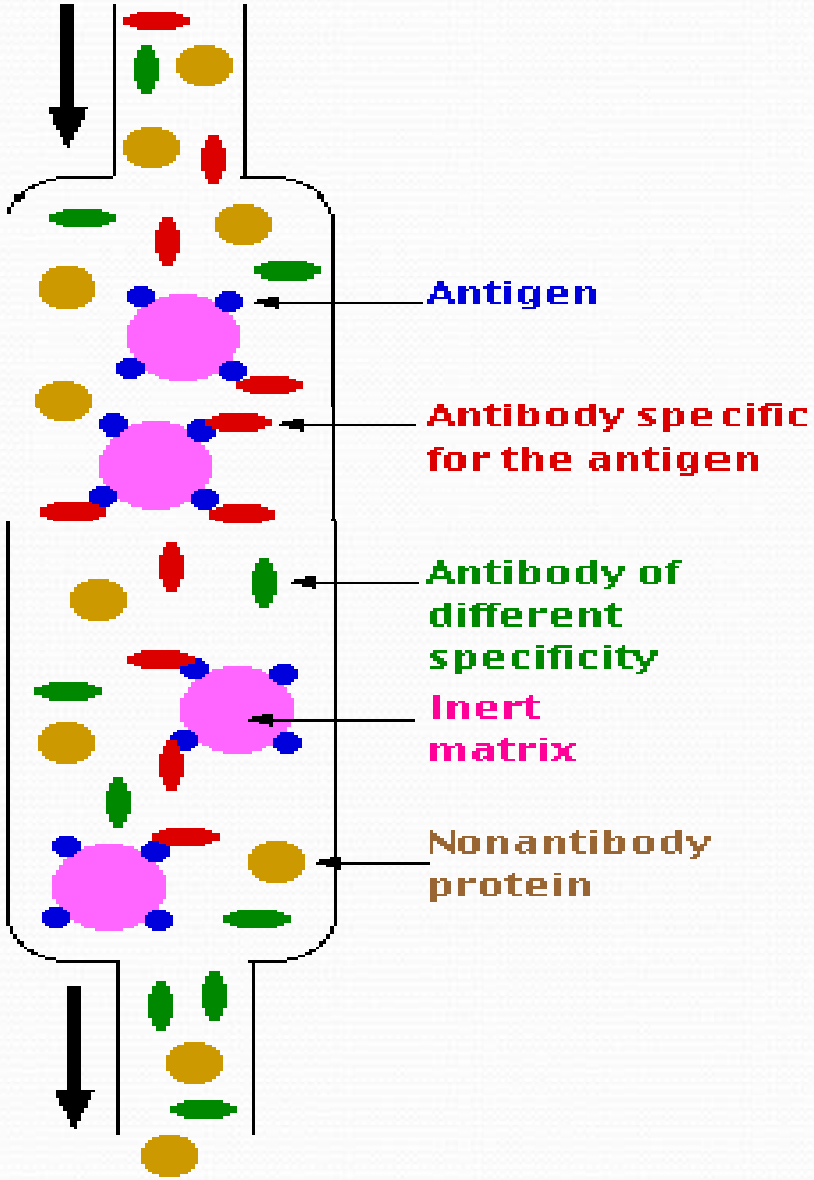
Vo = retention volume

M.wt = Molecular Weight

Applications of GPC to natural products

- **Determination of M. Wt. of peptides, proteins & polysaccharides.**
- **Desalting of colloids e.g. desalting of albumin prepared with 2% $(NH_4)_2SO_4$.**
- **Separation of mixture of mono- & polysaccharides.**
- **Separation of amino acids from peptides & proteins.**
- **Separation of proteins of different molecular weights.**
- **Separation of mucopolysaccharides & soluble RNA.**
- **Separation of myoglobin & haemoglobin.**
- **Separation of alkaloids & purification of enzymes.**

Affinity Chromatography



Ion Exchange Chromatography

Principle

- Process by which **ions** of an **electrolyte solution** are brought into contact with an **ion exchange resin**.

- The **ion exchange resin** is an insoluble polymer consisting of a "**matrix**" (Lattice or framework) that **carries fixed charges** (not exchangeable) and mobile **active ions** "**counter ions**" which are **loosely attached to the matrix.**

- In **water,** the **counter-ions move more or less freely in the framework &** can be **replaced by ions of the same sign present in the surrounding solution**.

