Swayam Course - Analytical Techniques

Week: 4, Module 9 - Ion-exchange Chromatography

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1. Learning outcomes

- Principle of ion-exchange chromatography
- Cation-exchange and anion-exchange media
- Column packing and process of ion-exchange chromatography
- Factors affecting ion-exchange chromatography
- Applications and precautions

2. Introduction

Ion exchange chromatography involves the separation of polar molecules and ions based on their charge. This separation technique can be used to purify charged molecules, ranging from small nucleotides to macromolecules, such as proteins. The stationary phase has ionic functional groups which interact with the analytes having opposite charge. Based on the charge of the analyte to be exchanged, ion-exchange chromatography is divided as - cation-exchange, used when analyte is positively charged (cation), and anion-exchange used when analyte is negatively charged (anion). The stationary phase of cation exchange chromatography contains negatively charged functional groups which retain positively charged analytes, while the stationary phase of anion exchange contains positively charged functional groups which retain negatively charged analytes. Ion exchange chromatography is a valuable method because of its mild separation conditions, low cost, high capacity, versatility, high resolving power, and wide range applicability.

In 1850, Thompson utilized ion-exchange chromatography to investigate adsorption of ammonium ions by the soil. However, the first practical description was given by Spedding and Powell in 1947. They gave a thorough description of the preparative resolution of rare earth metals by displacement ion-exchange chromatography. The method was further developed by Kraus and Nelson. They described several techniques for the analysis of ions such as fluoride, chloride, complexes involving nitrate or sulfate species etc. by anion-exchange chromatography. This was done in the initial part of the 6^{th} decade of the 19^{th} century. Ion exchange separation method for proteins was described by Peterson and Sober for the first time in 1956. But it was in 1975, that the present form of ion exchange chromatography got initiated. This was done by Small, Stevens and Bauman.

3. Principle

Ion-exchange chromatography resolves analytes based on of their charged groups. The stationary phase is a non-movable resin which has charged and ionizable functional groups or ligands. The charged groups of the analyte are attracted to the oppositely charged resin due to coulombic (ionic) interactions i.e. opposite charges attract each other. Analyte molecules are electrostatically attracted towards the oppositely charged resin and this leads to binding between them. Ion exchange chromatography is divided as - cation-exchange and anion-exchange chromatography. In cation-exchange chromatography, positively charged analytes get attached to a negatively charged resin. Whereas, considering anion exchange chromatography, the opposite happens i.e. negatively charged analytes get attached to a positively

charged resin. The analyte is charged because the pH used for chromatography is above (leading to negative charge) or below (leading to positive charge) the pI of the analyte. Bound analytes can then be eluted by escalating the ionic strength or changing the pH of the buffer being used for elution. The molecules that bind feebly to the stationary phase are eluted first.
Cation exchange resin binds positively charged cations as the stationary phase has negatively charged functional group:

$$E\text{-}F^{-}\,C^{+} + M^{+}\,N^{-} \rightleftarrows E\text{-}F^{-}\,M^{+} + C^{+} + N^{-}$$

Where, E is the exchanger having functional group F^{-} (fixed anion) C^{+} (replaceable cation) and the analyte is $M^{+}\,N^{-}$

Anion exchange resin binds negatively charged anions as the stationary phase has negatively charged functional group:

$$E\text{-}F^{+}\,A^{-} + M^{+}\,N^{-} \rightleftarrows E\text{-}F^{+}\,N^{-} + A^{-} + M^{+}$$

Where, E is the exchanger having functional group F^{+} (fixed cation) A^{-} (replaceable anion) and the analyte is $M^{+}\,N^{-}$

