ION EXCHANGE CHROMATOGRAPHY

Lab#6

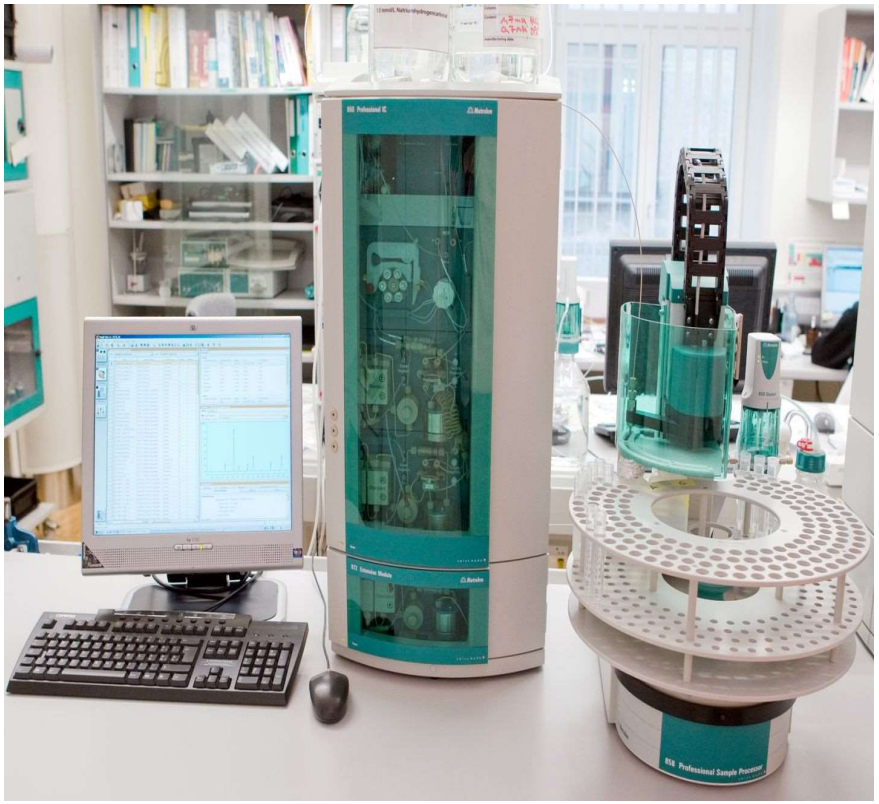
BCH 333

Ion Exchange Chromatography (IEC):

- One of the most used chromatographic method for protein purification.

- ***Its popularity stems from:***
  - The possibility of high-resolution protein separation.
  - The relative ease of use, reproducibility and availability.

- Separation based on charge.



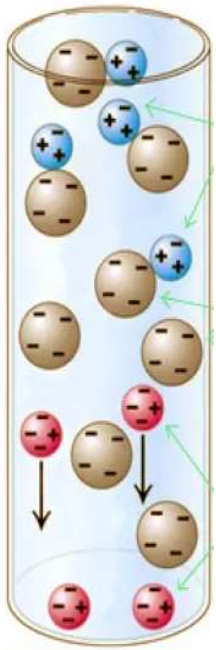
Ion-exchange chromatography

Ion Exchange Chromatography

Mobile phase:

(Buffer / salt)

Buffered aqueous solution, or salt.

