6Th Semester Theory

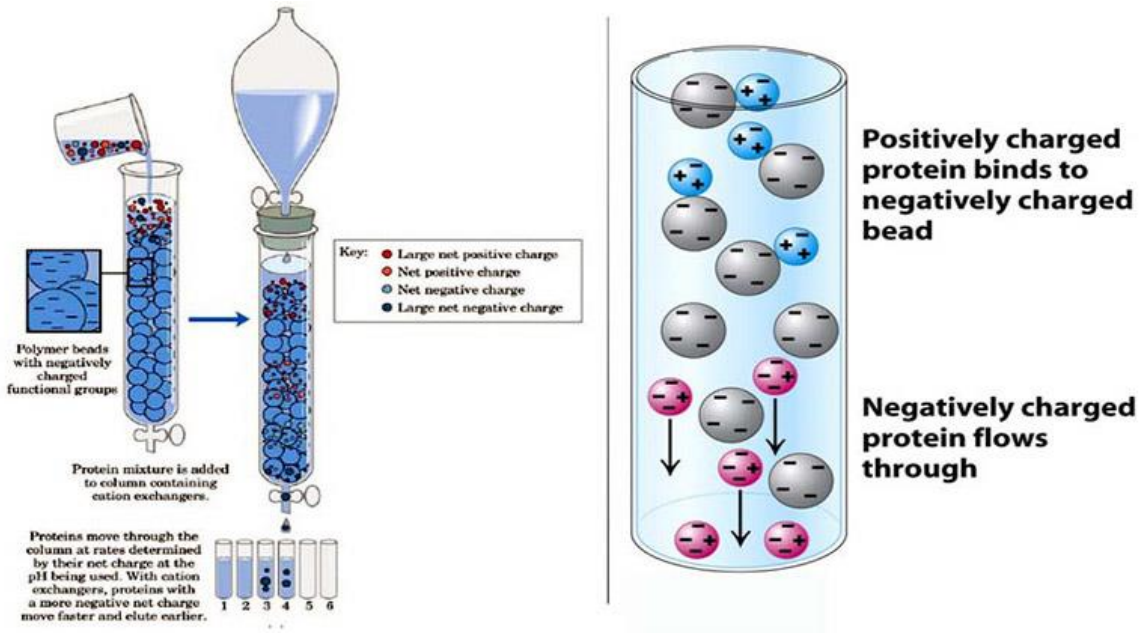
DSE4T : Research Methodology and Design
Tools techniques for research in Physiology and Health Science :

Ion Exchange Chromatography

- Chromatography is the separation of a mixture of compounds into its individual components based on their relative interactions with an inert matrix.
- Ion exchange chromatography (or ion chromatography) is a process that allows the separation of ions and polar molecules based on their affinity to ion exchangers.
- The principle of separation is thus by reversible exchange of ions between the target ions present in the sample solution to the ions present on ion exchangers.
- In this process two types of exchangers i.e., cationic and anionic exchangers can be used.

1. **Cationic exchangers** possess negatively charged group, and these will attract positively charged cations. These exchangers are also called "Acidic ion exchange" materials, because their negative charges result from the ionization of acidic group.
2. **Anionic exchangers** have positively charged groups that will attract negatively charged anions. These are also called "Basic ion exchange" materials.

- Ion exchange chromatography is most often performed in the form of column chromatography. However, there are also thin-layer chromatographic methods that work basically based on the principle of ion exchange.



Working Principle of ion exchange chromatography

This form of chromatography relies on the attraction between oppositely charged stationary phase, known as an ion exchanger, and analyte.

- The ion exchangers basically contain charged groups covalently linked to the surface of an insoluble matrix.
- The charged groups of the matrix can be positively or negatively charged.
- When suspended in an aqueous solution, the charged groups of the matrix will be surrounded by ions of the opposite charge.
- In this “ion cloud”, ions can be reversibly exchanged without changing the nature and the properties of the matrix.

Instrumentation of ion exchange chromatography

Typical IC instrumentation includes: pump, injector, column, suppressor, detector and recorder or data system.

1. **Pump**

The IC pump is considered to be one of the most important components in the system which has to provide a continuous constant flow of the eluent through the IC injector, column, and detector.

2. **Injector**

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. Liquid samples may be injected directly and solid samples need only to be dissolved in an appropriate solvent. Injectors should provide the possibility of injecting the liquid sample within the range of 0.1 to 100 ml of volume with high reproducibility and under high pressure (up to the 4000 psi).