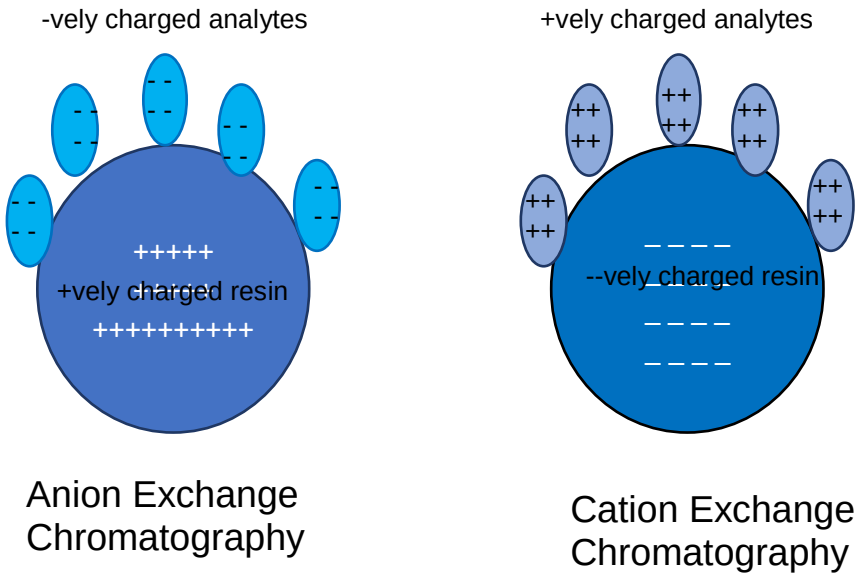

Figure: Classification of ion-exchange chromatography

4. Theory

Donnan Equilibrium
It is the equilibrium which exists among the charged species which are in solution outside and the charged species which are inside the exchanger.

Suppose an anion-exchange resin has the form $A^{+}B^{-}$. This is placed in an electrolyte $E^{+}B^{-}$. In accordance with the laws of thermodynamics, ion product inside = ion product outside the exchanger.

$$[E]_i[B]_i=[E]_o[B]_o$$

Where:
$[E]_i$ is concentration of E inside the resin
$[B]_i$ is concentration of B inside the resin
$[E]_o$ is concentration of E outside the resin
$[B]_o$ is concentration of B outside the resin

As electrolyte is electrically neutral and charge is balanced

$$[E]_o=[B]_o$$

Inside the resin, 3 differernt charged molecules exist. As the resin is also electrically neutral and charge is balanced

$$[A]_i+[E]_i=[B]_i$$

Where:
$[A]_i$ is concentration of A inside the resin

Substituting these into the initial equation

$$[E]_i([A]_i+[E]_i) = [E]_o*[E]_o$$

Hence, $[E]_o>[E]_i$.

Therefore, when kept inside an electrolyte, an ion exchanger will have more electrolyte outside it as compared to inside it. This is because the ions having similar charge as the resin are extruded i.e. A^+ extrudes C^+. The anion, B^-, is not extruded. Electrostatic barrier to the diffusion of the counterion inside the resin is absent. Anions enter freely in an anionic resin but cations are repelled from the resin.
This is what ion-exclusion chromatography is based on. As dilute electrolytes are extruded from the resin, they travel through the column more rapidly as compared to nonelectrolytes which can diffuse into the resin. For example, if KCl and a sugar solution is resolved in an ion-exchange column, KCl elutes earlier.