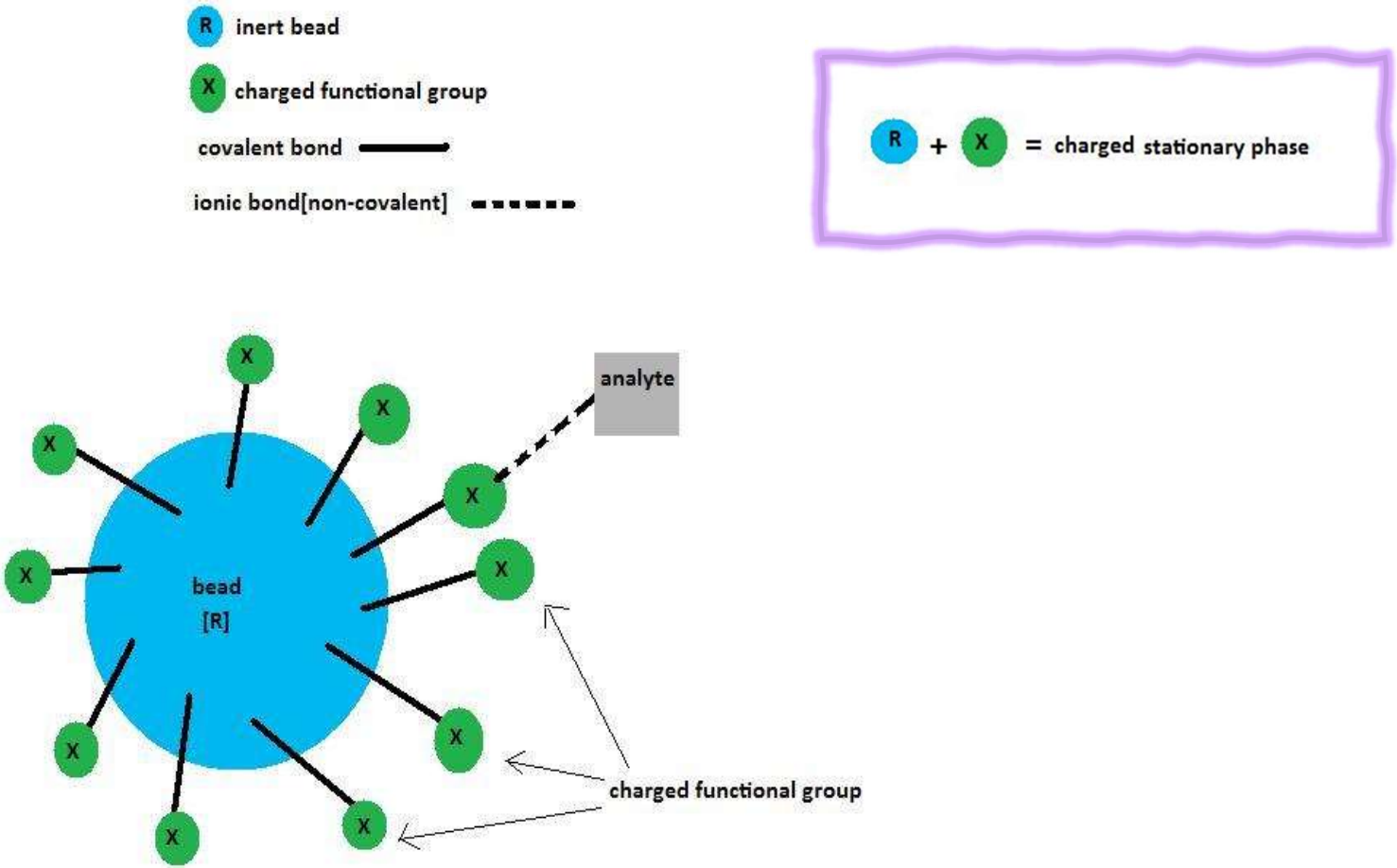


Stationary phase:

(Column matrix)

Is a resin or gel matrix beads with covalently bonded charged functional groups.
[charged beads]

Stationary phase (resin)



Stationary phase (Resin):

- Carries ***ionizable functional groups*** coupled to an ***inert*** matrix material (Resin).

- Because of the principles of electroneutrality, these immobilized charges are electrostatically associated with exchangeable counterions from the solution.