3. **Columns**

Depending on its ultimate use and area of application, the column material may be stainless steel, titanium, glass or an inert plastic such as PEEK. The column can vary in diameter from about 2mm to 5 cm and in length from 3 cm to 50 cm depending on whether it is to be used for normal analytical purposes, microanalysis, high speed analyses or preparative work.

Guard column is placed anterior to the separating column. This serves as a protective factor that prolongs the life and usefulness of the separation column. They are dependable columns designed to filter or remove particles that clog the separation column

4. **Suppressor**

The suppressor reduces the background conductivity of the chemicals used to elute samples from the ion-exchange column which improves the conductivity measurement of the ions being tested. IC suppressors are membrane-based devices

which are designed to convert the ionic eluent to water as a means of enhancing the sensitivity.

5. **Detectors**

Electrical conductivity detector is commonly use.

6. **Data system**

In routine analysis, where no automation is needed, a pre-programmed computing integrator may be sufficient. For higher control levels, a more intelligent device is necessary, such as a data station or minicomputer.

Procedure of ion exchange chromatography

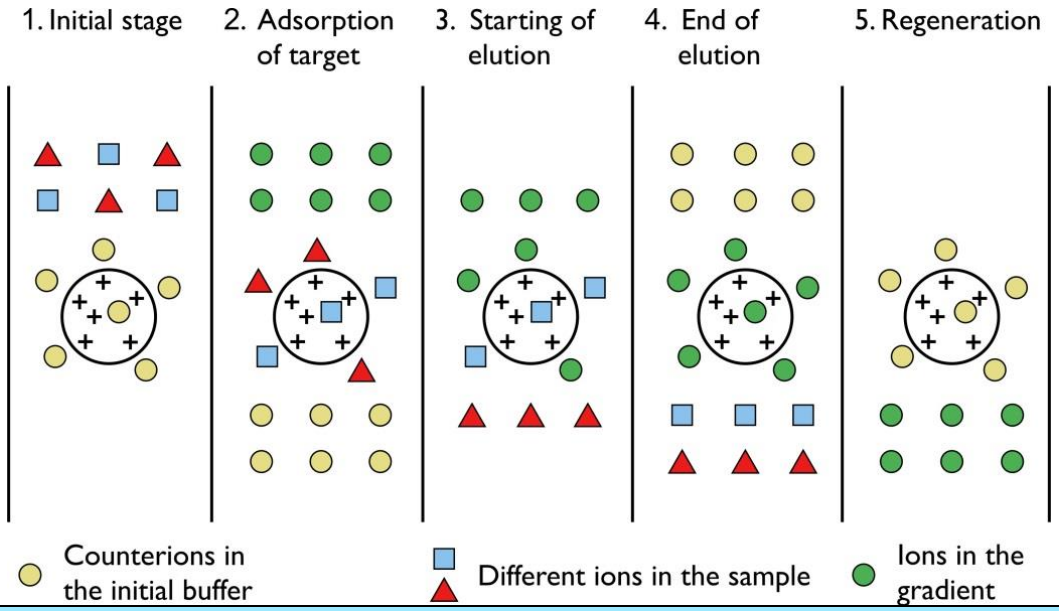
- Ion exchange separations are carried out mainly in columns packed with an ion-exchanger.
- These ionic exchangers are commercially available. They are made up of styrene and divinyl benzene. Example. DEAE-cellulose is an anionic exchanger, CM-cellulose is a cationic exchanger.
- The choice of the exchanger depends upon the charge of particle to be separated. To separate anions “Anionic exchanger” is used, to separate cations “Cationic exchanger” is used.
- First the column is filled with ion exchanger then the sample is applied followed by the buffer. The tris-buffer, pyridine buffer, acetate buffer, citrate and phosphate buffers are widely used.
- The particles which have high affinity for ion exchanger will come down the column along with buffers.
- In next step using corresponding buffer separates the tightly bound particles.
- Then these particles are analyzed spectroscopically.

Applications of ion exchange chromatography

- An important use of ion-exchange chromatography is in the routine analysis of **amino acid** mixtures.
- The 20 principal amino acids from blood serum or from the hydrolysis of proteins are separated and used in clinical diagnosis.
- This is most effective method for water purification. Complete deionization of water (or) a non-electrolyte solution is performed by exchanging solute cations for hydrogen ions and solute anions for hydroxyl ions. This is usually achieved by method is used for softening of drinking water.
- In the analysis of products of hydrolysis of nucleic acids. In this way, information is gained about the structure of these molecules and how it relates to their biological function as carriers of hereditary information.
- Chelating resins are used to collect trace metals from seawater.
- To analyze lunar rocks and rare trace elements on Earth.