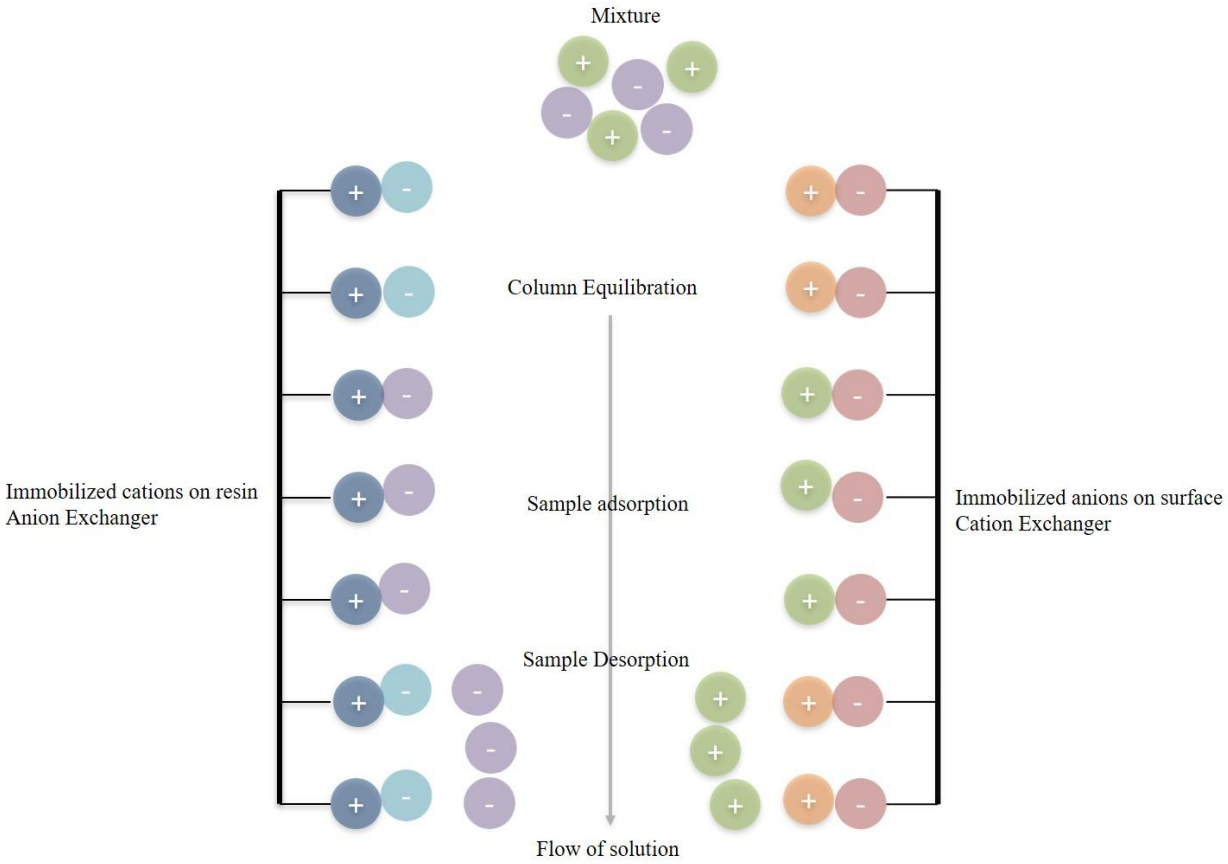

Figure: Ion exchange chromatography

5. Ion-exchange media

It is a non-soluble matrix with covalently attached functional groups which can get charged. Cation-exchange resins are negatively charged exchangers, which get attached to positively charged ions (cations). On the other hand, anion-exchange resins are positively charged, which get attached to negatively charged ions (anions). There are many cation-exchange and anion-exchange media available commercially for the purification of charged analytes. Ion exchange resins can be divided into strong ion exchange resins and weak ion exchange resins. A strong ion-exchange resin is charged fully over a broad range of pH, while a weak ion-exchange resin is ionized over a limited pH range. The charged functional groups may be crosslinked to polystyrene, sephadex, sepharose, cellulose or polyacrylic beads. Commonly used ion-exchange functional groups-

S. No.	Type of exchanger	Functional group	Commercially available media
1.	Strong cation-exchangers	Sulfonic acid (S)	RESOURCE™ S, Macro-Prep® High S, Macro-Prep® 25 S
2.	Weak cation-exchangers	Carboxymethyl (CM)	CM sephadex C-50, C-25, Macro-Prep® CM
3.	Strong anion-exchangers	Quarternary Ammonium (Q)	RESOURCE™ Q, Macro-Prep® High Q, Macro-Prep® 25 Q
4.	Weak anion-exchangers	Diethylaminoethyl (DEAE)	DEAE sephacel A-50, DEAE cellulose, Macro-Prep® DEAE