➔ ***So, the stationary phase surface displays ionic functional groups ($R\text{-}X^{+/-}$) that interact with analyte ions of opposite charge.***

Types of IEC :

1. *Cation exchange chromatography:*

Retains positively charged ions because the stationary phase displays a negatively charged functional group.

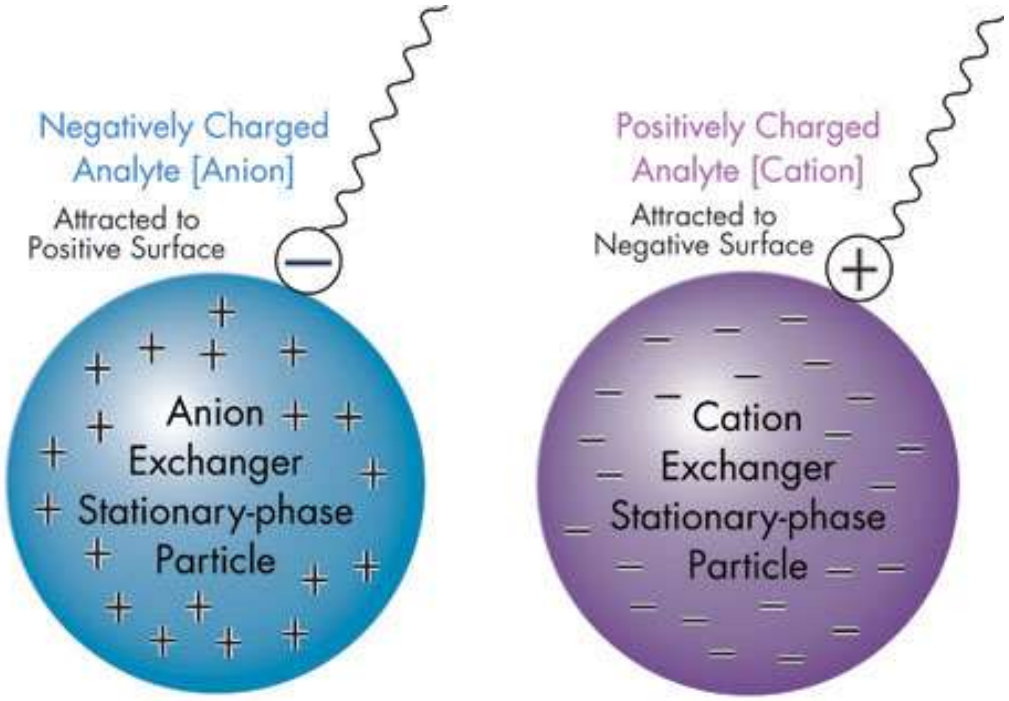
