UNIT-V

ION EXCHANGE CHROMATOGRAPHY

Points to be covered in this topic

- ❖ Introduction
- ❖ Classification
- ❖ Ion exchange resins
- ❖ Properties
- ❖ Mechanism of ion exchange resins
- ❖ Factor affecting ion exchange
- ❖ Methodology
- ❖ Applications

ION EXCHANGE CHROMATOGRAPHY

❖ INTRODUCTION

- Ion exchange chromatography can be defined as a **reversible process in which ions of similar charged are exchanged between solid and liquid.**
- The solid is known as an ion exchanger. It is an **adsorption chromatography, a useful and popular method for separation of a mixture** of similar charged substances into pure components. It is also known as cation anion exchange chromatography.

❖ DEFINATION

- These are the substances **capable of exchange of ions with the electrolytic solution.**
- These are porous solid, swelling in water without dissolving in it. The most common properties of all ion exchanger are as follows:

  a) They are almost insoluble in water and organic solvents.

  b) They are complex in nature (Polymeric in nature)

  c) They have active or counter ions that will exchange reversibly with other ions in a surrounding solution without any substantial change in the material.

❖ TYPE OF ION EXCHANGERS

- There are three classes of ion exchangers, these includes

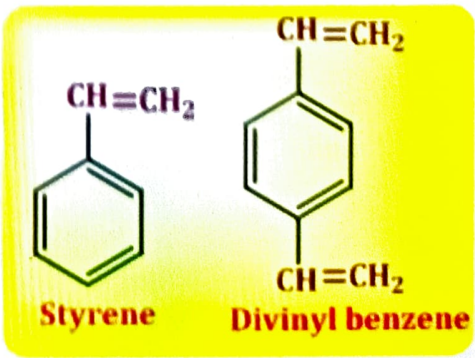
  1. Resins

  2. Gels

  3. Inorganic exchangers

❑ Resins

- Resins are **amorphous particles of organic materials which are composed of polystyrene and divinely benzene.**
- Polystyrene contains sites for **exchangeable functional groups.**
- Divinyl benzene acts as **a cross linking agents**



- and offers adequate strength i. e., mechanical stability.

➢ Classification of resins

I. According to their chemical nature, they can be classified as:

a) **Strong cation exchange resin:** These types of resins are useful for the chromatographic separation of amino acids, rare earths and other substances that contains sulphonic acid groups as the ionisable groups.

b) **Weak cation exchange resin:** These resins are based on polymers of methacrylic acid and possess carboxyl groups.

c) **Strong anion exchange resin:** These resins with positively charged quaternary ammonium groups attached to cross linked polystyrene frame work belong to this class. Trimethyl ammonium groups are used for this resin.

d) **Weak anion exchange resin:** The tertiary amine resins and polyamine resins having a mixture of primary, secondary and tertiary amine groups on the polystyrene net work are well known.

II. According to the source, they are classified as

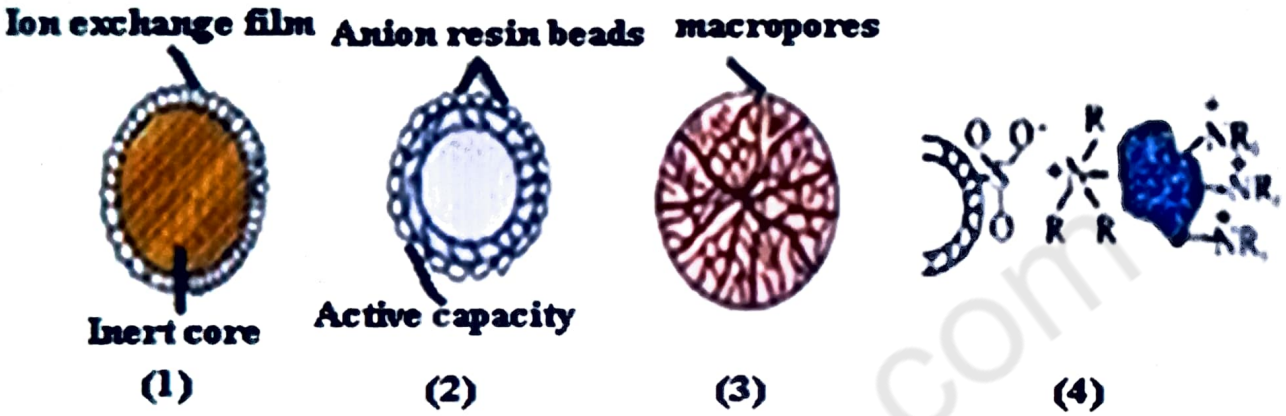
1. Natural source: These are of two types
   - a) Cation: E.g: Zeolites, clay etc.
   - b) Anion: E.g: Dolomite
2. Synthetic source: These are of two types
   - a) Inorganic resins
   - b) Organic resins : Most widely used

III. Structural types of ion exchange resins

1. **Pellicular type with ion exchange film:** The particle size is ranging from 30 -40 mm with 1 - 2 mm film thickness. These have very low ion exchange capacity to separate the ions. Their ion exchange efficiency is 0.01 - 0.1 meq/g of ion exchange capacity.
2. **Porous resin coated with exchanger beads:** The particle size ranges

from 5 – 10 m. They are totally porous in nature and highly efficient. Their exchange capacities are from 0.5 – 2 meq/g of ion exchange resin.

3. **Macro reticular resin bead:** A reticular network of the resin is seen superficially on the resin beads. They **are not highly efficient and have very low ion exchange capacities.**
4. **Surface sulfonated and thermostatically bonded with anion:** It is less efficient and also has less exchangeable capacity.



Structural types of ion exchange resins.