Table: Some commonly used Ion-exchange media

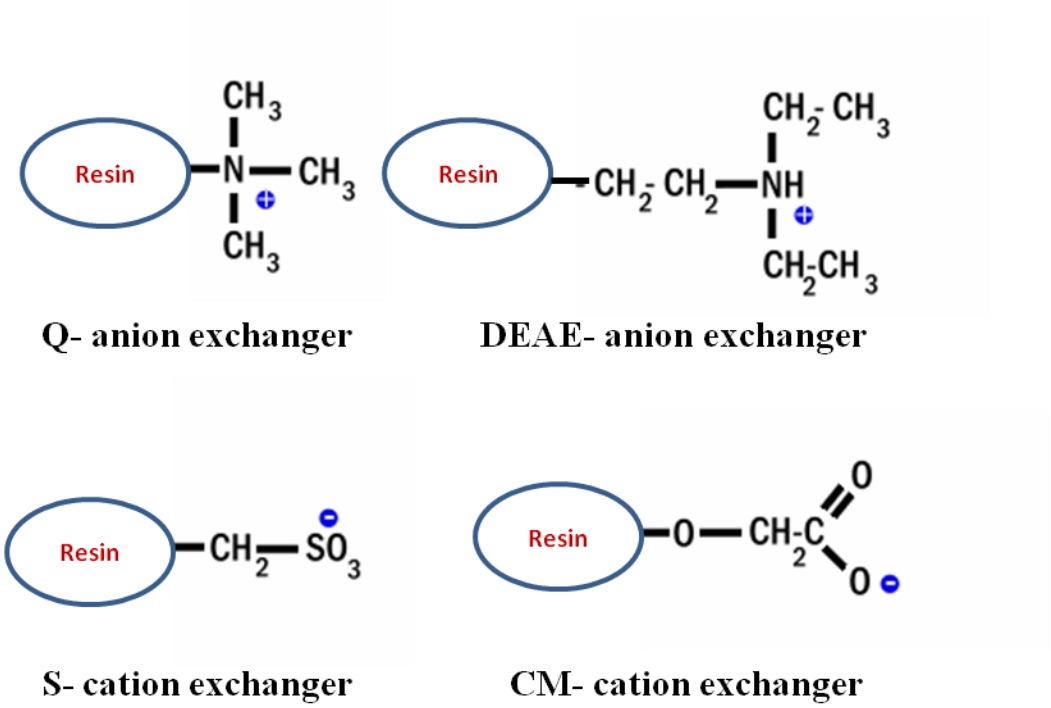

Figure: Chemical structures of common ion-exchange media

- The selection of the ion exchanger is determined by the analytes and their requirements of the separation.
- Many analytes, specially proteins, are stable within a fixed pH range.
- Hence, the resin used must function in this range.
- In general, if an analyte is stable below its pI (net positive charge) a cation exchanger is to be utilized, while if it is stable at a pH greater than its pI (net negative charge) an anion exchanger should be used.
- Based on the stability of the protein or other molecule which needs to be separated and the effect of pH on its charge, a strong or weak resin can be used.
- Weak electrolytes requiring extreme pH for ionisation can be resolved only on strong exchangers, as they only function over a broad pH range.
- On the other hand, for strong electrolytes, weak exchangers are better as risk of protein denaturation is lower.
- They also don't bind to weakly charged impurities.

- Generally, cationic buffers are used with anion exchange resins, and anionic buffers are used with cation exchange resins.

6. Packing of a column

Swelling the gel

- Add appropriate amount of filtered ddH_2O to the gel matrix depending on the swelling capacity of the beads as per the manufacturer's recommendations.
- Once the gel has swollen and settled down in the beaker, decant the ddH_2O on the top.
- Add appropriate buffer to the gel, mix gently (do not use magnetic stirrer for mixing) and wait for it to settle down.
- Decant and resuspend the gel in an equal volume of the buffer and degas the gel in order to remove any trapped air.
- All buffers and the gel should be degassed to avoid air bubbles. Air bubbles cause uneven packing of the column and give rise to poor resolution.
- All buffers should be filtered before use.

Pouring the gel into the column

- Mount the glass column vertically on a metal stand and adjust the bottom adapter.
- Add some buffer to the empty column and permit it to pass through outlet tube so that any trapped air is removed.
- Block the outlet tubing and pour the gel slurry to fill the column up to the required height.
- Gel slurry should be poured with the help of a glass rod in such a way that the glass rod touches the inner wall of the column.
- This avoids the introduction of any air bubble.
- A gel reservoir should be used for smooth and continuous pouring of the gel.
- Allow it to settle down and decant any excess buffer or gel.
- Put the top adapter.

Equilibration of the column

- Attach the top tubing to the peristaltic pump and buffer reservoir.
- Keep the bottom tubing to waste.
- The operating pressure should be as per the manufacturer's recommendations for the gel.
- Choose the buffer as per purification needs and equilibrate the column with 2-3 bed volumes of this buffer.

7. Process of ion-exchange chromatography

Buffer selection

- Buffer selection is an important part to achieve good separation in ion-exchange chromatography.
- Buffer should be selected in such a way that its ionic strength and pH are well-matched with protein activity and stability.
- The pH should be such that it does not interfere with the binding of the proteins of interest to the resin.
- The pH should not be too low or too high as that would require high ionic strength for elution and may interfere with elution. This can cause precipitation of proteins and hence, should be avoided.
- Generally, at an ionic strength of around 0.1 M, proteins start to detach from ion-exchange resin at a pH of about pI +/- 0.5-1 pH units.