Advantages of ion exchange chromatography

1. t is one of the most efficient methods for the separation of charged particles.
2. It can be used for almost any kind of charged molecule including large proteins, small nucleotides and amino acids.
3. Ion exchange is used for both analytical and preparative purposes in the laboratory, the analytical uses being the more common.
4. Inorganic ions also can be separated by ion-exchange chromatography.

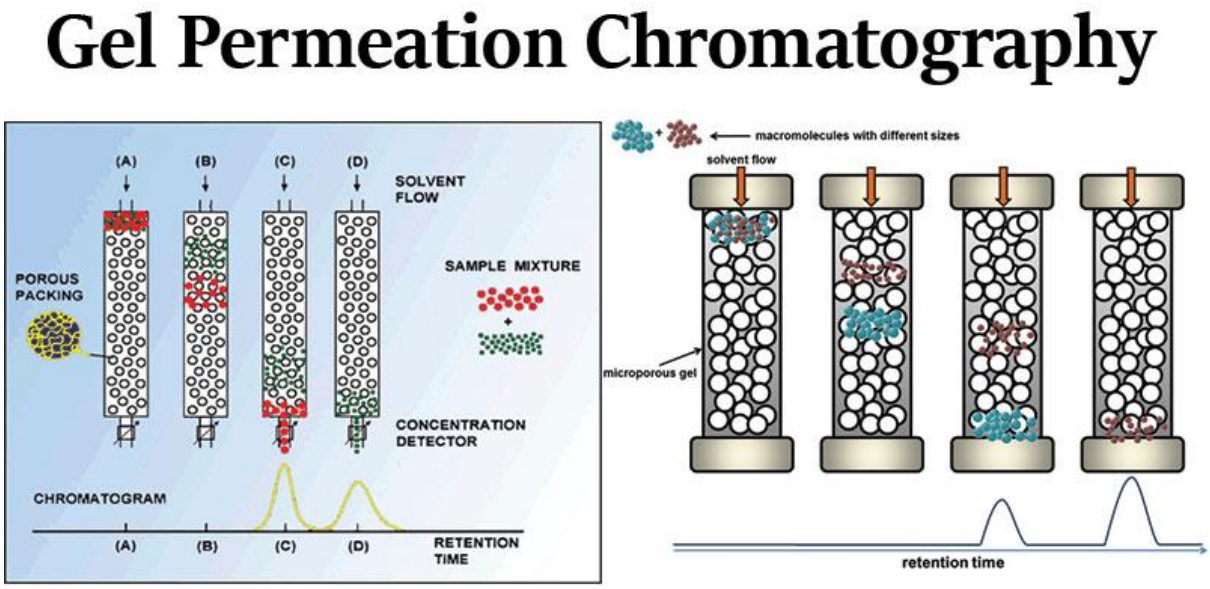


Limitations of ion exchange chromatography

- Only charged molecules can be separated.
- Buffer Requirement

Gel Permeation Chromatography

- Gel permeation chromatography is also called as gel filtration or size exclusion chromatography.
- In size exclusion chromatography, the stationary phase is a porous matrix made up of compounds like cross-linked polystyrene, cross-like dextrans, polyacrylamide gels, agarose gels, etc.
- The separation is based on the analyte molecular sizes since the gel behaves like a molecular sieve.
- This technique is used for the separation of proteins, polysaccharides, enzymes, and synthetic polymers.
- As a technique, size exclusion chromatography was first developed in 1955 by Lathe and Ruthven.



Principle of Gel Permeation Chromatography

- It is a technique in which the separation of components is based on the difference in molecular weight or size.
- The stationary phase used is a porous polymer matrix whose pores are completely filled with the solvent to be used as the mobile phase.
- The molecules in the sample are pumped through specialized columns containing such microporous packing material (gel).
- The basis of the separation is that molecules above a certain size are totally excluded from the pores, while smaller molecules access the interior of the pores partly or wholly.

- The flow of the mobile phase hence will cause larger molecules to pass through the column unhindered, without penetrating the gel matrix, whereas smaller molecules will be retarded according to their penetration of the gel.