➢ Properties of ion exchange resin

- It must be **chemically stable.**
- It should be **insoluble in common solvents.**
- The resin must be **sufficiently hydrophilic.**
- It should have **a sufficient degree of cross linking.**
- The swollen **resin must be denser than water.**
- It must contain **sufficient numbers of ion exchange groups.**

2. Gels:

- Ion exchange gels are used for the **separation of large molecules like proteins, nucleic acids.**
- These are **much softer than polystyrene resins**
- Dextron and its relatives are **called as gels**
- Cellulose and dextran ion exchangers which are polymers of sugar glucose **possess large pore sizes and lower charge densities.**

3. Inorganic exchangers

- The combinations of **hydrous oxides of highly charged ions,** with one oxide more acidic than the other have been found to have ion exchanging properties.
- The amorphous precipitates have **higher exchange capacities than the crystalline compounds because of the greater surface area of the former** type of compounds.
- **E.g.: Titanium Arsenic has been used to absorb alkaloids, Hydrous antimony peroxide has**
- been used to study exchange equilibrium of K and R_b ions with hydrogen and other ions.

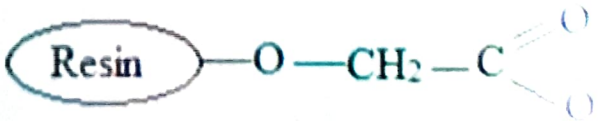
❖ MECHANISM OF ION EXCHANGE PROCESS

- The mechanism of separation is by **reversible exchange of ions between the ions present in the solution and those present in the ion exchange resin.**
- Ion exchange separations are mainly carried out in columns packed with an ion exchanger.
- **There are two types of ion exchanger, as follows:**

a) Cationic exchangers:

- It possesses **negatively charged groups and these will attract positively charged groups.** These exchangers are also called acidic ion exchange materials since their **negative charges result from the proteolysis of acidic groups.**
- Commonly used cation exchange resins are **S-resin, sulfate derivatives; and CM resins, carboxylate derived ions**

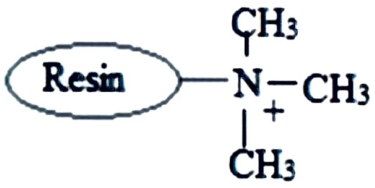
(S-Cation exchanger)



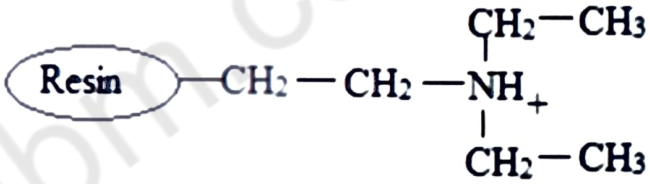
(CM-Cation exchanger)

b) Anionic exchangers:

- It has positively charged groups, which will **attract negatively charged molecules.**
- This exchanger is termed as basic ion exchange materials since their positive charges generally result from the **association of protons with basic groups.**
- Based upon the affinity of ions towards the matrix the **ions like cation and anion are separated. The ions that have less affinity towards matrices** will elute first and the ions that **have more affinity towards matrices it will elute later.**
- Commonly used anion exchange resins are Q-resin, a **Quaternary amine; and DEAE resin, Di Ethyl Amino Ethane.**



(Q-Anion exchanger)



(DEAE-Anion exchanger)

- **The actual ion exchange mechanism is thought to be composed of five distinct steps:**

1. Diffusion of the ion to the exchanger surface. This **occurs very quickly in homogeneous solutions.**
2. Diffusion of the ion through the matrix to the exchanger site. This is **dependent upon the degree of cross linkage of the exchanger and the concentration of the solution.**
3. Exchange of ions at the exchange site occurs. This **occur instantaneously in an equilibrium process as follows:**

$$Resin - SO_3H + Na^+ \longrightarrow Resins - SO_3Na + H^+$$

$$Resin - N(CH_3)_3OH + Cl^- \longrightarrow Resin - N(CH_3)_3Cl + OH^-$$

4. Diffusion of the exchanged ion through the exchanger to the surface
5. Selective desorption by the **eluent and diffusion of the molecule into**

the external solution takes places

❖ FACTORS AFFECTING ION EXCHANGE

- The factor affecting ion exchange are as follows:

a) Nature of ion exchange resin:

- Cross linking & swelling is important factor which depends on the **proportion of cross linking agent (Divinyl benzene & polystyrene).**
- When more cross linking agent is present they are more rigid, but swells less. When **swelling is less , separation of ions of different sizes is difficult** as they nan not pass through the pores present & it becomes selective to ions of different sizes. When **less crosslinking agent is present, they are less rigid but swells more.**
- When swelling is **more separation will not be efficient as exchange of functional groups does not take place due to wide pore hence optimum quantity of cross linking agent** should be added to the polymeric ion exchange resins for the separation to be effective.

b) Nature of exchanging ions:

i) Valency of ions: At low concentration & at ordinary temperature extent of exchange increase with increase in valency.

$Na^{+} < Ca^{2+} < Al^{3+} < Th^{4+}$

ii) Size of ions: For similar charged ions, exchange **increases with decrease in the size of hydrated ion.**