- Thus, pH of the elution buffer should be a minimum of 0.5–1 unit less than the pI of the protein to be separated when utilizing a cation exchanger or 0.5–1 unit greater than the pI when utilizing an anion exchanger.
- Commonly used buffers in cation exchange include citrate, HEPES, MES and phosphate buffers etc.
- Commonly used buffers in anion exchange include N-methyl piperazine, bis-tris, tris, phosphate, piperidine etc.

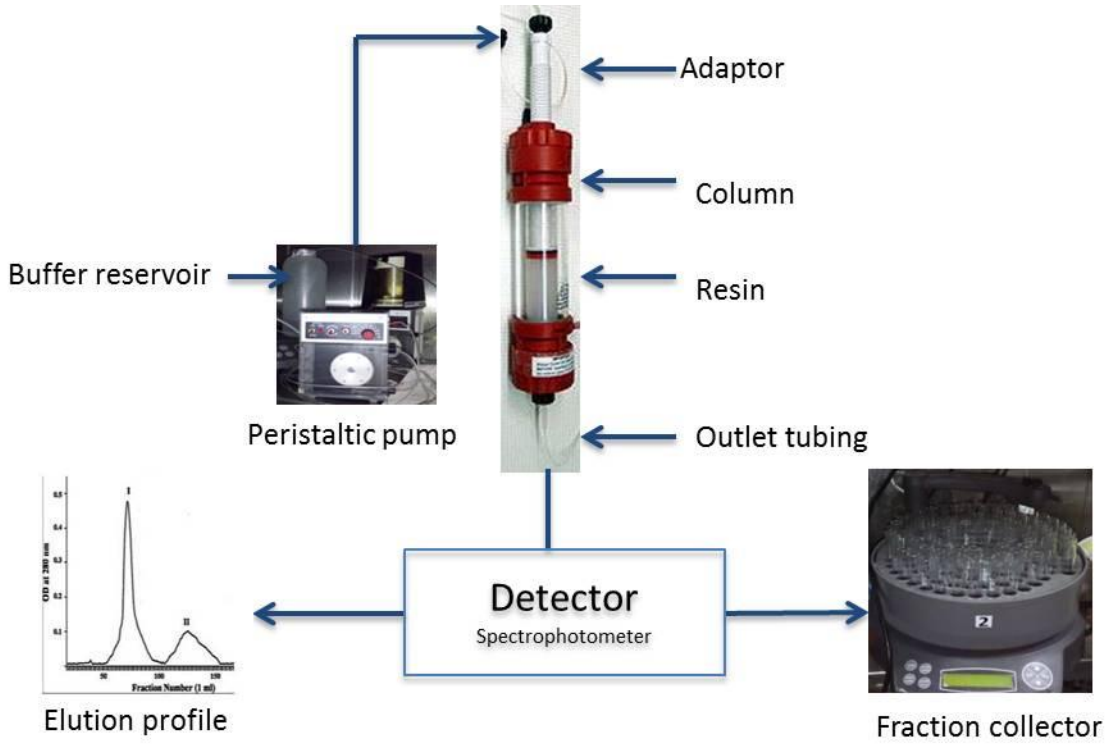

Figure: Schematic representation of ion-exchange chromatography process

Sample preparation & loading

- The sample should be prepared in a buffer identical to the one used for equilibration of the column.
- It should be diluted and free of salt as this increases the efficiency of binding.
- If it has high salt concentration, it should be diluted with equilibration buffer, prior to loading onto the column. This will lower the salt concentration so that it does not hinder functional group binding.
- It should be filtered prior to loading and be free of any debris.
- Concentration of the sample to be loaded should not exceed 20 mg/ml protein.
- Sample should be loaded directly to the column via a peristaltic pump. If it is loaded with a syringe, the surface of the gel should not be disturbed.
- The amount of sample that can be loaded on to a column depends on the binding ability of the resin. The total amount of protein should not exceed the total binding capacity of the resin packed in the column.

Flow rate

- Ideally, the flow rate should be slow when loading the sample as it will aid better binding.
- Washing of the column and elution of attached proteins can be done comparatively at a higher flow rate.

Elution

- Once optimum binding has been achieved, the column should be cleaned with 2-3 bed volumes of equilibration buffer to completely separate unbound contaminants.
- The attached proteins can be eluted using high salt concentration in the equilibration buffer, either by the gradient (e.g. 0.1 – 0.5 M NaCl) or step wise (such as 0.1M, 0.3M, 0.5M NaCl) elution.
- The difference between them is that salt concentration is increased gradually in gradient method while it is increased in discrete steps in the step wise method.
- Elution with long range gradients result in maximum separation between peaks, however separation time increases and peaks are broader.
- In case of elution with steep gradients, separations are faster with sharp peaks. However, resolution may be low as peaks are eluted close to each other.
- After completion of each run, the column should be washed thoroughly with a high salt concentration to remove any analytes left in the gel.
- Ideally, it should be done with 2-3 bed volumes of buffer.