Components/ Instrumentation of Gel Permeation Chromatography

1. Stationary Phase
2. The Mobile Phase
3. The Columns
4. The Pump
5. Detectors

A. Stationary phase

It is composed of semi-permeable, porous polymer gel beads with a well-defined range of pore sizes.

It has the following properties:

- Chemically inert
- Mechanically stable
- With ideal and homogeneous porous structure (wide pore size give low resolution).
- A uniform particle and pore size.

Examples of gel:

1. **Dextran** (Sephadex) gel: An α 1-6-polymer of glucose natural gel
2. **Agarose** gel: A 1,3 linked β-D-galactose and 1,4 linked 3,6-anhydro-α, L-galactose natural gel
3. **Acrylamide** gel: A polymerized acrylamide, a synthetic gel

B. The Mobile Phase

It is composed of a liquid used to dissolve the bio-molecules to make the mobile phase permitting high detection response and wet the packing surface.

C. Columns

Any of the following kinds may be used:

- Analytical column- 7.5–8mm diameters.
- Preparative columns-22–25mm
- Usual column lengths-25, 30, 50, and 60 cm.
- Narrow-bore columns- 2–3mm diameter have been introduced

D. Pumps

They are either syringe pumps or reciprocating pumps with a high constant flow rate.

E. Detectors

The detectors may be concentration sensitive detectors, bulk property detectors, refractive index (RI) detector, etc.

Steps in Gel Permeation Chromatography

It involves three major steps:

A. Preparation of column for gel filtration

It involves:

1. Swelling of the gel
2. Packing the column semi-permeable, porous polymer gel beads with a well-defined range of pore sizes.
3. Washing: After packing, several column volumes of buffer solution is passed through the column to remove any air bubbles and to test the column homogeneity.

B. Loading the sample onto the column using a syringe

C. Eluting the sample and detection of components

Applications of Gel Permeation Chromatography

1. Proteins fractionation
2. Purification
3. Molecular weight determination.
4. Separation of sugar, proteins, peptides, rubbers, and others on the basis of their size.
5. Can be used to determine the quaternary structure of purified proteins.

Advantages of Gel Permeation Chromatography

- Short analysis time.
- Well defined separation.
- Narrow bands and good sensitivity.
- There is no sample loss.
- The small amount of mobile phase required.
- The flow rate can be set.

Limitations of Gel Permeation Chromatography

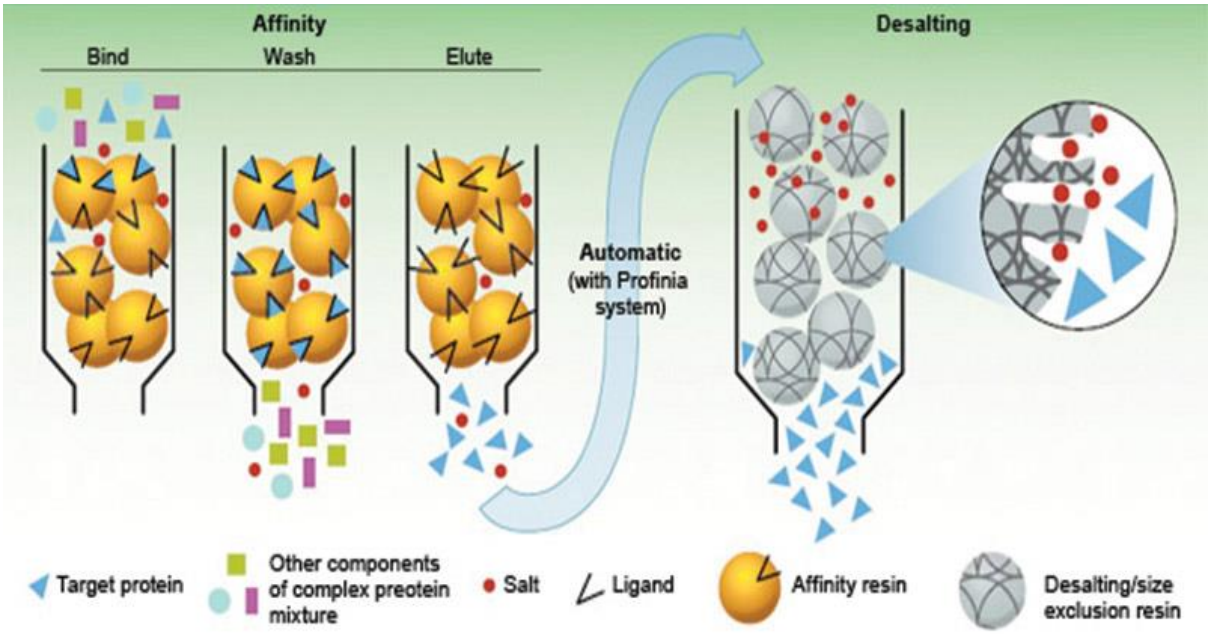
- The limited number of peaks that can be resolved within the short time scale of the GPC run.
- Filtrations must be performed before using the instrument to prevent dust and other particulates from ruining the columns and interfering with the detectors.
- The molecular masses of most of the chains will be too close for the GPC separation to show anything more than broad peaks.