$Li^{+} < H^{+} < Na^{+} < NH^{4+}$

iii) Polarizability: Exchange is preferred for greater polarizable ion

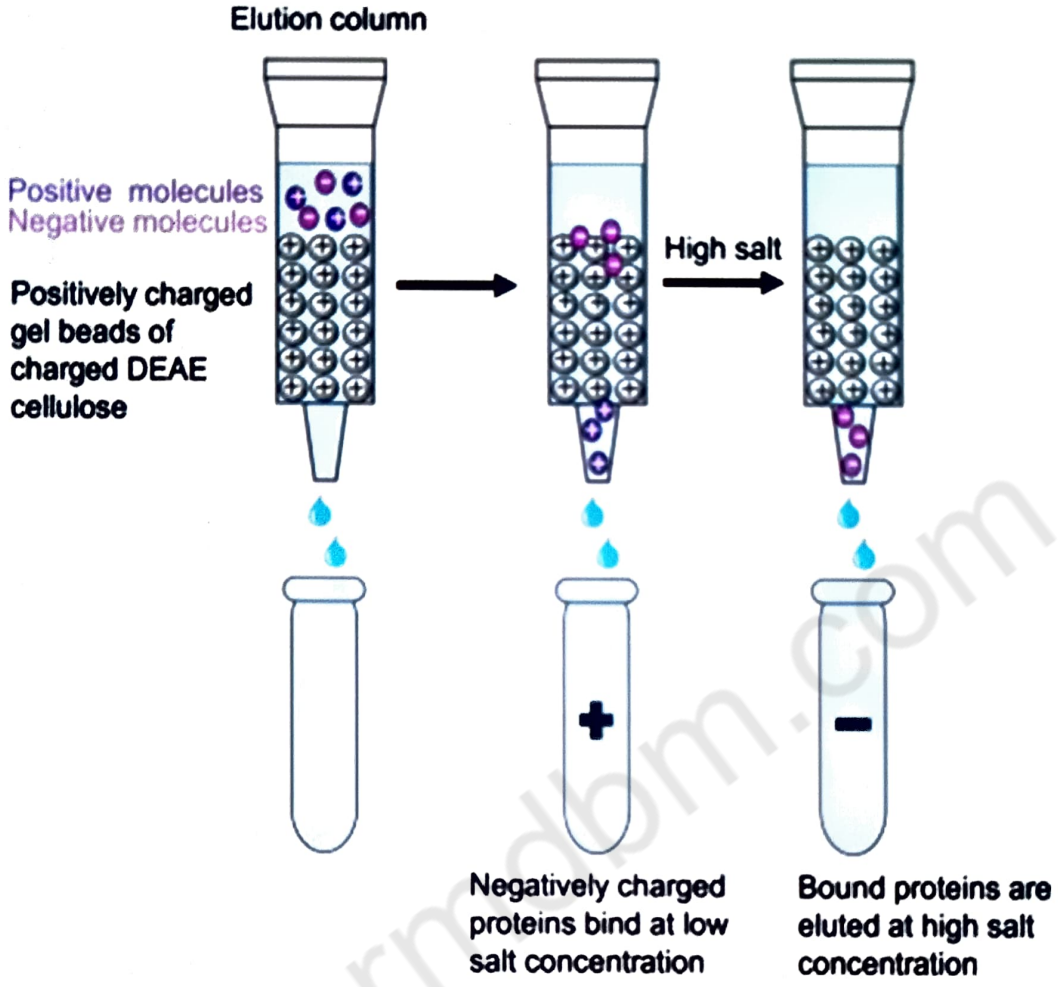
$I^{-} < Br^{-} < Cl^{-} < F$

iv) Concentration of solution: In dilute solution, polyvalent anions are generally adsorbed preferentially.

v) Concentration & charge of ions: If resin has higher +ve charge & solution has lower +ve charge, exchange is favoured at higher concentration. If the resin has lower +ve charge and solution has high +ve charge, then exchange is favoured at low concentration.

❖ METHODOLOGY

- General components and methodology of an ion-exchange chromatography are as follows



i) Column:

- Column used in the laboratories are made up of glass but those used in industries are made up of either high quality stainless steel or polymer, which are resistant to strong acids and alkalis.
- The geometry of column depends completely on the separation factor. The separation is improved by **increasing the length of the column but the length cannot be increased beyond a critical length.**
- A Dimension of column is 20:1 to 100:1 for the higher efficiency can be used.

ii) Packing of the column:

- In this wet packing method is used.
- The resins is mixed with the **mobile phases and packed in the column uniformly.**

- The sample to be separated is dissolved in the mobile phases and introduced all at once into the column.

iii) Application of the sample:

- After packing the column, the solution to be analyzed is added to the top of the column and allowed to pass through the bed of ion exchanger.
- For this purpose the **syringe or pipette is utilized.**

iv) Mobile phase:

- The organic solvents are less useful so they are not used these days.
- Only different strength of acids, alkalis and buffer are used as eluting solvent. **E.g.: 0.1 N HCl, 1N NaOH, phosphate buffers, acetate buffers, borate buffers, phthalate buffers, etc.**

v) Developments of the chromatogram and elution:

- After introduction of the sample, development of the chromatogram is done by using different mobile phases.
- The aqueous salt solution is adjusted to a constant ionic strength.
- The choice of the mobile phase depends on the selectivity of the resin for the solute ions. **Following two types of elution techniques are used:**
  - a) **In Isocratic elution technique,** the same solvent composition is used **i.e., same strength of acids or alkalis or buffers are used.**
  - b) **In gradients elution techniques,** initially less acidic or basic mobile phase is used. Then, **acidity or basicity is increased at regular intervals. The different fraction** of the elution is collected volume wise or time wise and analyzed.

vi) Analysis of the elute or Detection:

- Different fractions are collected with respect to the volume or time is analyzed for their contents.
- Several methods of analysis can be used which depends upon the nature and quantity of the ionic species are:
- **Conductometric method**
- **Amperometric methods**
- **Flame photometric method**
- **Polarography**

- UV. Spectroscopy
- Radiochemical methods using Geiger Muller counter, ionization chamber method.

vii) Regeneration of ion exchange resin:

- The ion exchange resin after **separation may not be useful for next separation as exchange functional groups are lost**. But due to the cost of ion exchange resins, they cannot be disposed off. Hence reactivation, regeneration of the resins is most important.
- It refers to the replacement of the **exchangeable cations or anions present in the resin.**
- The charging of the column with strong acid like hydrochloric acid is used for regeneration of the cation exchange resin while **strong alkali like sodium hydroxide or potassium hydroxide used for regeneration of the cation exchange resin.**

❖ APPLICATIONS

1. **Softening of hard water:** Hardness of water is due to the **presences of Ca^{2+}, Mg^{2+} and other divalent ions may be removed by passing** the hard water through the cation exchanger charged with Na^{+} ions .
2. **Complete demineralization of water:** This requires complete removal of ions i.e., both cations and anions. For this, water **is passed through an acidic cation exchanger then metallic cations are exchanged with H^{+} ions.**
3. **Purification of organic compounds:** Many natural products extracted in water have been found to contain ions originally present in water. Those ions can be **removed by using ion exchange process.**
4. **Separation of amino acids:** Ion exchange methods can be used to separate **the complex mixture of 18 amino acids obtained by the acid hydrolysis of proteins.**
5. **Purification and recovery of pharmaceuticals:** The process is used for purification and recovery of antibiotics, vitamins, alkaloids, hormones and other chemicals of **pharmaceutical importance during their**

manufacturing process.