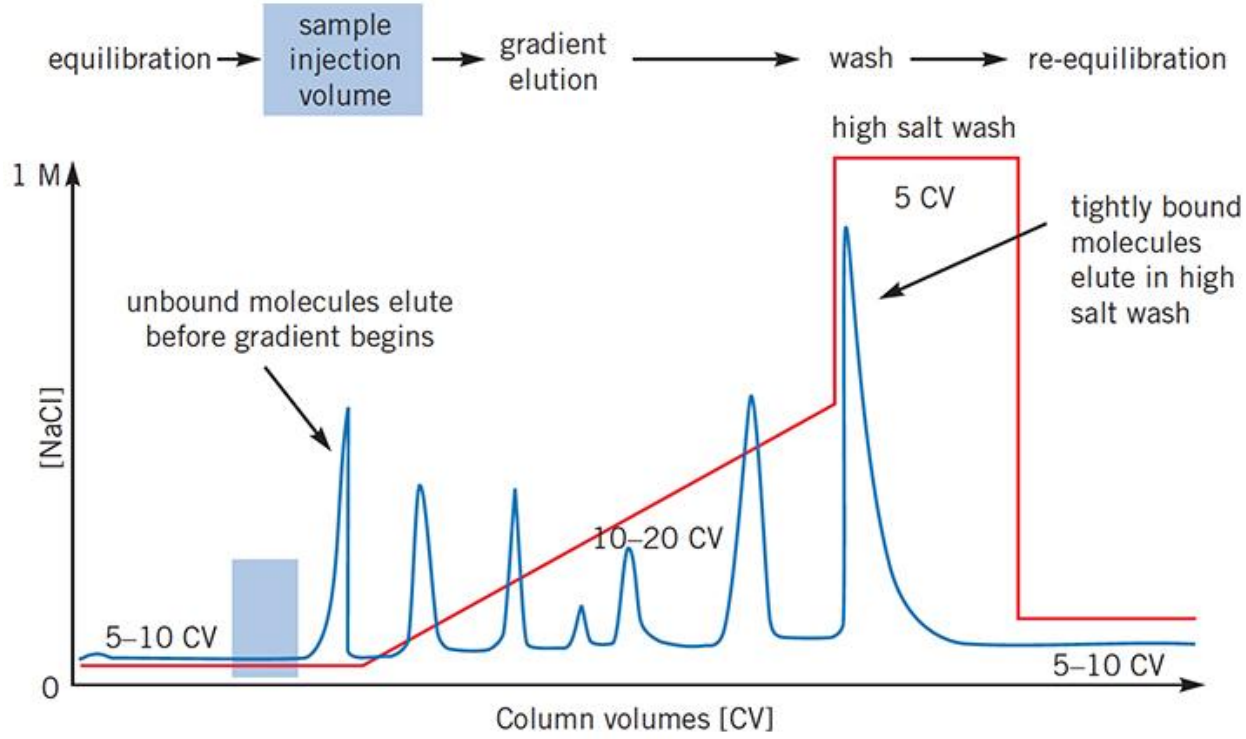

Figure: Chromatogram for various stages of ion exchange chromatography

8. Factors affecting ion-exchange chromatography

There are numerous factors which affect the separation quality in ion-exchange chromatography. The important factors including media, composition of buffer, sample preparation -volume and concentration, sample loading, flow rate, length of tubing and fraction size.

9. Applications

Ion-exchange chromatography is widely used in water treatment plants, food and chemical industries, pharmaceuticals and research.

1) Water treatment - This method is widely applied for water softening, demineralization and de-alkalization. Additionally, ion-exchange technique is used for the selective removal of contaminants from underground water.
2) Food industry – It is used for whey-demineralization, acid and colour removal from fruit juices and to concentrate polyphenols.
3) Chemical industry – It is commonly used for recovery and removal of metals, production of chlorine and purification of hydrogen peroxide, etc.
4) Pharmaceuticals – In pharmaceutical industry, ion-exchange chromatography is routinely applied for purification of antibiotics and other drugs.
5) Research – Ion-exchange chromatography is widely applied in research for purification of various molecules, including proteins, nucleotides and small molecules.