- The "**matrix**" (framework) **of a** "**cation exchanger**" is considered as a crystalline non-ionized "**polyanion**" & the **matrix of an** "**anion exchanger**" as a non-ionized "**polycation**".

Cation Exchangers

- **Active ions (counter ions) are cations.**
- The **polar groups attached to the matrix are acidic** (sulphonic acids, carboxylic acids, phenols, phosphoric acids) e.g. a cation exchanger in the free carboxylic acid form:

$X\text{-}COO^- H^+$

X = Frame work (matrix)

$-COO^-$ = Fixed charge (anionic),

Non-exchangeable

H^+ = Counter ion (cation), Exchangeable

They are usually (but not always) supplied in the Na^+ form: **X-COO$^-$Na+**

or $\overline{Na}^+$, Where — = Matrix

e.g. exchange with $CaCl_2$ aqueous solution

$$2\ \overline{Na}^+ + Ca^{++}\ Cl_2 \longrightarrow \overline{Ca}^{++} + 2\ Na^+\ Cl^-$$

Anion Exchangers

- **Active ions (counter ions) are anions.**
- The polar groups attached to the matrix are tertiary or quaternary ammonium groups (basic).
- e.g. Anion exchanger in the quaternary ammonium form:

X. $NR_3^+OH^-$

X = Framework (matrix)

$-NR_3^+$ = Fixed charge (cationic)

Non exchangeable

$-OH^-$ = counter ion (anion), Exchangeable

or $\overline{\overline{Cl}}^-$ (where, ——— is the matrix)

e.g. exchange with Na_2SO_4 solution

$$2\,\overline{\overline{Cl}}^- + Na_2^{+2}\,SO_4^{-2} \longrightarrow SO_4^{-2} + 2\,Na^+Cl^-$$

- They are supplied as the chloride rather than the hydroxide as the chloride form is a more stable. Represented as: **X. $NR_3^+Cl^-$**

Regeneration of the resin

- **Ion exchange process is generally reversible e.g in the following:**

- $2\,Na^{+} + Ca^{++}\,2Cl^{-} \rightarrow Ca^{++} + 2\,Na^{+}\,Cl^{-}$

- **The cation exchanger could be exhausted after exchanging all Na^{+} for Ca^{++}, the exchanger could be regenerated (loaded again with Na^{+}) by contacting it with excess Na^{+} ions e.g. a solution of NaCl.**

Types of Exchangers

- **Ion Exchangers**

  These are either **cation** or **anion exchangers** of either **organic** or **inorganic** nature.

  A- Inorganic ion exchangers

  Common clays, soils, minerals e.g. zeolites used for "softening water".

  Disadvantage: low ion-exchange capacity.

  Advantages:

- Great resistance to high temperatures.
- High volume capacity.
- Great selectivity towards simple inorganic ions.

B- Organic ion exchangers

- **They may be natural or synthetic.**
- **<u>Preparation of organic synthetic ion exchangers</u>** :
- Polycondensation of **phenols**, **sulpho-** & **carboxy- derivatives with formaldehyde** → **cationic** exchangers.
- Polycondensation of **aromatic amines with formaldehyde** → **anionic** exchangers.
- These techniques yield products **linear** in structure & relatively **soluble in water** which are now replaced by resin materials based on **styrene divinyl benzene copolymers** and **polyacrylate**.

Applications of Ion Exchange Chromatography

1- Water softening:

Removal of Ca^{2+}, Mg^{2+} & other multivalent ions causing hardness of water by filtration through a layer of strong cation resin.

2-Water demineralization:

Removal of cations & anions dissolved in water. Usually carried by the two step technique in which two columns of strongly acid cation exchanger in $[H^+]$ form & strongly basic anion exchanger in $[OH^-]$ form are used in sequence.

3- Neutralization:

Cationic exchanger in $[H^+]$ can be used to neutralize alkali hydroxide & anionic exchanger in $[OH^+]$ form to neutralize the acidity.

4- Separation of electrolytes from non-electrolytes.

5- Separation of carbohydrates & their derivatives:

- **Uronic acids** separated on anion exchanger.
- **Sugars** converted into ionized form by using borate& separated on strong anion exchanger.
- **Hexosamines** separated on strong cation exchanger.

Thank You

.....Keep Learning!