6. **Biochemical separations:** Used for biochemical separations like some drugs or metabolites from blood, urine or other biological fluids.

7. **Other applications:**

- For the measurement of various **active ingredients in medicinal formulations.**
- For the measurement of drugs and their **metabolites in serum and urine, for residue analysis in food raw materials.**
- For the measurement of **additives such as vitamins and preservatives in food and beverages.**

UNIT-V

GEL CHROMATOGRAPHY

Points to be covered in this topic

- ❖ Introduction
- ❖ Theory
- ❖ Instrumentation
- ❖ Application

GEL CHROMATOGRAPHY

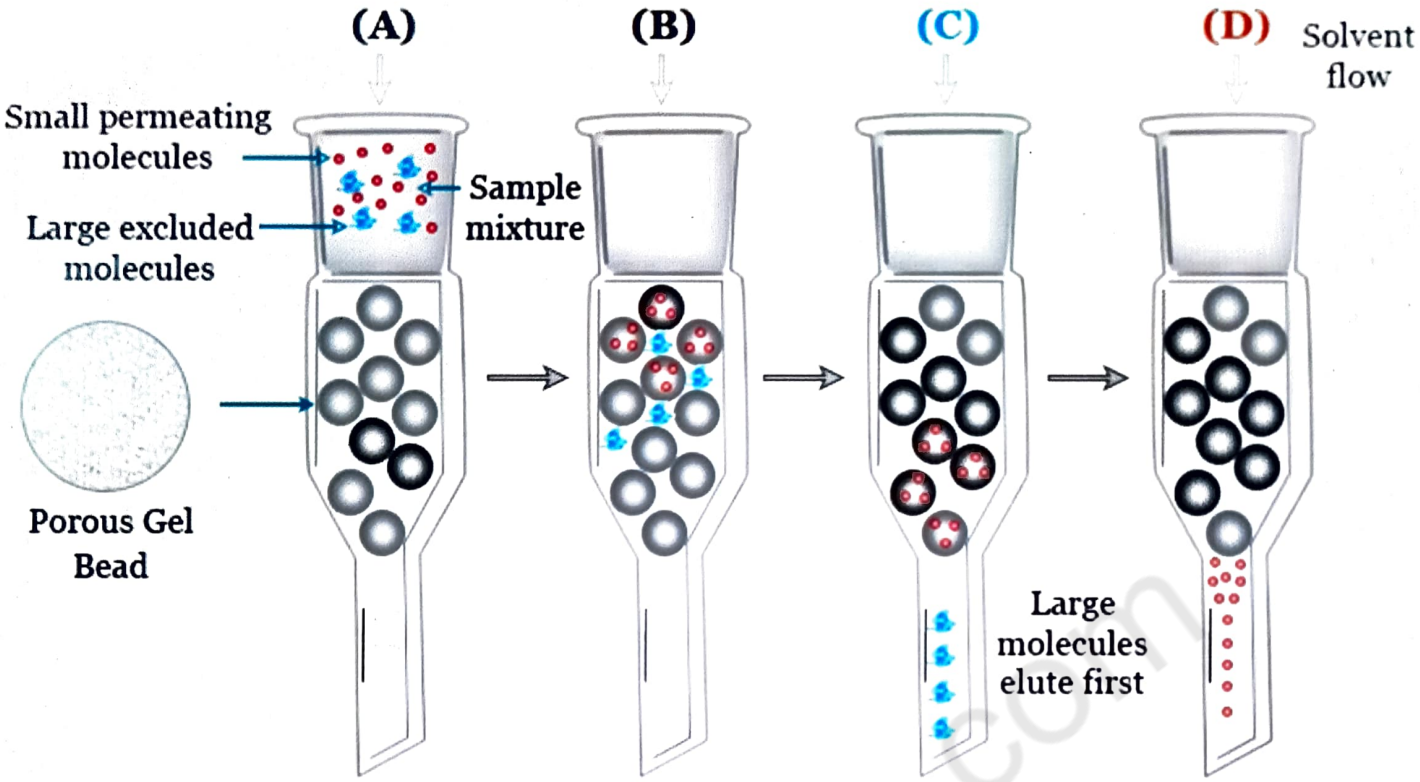
❖ INTRODUCTION

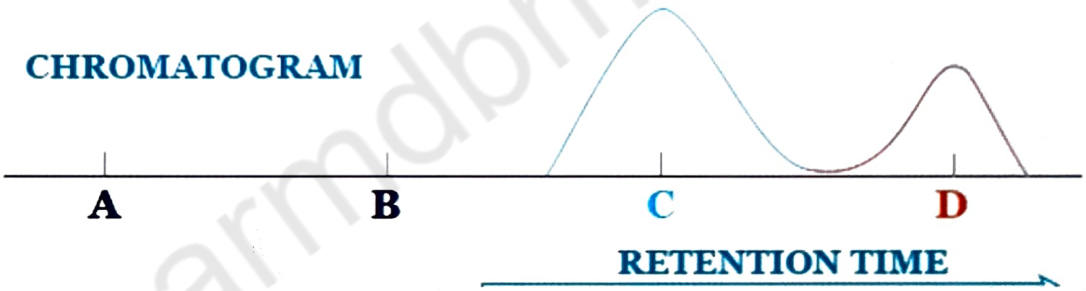
- Gel chromatography also called as **Gel permeation chromatography, Exclusion chromatography and Molecular sieve chromatography.**
- It is a simple and reliable method for **separating molecules according to their size.**
- It uses a porous material as the **stationary phase & a liquid as a mobile phase.** The diameter of the pores of the porous material are of the order **50-300 A^0, which is similar to the size of many molecules.**
- The molecules penetrate the pores according to their size. **Small molecules penetrate more rapidly than larger molecule.**
- The result is difference in the rate at which the molecule pass down the column, **the smaller molecules travelling faster than the larger molecule and become separated.**