10. Precautions

1) Selection of appropriate media is very essential. It should be selected according to the pI and other chemical properties of the molecules to be separated.
2) Column length should be less and diameter should be more for better separation results.
3) Flow rate should be appropriate. It should not be very slow such that it allows diffusion of sample or very fast such that it results in poor resolution.
4) Sample should be soluble, so that it does not precipitate before loading.
5) Sample should be prepared in the same buffer with which the column has been equilibrated.
6) Avoid any air bubbles in the resin, as they might disturb the separation.
7) Tubing length should be minimum to avoid mixing of separating analytes.

11. Explanatory example

Suppose we have a mixture of equal amounts of three proteins - albumin, pepsin and lysozyme and we need to separate pepsin. The pI's of these proteins are 5.7, 3.4 and 9.3 respectively. Suppose, a buffer with pH 8 is chosen. A pre-made CM sephadex column is used. It is a weak cation exchanger with high binding capacity. It is produced by introducing carboxymethyl functional groups onto the cross-linked dextran matrix. These groups are attached to glucose units in the matrix by stable ether linkages.

For albumin and pepsin, pH>pI.
Hence, they will have net negative charge.
For lysozyme, pH<pI.
Hence, it will have net positive charge.

After column preparation according to the method described in the text above and as per manufacturers recommendations, the sample is loaded into the column and fractions collected.
The following chromatogram is obtained.

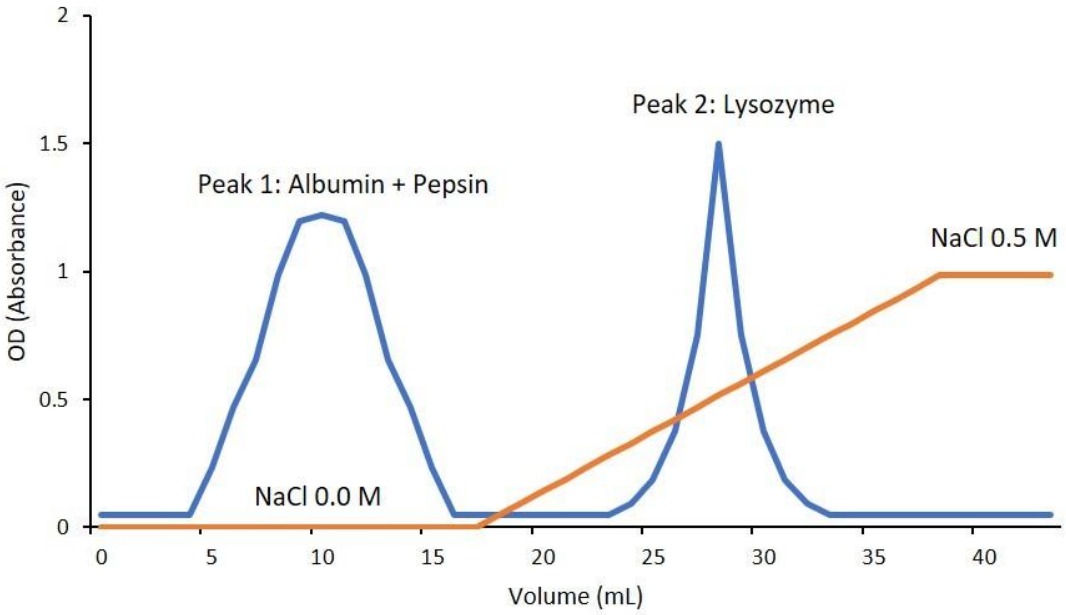

Figure: Chromatogram showing Ion Exchange Chromatography - Albumin, Pepsin, Lysozyme

As albumin and pepsin are negatively charged, they don't bind to the resin and are eluted out first without needing salt to break interactions. Lysozyme is bound to the resin and hence is eluted only when salt is added, which breaks the interactions between the molecule and the resin. Now, lysozyme is separated.

A buffer with pH 6.5 is chosen for the unresolved mixture of albumin and pepsin. A pre-made DEAE column is used. It is a weak anion exchanger using Diethylaminoethyl as ligand.

For albumin and pepsin, pH>pI.
Hence, they will have net negative charge.

After column preparation according to the method described in the text above and as per manufacturers recommendations, the sample is loaded into the column and fractions collected.
The following chromatogram is obtained.