Affinity Chromatography

- Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.
- It is a separation technique in which a mobile phase carrying a mixture is caused to move in contact with a selectively absorbent stationary phase.
- Affinity chromatography is a type of liquid chromatography for the separation, purification or specific analysis of sample components.
- It utilizes the reversible biological interaction or molecular recognition called affinity which refers to the attracting forced exerted in different degrees between atoms which cause them to remain in combination.

Example: Enzyme with and inhibitor, antigen with an antibody etc.

- It was discovered by Pedro Cuatrecasas and Meir Wilcheck.



Principle of Affinity Chromatography

- The stationary phase consists of a support medium, on which the substrate (ligand) is bound covalently, in such a way that the reactive groups that are essential for binding of the target molecule are exposed.
- As the crude mixture of the substances is passed through the chromatography column, substances with binding site for the immobilized substrate bind to the stationary phase, while all other substances is eluted in the void volume of the column.

- Once the other substances are eluted, the bound target molecules can be eluted by methods such as including a competing ligand in the mobile phase or changing the pH, ionic strength or polarity conditions.

Components of Affinity Chromatography

1. **Matrix**

- The matrix is an inert support to which a ligand can be directly or indirectly coupled.
- In order to for the matrix to be effective it must have certain characters:
- Matrix should be chemically and physically inert.
- It must be insoluble in solvents and buffers employed in the process
- It must be chemically and mechanically stable.
- It must be easily coupled to a ligand or spacer arm onto which the ligand can be attached.
- It must exhibit good flow properties and have a relatively large surface area for attachment.
- The most useful matrix materials are agarose and polyacrylamide.

2. **Spacer arm**

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

3. **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.

Steps in Affinity Chromatography

- Affinity medium is equilibrated in binding buffer.
- Sample is applied under conditions that favor specific binding of the target molecule(s) to a complementary binding substance (the ligand). Target substances bind specifically, but reversibly, to the ligand and unbound material washes through the column.
- Elution is performed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Target protein is collected in a purified, concentrated form.
- Affinity medium is re-equilibrated with binding buffer.

These events can be summarized into the following three major steps:

1. **Preparation of Column**

- The column is loaded with solid support such as sepharose, agarose, cellulose etc.
- Ligand is selected according to the desired isolate.
- Spacer arm is attached between the ligand and solid support.

2. **Loading of Sample**

- Solution containing a mixture of substances is poured into the elution column and allowed to run at a controlled rate.

3. **Elution of Ligand-Molecule Complex**

- Target substance is recovered by changing conditions to favor elution of the bound molecules.

Applications of Affinity Chromatography

- Affinity chromatography is one of the most useful methods for the separation and purification of specific products.
- It is essentially a sample purification technique, used primarily for biological molecules such as proteins.

Its major application includes:

- Separation of mixture of compounds.
- Removal of impurities or in purification process.
- In enzyme assays
- Detection of substrates
- Investigation of binding sites of enzymes
- In in vitro antigen-antibody reactions
- Detection of Single Nuceotide polymorphisms and mutations in nucleic acids

Advantages of Affinity Chromatography

- High specificity
- Target molecules can be obtained in a highly pure state
- Single step purification
- The matrix can be reused rapidly.
- The matrix is a solid, can be easily washed and dried.
- Give purified product with high yield.
- Affinity chromatography can also be used to remove specific contaminants, such as proteases.

Limitations of Affinity Chromatography

- Time consuming method.
- More amounts of solvents are required which may be expensive.
- Intense labour

- Non-specific adsorption cannot be totally eliminated, it can only be minimized.
- Limited availability and high cost of immobilized ligands.
- Proteins get denatured if required pH is not adjusted.