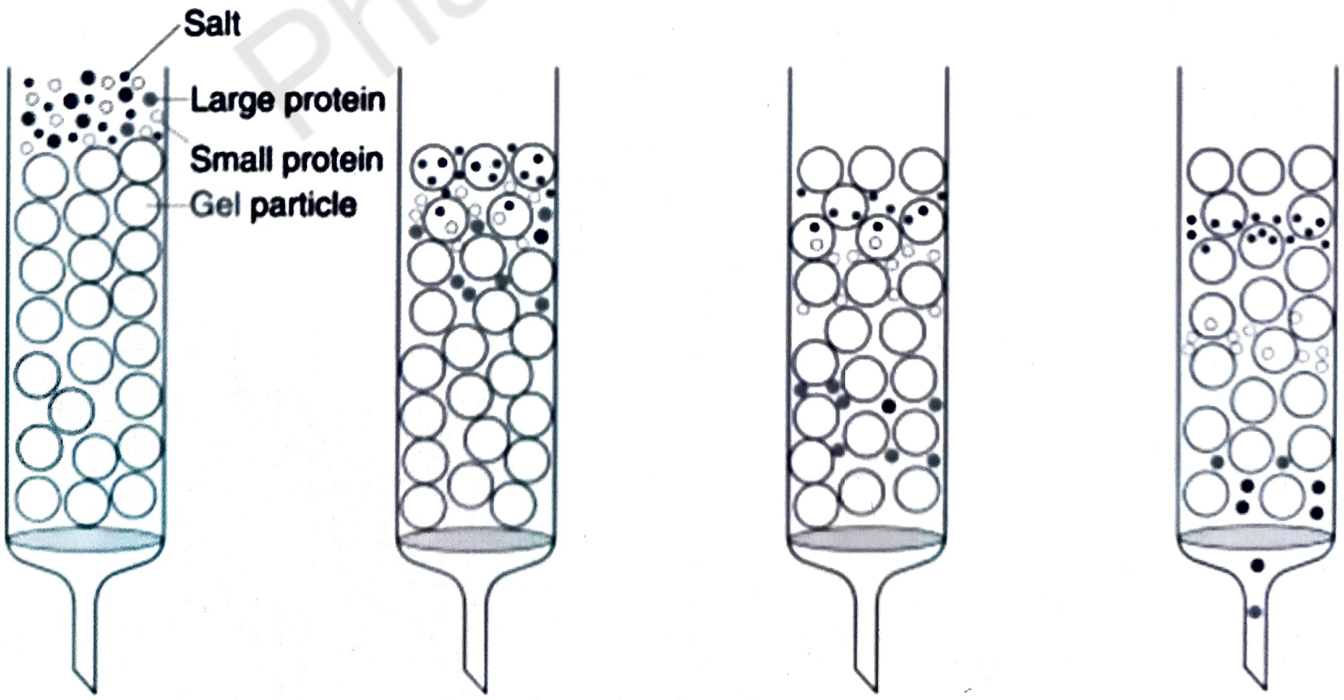
❖ PRINCIPLE

- A mixture of molecules dissolved in liquid (the mobile phase) is applied to a **chromatography column which contains a solid support in the form of microscopic spheres, or "beads" (the stationary phase).**
- The mass of beads within the column is often referred to as the column bed. The **beads act as "traps" or "sieves"** and function to filter small molecules which become temporarily trapped within the pores. Larger molecules pass around or are **"excluded" from the beads.**
- Large sample molecules cannot or can only partially penetrate the pores, whereas smaller molecules can access most or all pores.
- Thus, **large molecules elute first, smaller molecules elute later,** while molecules that can access all the pores elute last from the column. Particles of different sizes will elute (filter) through a stationary phase at different rates.

Gel Permeation Chromatography (GPC)







❖ THEORY

- Total volume of column packed with a gel that has been swelled by water or other solvent is given by following equation:

$$V_t = V_g + Vl + Vo$$

Where, V_t = Total bed volume

V_g = Volume occupied by solid matrix of gel

V_l = Volume of solvent held in pores or interstices

V_o = Free volume outside the gel particles

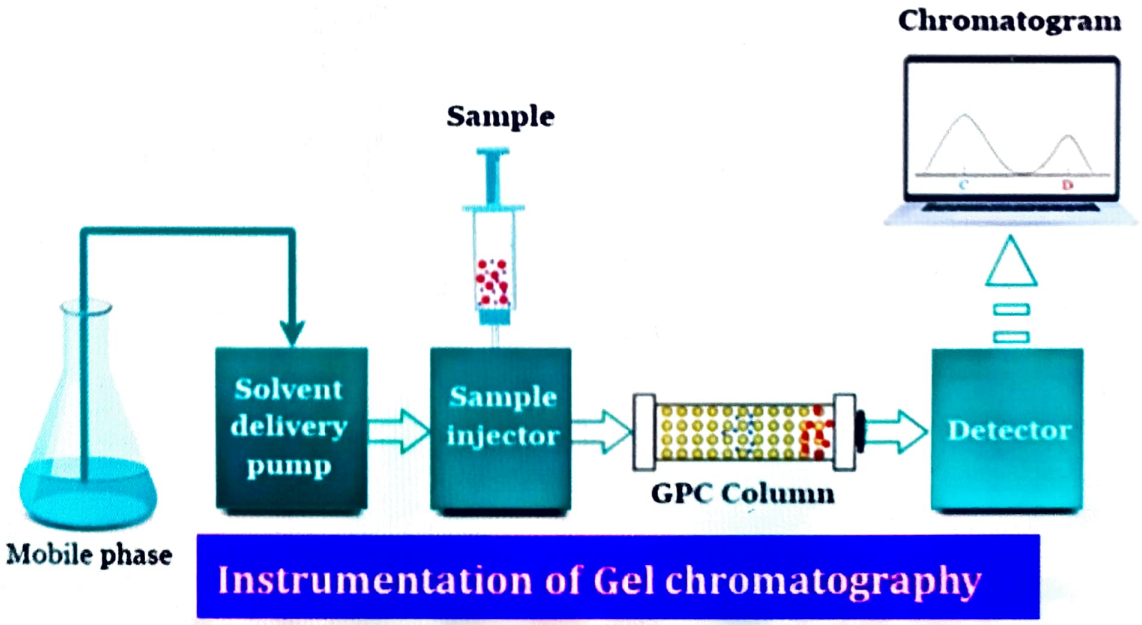
- If conditions are assumed such that time taken for solute molecules to diffuse into pore is less as compared to time spent by molecule near pore and Separation process independent of diffusion process then under these conditions:

$$V_e = V_o + K_d \cdot V_l$$

- V_e = Volume of effluent flowing through column between point of sample injection & sample emergence from column
- K_d = Distribution coefficient
- For large molecules: $K_d = 0$, $V_e = V_o$,
- For molecules that can penetrate all the pores: $Kd = 1$, $V_e = V_o + V_l$

❖ INSTRUMENTATION

- The important **practical requirements for gel chromatography are as follows:**



Instrumentation of Gel chromatography

1. Stationary Phase:

- Stationary Phase is **Semi-permeable, porous beads** with well defined range of pore sizes.
- **Beads are cross-linked polymers.**
- Smaller pore sizes are used for rapid desalting of proteins or for protein purification. Intermediate pore sizes are used to separate relatively small proteins. **Very large pore sizes are used for purification of biological complexes.**
- **Stationary phase used for gel exclusion chromatography include dextran, polyacrylamide and dextran poly acrylamide.**

✓ **Properties of gel beads:**

a) They should be **chemically inert**

b) They should be **mechanically stable**

c) They should have ideal and homogeneous porous structure (wide pore size give low resolution).

d) They should have **uniform particle and pore size.**

e) The pore size of **the gel must be carefully controlled.**

2. Mobile phase

- It composed of **a liquid used to dissolve the biomolecules to make the mobile** phase permitting high detection response and wet the packing surface.
- The choice of mobile phase to **be used in any separation will depend on the type of separation to be achieved and component to be separated.**
- The most common eluents in for polymers that dissolve at **room temperature. e.g. Tetrahydrofuran, Chloroform, Dimethyl formamide.**

3. Column

- The column consist of a straight tube with a **bed support at the bottom.**
- The bed support allows only the liquid to pass through **without disturbing the bed material.**
- The simplest column consist of a straight tube containing some glass wool into the bottom. **The glass wool is then covered with thin layer of quartz**

- or glass beds.

✓ **Commercially Available Columns**

- Analytical column- **7.5–8mm diameters.**
- Preparative **columns-22–25mm for.**
- Usual column **lengths-25, 30, 50, and 60 cm.**
- Recently, **narrow bore columns- 2–3mm diameter** have been introduced, which save time and solve.

4. Pump

- A **highly constant flow rate has to be maintained during the entire chromatogram.** A change of the flow rate of only 0.1% can cause an error in molar mass of up to 10%.
- Most pumps can only reproduce the **flow rate to 0.2–0.3%. In-line filters in the solvent reservoir may prevent particles** from coming into the pump heads, which might damage the check valves or the pump seals.
- **Various pump used are : Syringe pumps, Reciprocating pumps.**

5. Detectors:

- Various type of detector used are as follows

A. Concentration sensitive detectors

- **Bulk Property Detectors-** Refractive Index (RI) Detector
- **Solute Property Detectors-** Ultraviolet (UV) Absorption Detector
- **Evaporative Detectors-** Evaporative Light Scattering Detector (ELSD)

B. Molar mass sensitive detectors

a) Light Scattering Detectors

- Low Angle Light Scattering (LALS) Detectors
- Multi-angle Light Scattering (MALS) detectors

b) Viscosity Detectors- Differential Viscometers

- Other :- Flame Ionization Detector (FID), **A Mass Spectrometer or Fourier Transform Infrared (FTIR) Spectrometer**

(FTIR) Spectrometer

❖ APPLICATION

- **Main application are as follows:**

1. **Purification:** The main application of gel chromatography is the **fractionation & purification of biological macromolecule.** i.e. protein, polysaccharide, nucleic acid etc.
2. **Molecular weight determination:** When a polymer sample of mixed molecular weight flows down the column there will be a **separation of the solute according to their molecular weight.** The chromatogram results from a separation based on the size of the sample molecule. It represents distinct molecular weight distribution.
3. **Protein-building studies:** It is used to study the **reversible binding of a ligand to a macromolecule such as protein including receptor protein.**
4. **Solution concentration:** Solution of **high molecular weight substances can be concentrated by addition of dry Sephadex G-25.**
5. **Desalting:** By use of a column of, **Sephadex G-25, solutions of high molecular weight compound may be desalted.** The separation of large molecule of biological origin from inorganic & ionisable species is termed as desalting