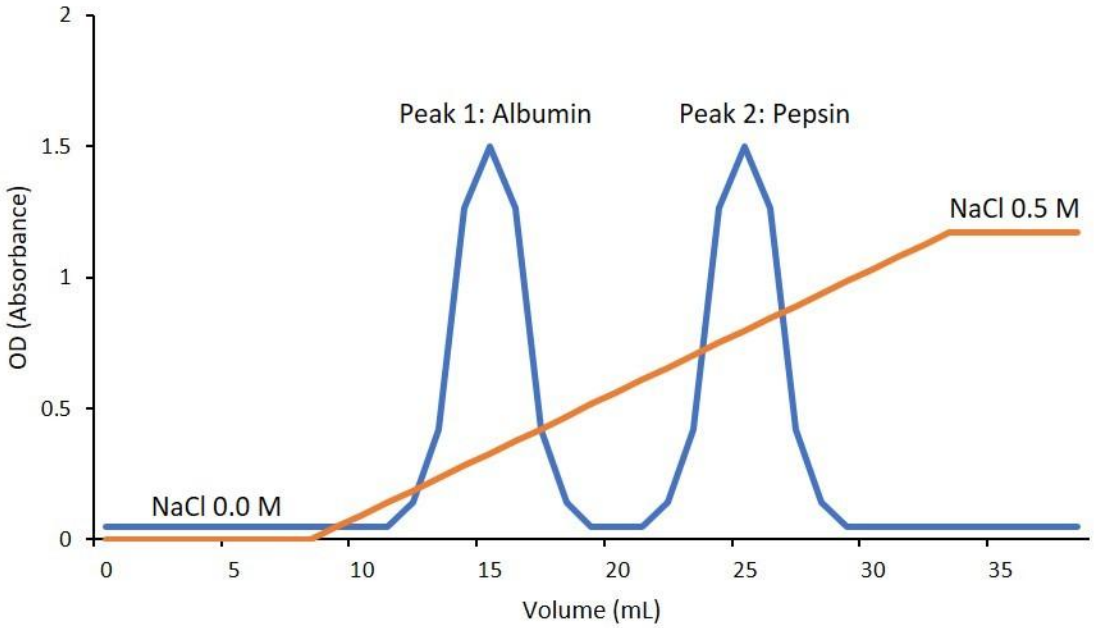

Figure: Chromatogram showing Ion Exchange Chromatography - Albumin, Pepsin

As albumin and pepsin are negatively charged, they both bind to the resin. But pepsin will have greater negative charge and will therefore bind to the resin more strongly as compared to albumin. To break these stronger interactions, a higher salt concentration is required. Hence, albumin elutes first at low salt concentration while pepsin elutes later at higher salt concentration.

12. Comparison of three chromatographic techniques

S. No.	Ion exchange chromatography	Gel filtration chromatography	Affinity chromatography
1.	It is based on separating molecules pased on their charge which can be changed by adjusting the pH in relation to their pI.	It is based on separating molecules based on their molecular size and weight alone.	It is based on specific interactions between the molecule of interest and a specifically designed ligand.
2.	The pI of the molecules of interest should be different.	The molecular weight/size of the molecules of interest should be different.	Specific ligands of the molecules of interest should be available.
3.	It can also be used for protein purification.	It can also be used to determine the molecular weight of the components of a mixture.	It can also be used to study protein-protein interactions.
4.	It is used in water treatment and other applications like pharmaceutical research	It can be used for purification, concentration and desalting.	It is used to deplete high abundant proteins like albumin from a sample. It

	food industry, chemical industry etc.		can also be used to enrichment specific proteins or protein groups and in the study of protein-protein interactions or the purification of specific antibodies and proteins.
5.	Upon elution, purified protein will always be in salt, which will have to be removed for further experimentation.	Upon elution, purified protein will be diluted and will have to be concentrated for further experimentation.	Upon elution, protein will be bound tightly to the ligand and may require severe conditions for separation which can cause denaturisation.

13. Summary

- Ion-exchange chromatography is used to separate macromolecules on the basis of their charge. It can be subdivided into cation-exchange and anion-exchange, depending on type of analytes they exchange.
- Wide range of weak and strong anion and cation exchange media are available to choose from as per one's needs.
- Factors that affect separation and resolution may include buffer, media, flow rate, etc.
- Ion-exchange chromatography is commonly applied in water treatment plants, food and chemical industry, pharmaceuticals and research.