UNIT-V

AFFINITY CHROMATOGRAPHY

Points to be covered in this topic

- ❖ Introduction
- ❖ Theory
- ❖ Instrumentation
- ❖ Applications

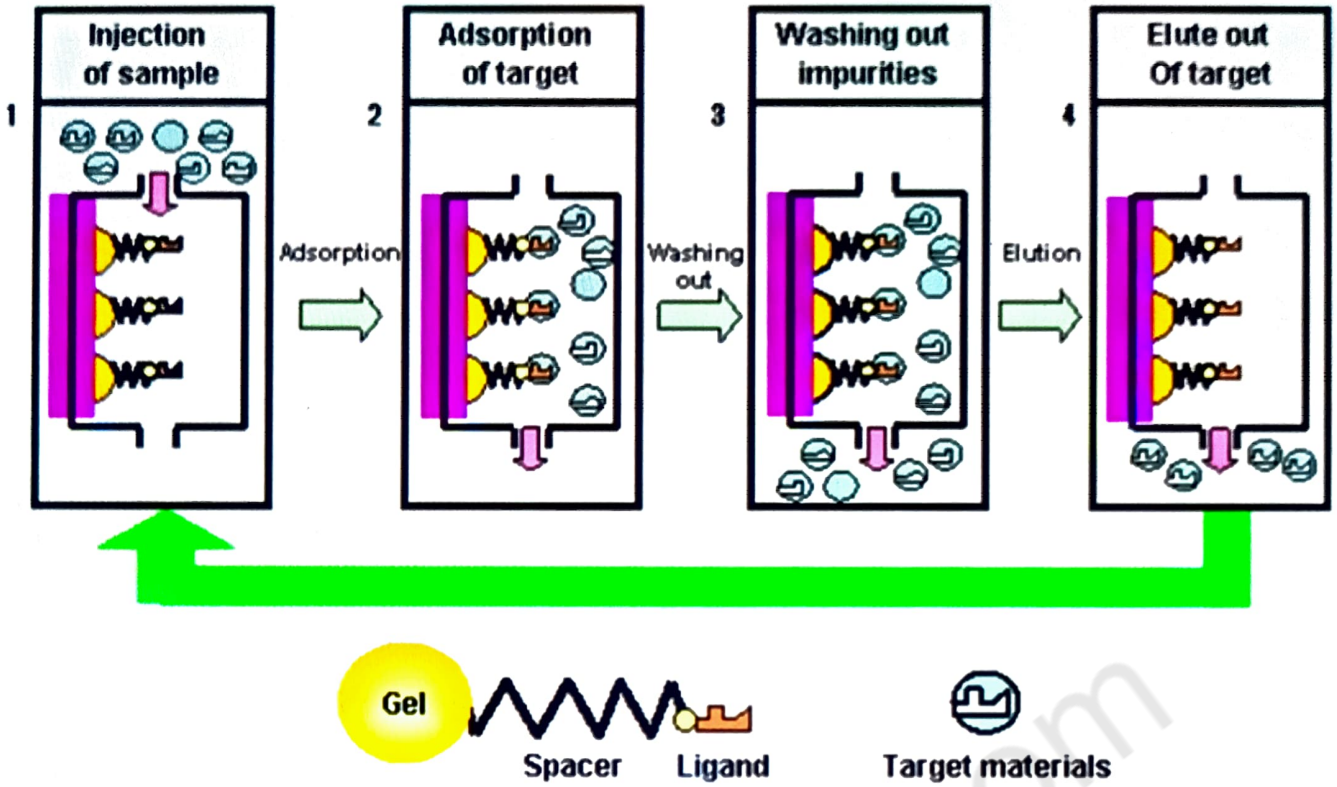
AFFINITY CHROMATOGRAPHY

❖ INTRODUCTION

- Affinity chromatography is a method of **separating biochemical mixtures based on a highly specific interaction such** as that **between antigen and antibody, enzyme and substrate or receptor and ligand.**
- Affinity chromatography, also known as **bioselective adsorption, is a protein purification technique.** It is widely used as a **means of separation and purification with specific properties.**
- Biological macromolecules, such as **enzymes and other proteins, interact with other molecules with high specificity through several different types of bonds and interaction.** Such interactions include hydrogen bonding, ionic interaction, disulfide bridges, hydrophobic interaction, and more.
- The high selectivity of affinity chromatography is caused by allowing the desired molecule to interact with the stationary phase and be bound within the **column in order to be separated from the undesired material** which will not interact and elute first.
- The molecules **no longer needed are first washed away with a buffer while** the desired proteins are let go in the presence of the eluting solvent (of higher salt concentration).

❖ PRINCIPLE

- **The principle of affinity chromatography is as follows:**
- The stationary phase is **first loaded into a column with mobile phase containing a variety of biomolecules from DNA to proteins** (depending on the purification experiment).
- Then, the **two phases are allowed to bind.**
- A **wash buffer is then poured** through a column containing **both bound phases.**
- The **wash buffer removes non-target biomolecules** by disrupting their weaker interactions with the stationary phase.



- Target biomolecules have a much higher affinity for the **stationary phase, and remain bound to the stationary phase,** not being **washed away by wash buffer.**
- An **elution buffer is then poured** through the column containing the remaining target biomolecules.
- The elution buffer **disrupts interactions between the bound target biomolecules with the stationary to a much greater** extent than the wash buffer, effectively removing the target biomolecules.
- This purified solution contains elution **buffer and target biomolecules, and is called elution.**

❖ INSTRUMENTATION

- The important practical requirements for affinity chromatography are as follows:

1. Matrix

- The matrix is an inert support to which a **ligand can be directly or indirectly coupled.**
- The most useful **matrix materials are agarose and polyacrylamide.**
- **The matrix to be effective it must have certain characters which** are as

follows:

a) Matrix should be **chemically and physically inert.**

b) It must be **insoluble in solvents and buffers employed** in the process.

c) It must be **chemically and mechanically stable.**

d) It must be easily coupled to **a ligand or spacer arm onto which the ligand can be attached.**

e) It must exhibit **good flow properties and have a relatively large surface area** for attachment.

2. Ligand

- It refers to the molecule that **binds reversibly to a specific target molecule.**
- The ligand can be selected **only after the nature of the macromolecule to be isolated is known.**
- When a hormone receptor protein is to be purified by affinity chromatography, the **hormone itself is an ideal candidate for the ligand.**
- For antibody isolation, an **antigen or hapten may be used as ligand.**
- IF an enzyme is to be purified, a substrate analog, **inhibitor, cofactor, or effector may be used as a the immobilized ligand.**

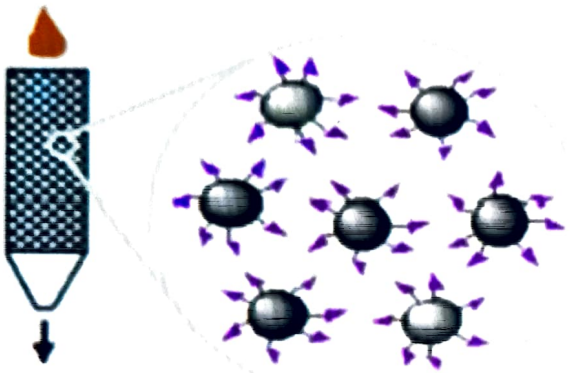
3. Solvents

- The primary buffer in affinity chromatography is the one in which the matrix resides. This buffer should **not degrade the matrix in any way.** The buffer should also have a **reliable effect on the sample.** The ideal buffer minimizes nonspecific interactions while maximizing the **specific interaction between the sample and the ligand.**
- The other major solvent to consider in **affinity chromatography is the elution buffer.**
- The purpose of the elution buffer is to wash away unbound proteins initially and at **higher concentration release the desired protein from the ligand.**

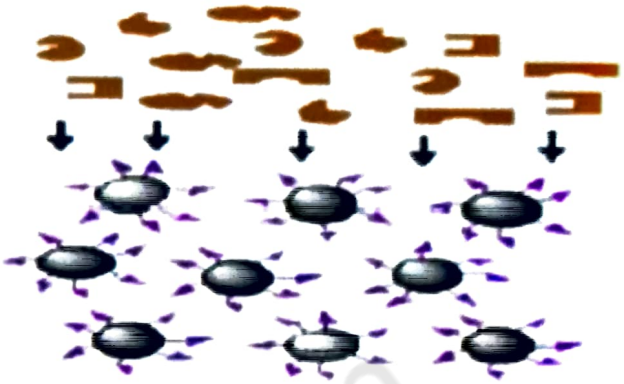
4. Spacer arms

- It is used to improve binding **between ligand and target molecule by overcoming any effects of steric hindrance.**

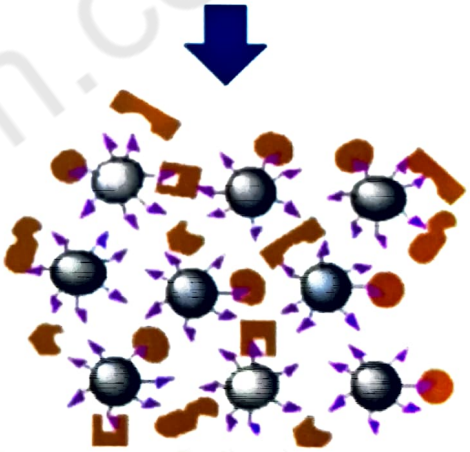
- Since the success of affinity chromatography resides in its ability to bind an active site to its corresponding ligand, if the **protein binding region cannot join with the immobilized ligand the technique is effectively useless.**
- **Steps involving in affinity chromatography**



1. Loading of affinity column

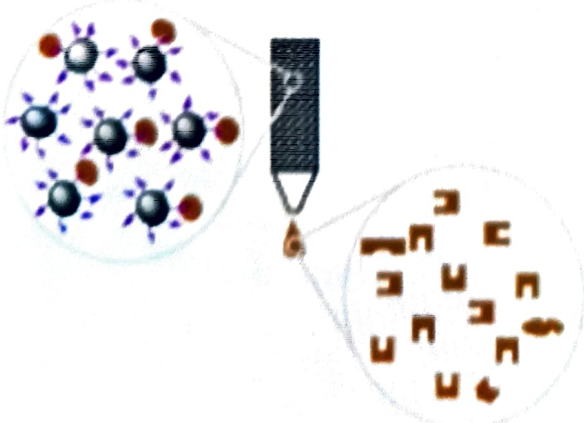


2. Proteins sieve through matrix of affinity beads

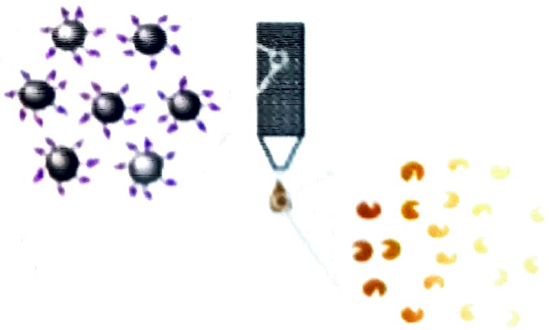


3. Proteins interact with affinity ligand with some binding loosely and others tightly

4. Wash off proteins that do not bind.



5. Wash off proteins that bind loosely



6. Elute proteins that bind tightly to ligand and Collect purified protein of interest

❖ APPLICATIONS

1. Protein purifying

- Affinity chromatography has a **large range of protein purifying applications.** Extra cellular and other receptor proteins can also be purified by affinity chromatography.
- It allows **protein purification in a relatively short amount** of time with a high yield.

2. Isolation of enzyme

- Enzymes can be isolated by a **host at different ligands fit for bioselective adsorption.** For example, adenosine monophosphate (AMP) can be immobilized and **used to bind those proteins exhibiting an affinity for AMP, ADP or ATP.**

3. Immobilized Metal Affinity Chromatography In Proteomics

- It has been proved that the progress of proteomics is mostly determined by the **development of advanced and sensitive protein separation technologies**.
- Immobilized metal affinity chromatography (IMAC) is a **powerful protein fractionation method** used to enrich metal-associated proteins and peptides

4. Affinity Chromatography To The Study Of Drug–Melanin Binding Interactions

- Affinity chromatography using chromatographic stationary phases based on **physically adsorbed or chemically bonded melanin provides a useful tool** for studying the interactions of small molecules and metal ions with melanin

5. Purification of lectins by Biospecific affinity chromatography

- Biospecific adsorbents which can be used for the purification of lectins are easily prepared by a one-step reaction between **Epoxy-activated Sepharose 6 B and Lectin-specific sugars.**

6. Purification of plasma proteins for therapeutic use

- Affinity chromatography is a powerful technique for the purification of many proteins in human plasma. It is being used in the production of various licensed therapeutic plasma products, such as : **Factor VIII, Factor IX, Von Will brand Factor, Protein C, Antithrombin III, and Factor XI.**