$$[R\text{-}X^{-}]$$

2. *Anion exchange chromatography:*

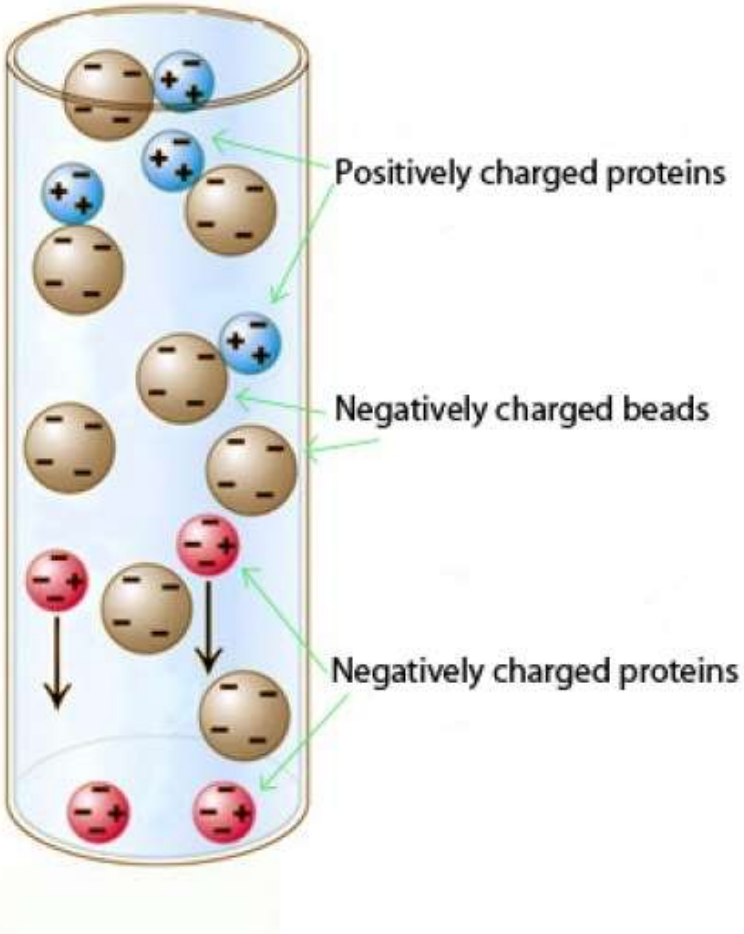
Retains negatively charged ions because the stationary phase displays a positively charged functional group.

$$[R\text{-}X^{+}]$$

Types of IEC cont':



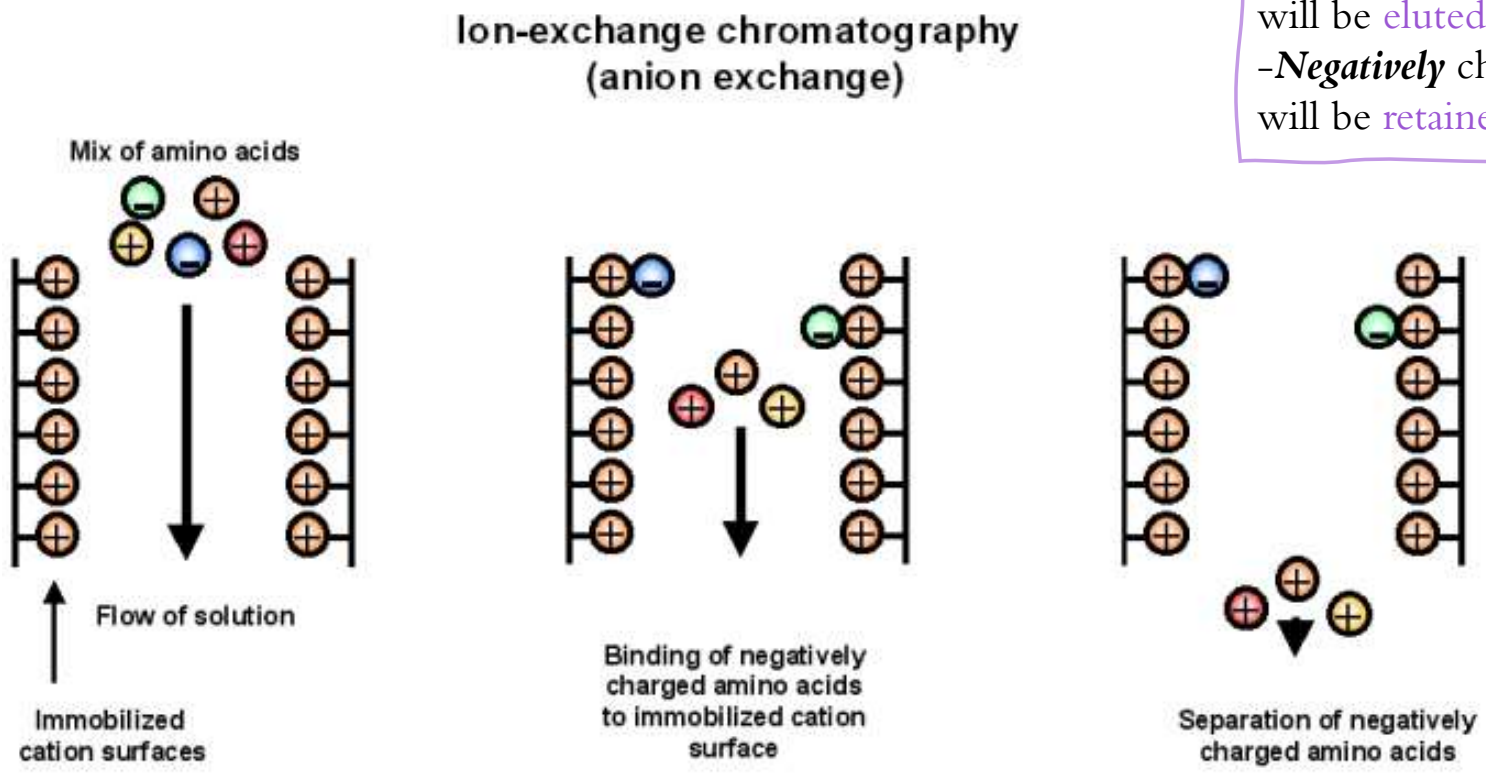
1. Cation exchange chromatography:



-***Negatively*** charged proteins will be eluted.
-***Positively*** charged protein will be retained.

2. Anion exchange chromatography:

- ***Positively*** charged proteins will be eluted.
-***Negatively*** charged protein will be retained.



Analyte with charge that is

Opposite to stationary phase (beads):

- Adsorbed and retained by an ion exchanger (column).
- The binding of the charged compounds is ***reversible.***

Same to stationary phase (beads) or Neutral:

- Pass through the void volume and are eluted from the column.

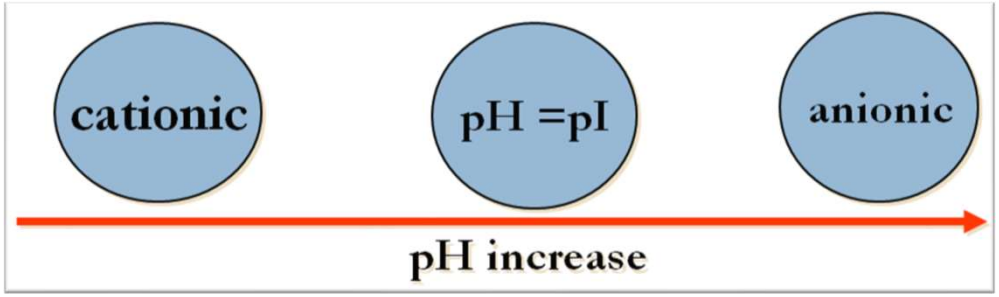
Molecules with charges same as stationary phase charge will elute first.

…..But what about the retained one?

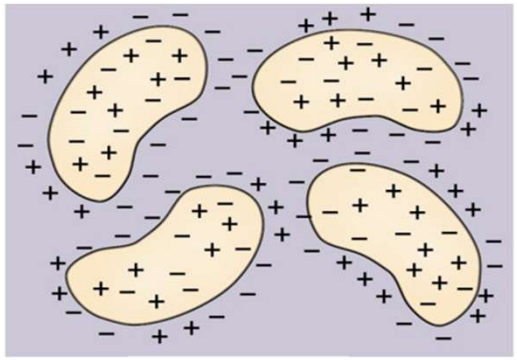
Protein net charge at different pH:

- The charge of proteins is related to the pH of the buffer:

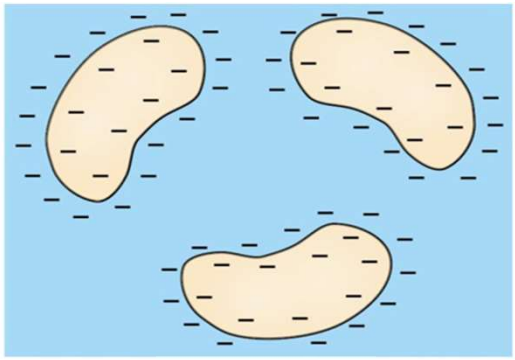
- At buffer with pH = pI → Net charge of protein molecule is ***ZERO***
- At buffer with pH higher than pI *(pH>pI)* → Net charge of protein molecule is ***Negative.***
- At buffer with pH lower than pI *(pH<pI)* → Net charge of protein molecule is ***Positive.***



The pH at which the positive charges equal the negative charges (in other words, the net charge of the protein is zero) defines that protein's ***isoelectric point (pI).***

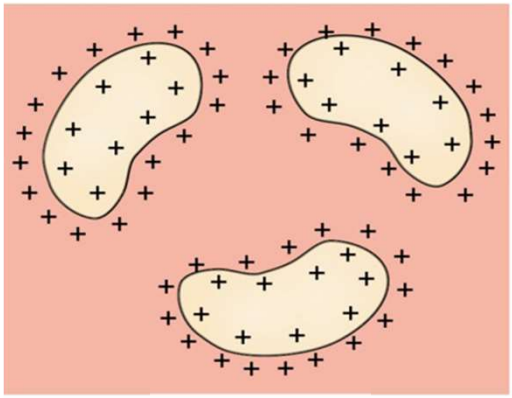


pH = pI



pH > pI

The charge of proteins is related to the pH of the buffer.



pH < pI

The analytes (+ or -) retained on the stationary phase can be eluted by two methods:

1.Using buffer:

Will change the analytes net charge to:

➔having net charged same as the stationary phase.

or

➔ Neutral net charged.

2. Increasing the ionic strength:

By adding salts [e.g.NaCl]

➔to compete the bounded protein to bind with the charged beads.

Elution of the retained proteins:

1. *Elution of the analyte by using buffers:*

- At a given pH (dependent on the composition of the mobile phase), a protein will possess an overall ***net charge.***

- ***At pH of buffer lower than pI*** of the analyte ➔ the net charge will be more ***positive.***
- ***At pH of buffer higher than pI*** of the analyte ➔ the net charge will be more ***negative.***

- So, we can change the net charge of the analyte in a way that it will elute from the column if it has the same charge of the stationary phase, by using the suitable pH of the buffer and knowing the pI of the analyte.

- Or by using buffer has pH that will cause the net charge of the analyte equal to zero, leading to the analyte to be eluted.

Elution of the retained proteins cont':

2. ***Elution of the analyte by using salts:***

- In cation exchange chromatography, to elute the positively charged analyte we could ***displace*** the positively charged analyte by the addition of the positively charged sodium ions of NaCl.

➢ The sodium ions [+] of NaCl, will compete with the analyte [+] to bind to the negatively charged stationary phase [-].

➔ This will cause the elution of the analyte from the column.

➔ What about anion exchange chromatography?

Choosing the proper pH in IEC:

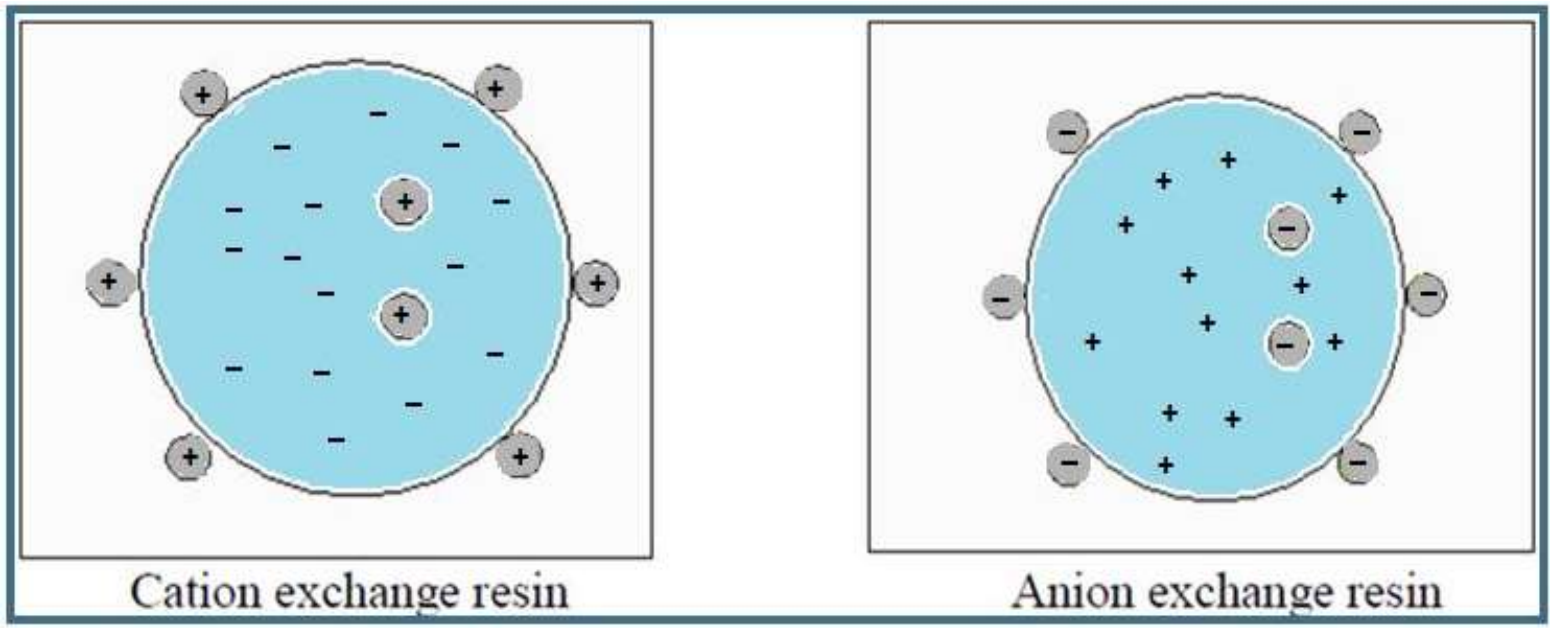
- For IEC, a good rule to follow when separating a protein whose isoelectric point is known is to select a working pH which is ***1 unit away from the pI of the protein.***

- At this pH, the protein will possess a high enough net charge to bind well to the ion exchange column.

Choosing the proper matrix in IEC:

- If the protein possesses a net charge, an ion exchanger needs an opposite charge in order to bind that protein.
- Ion exchangers are typically composed of a charged (ion exchange) group attached covalently to an insoluble matrix.
- A ***positively*** charged group, such as DEAE (diethylamino ethyl), defines the matrix as an anion exchange matrix whereas a ***negatively*** charged group, such as CM (carboxymethyl) makes a cation exchange matrix.
- ***DEAE:*** -CH2-CH2-N^{+}H(CH2CH3) 2.
- ***CM:*** - CH2 – COO^{-}

➔Thus, if a protein has a negative net charge at a given pH, an anion exchange matrix should be used for its purification.


Cation exchange resin
Anion exchange resin
the analyte [e.g:protein]

Advantages of IEC:

- The ion exchange principle permits the protein to bind even when a large buffer volume is applied, making this method especially useful for an initial purification step from a crude extract.

- Ion-exchange chromatography separates molecules based on ***their charged groups***, which cause the molecules to interact electrostatically with opposite charges on the stationary phase matrix.

Practical part

OBJECTIVES:

- Separating mixture of two proteins, myoglobin and cytochrome C based on their charges by cation exchange chromatography.

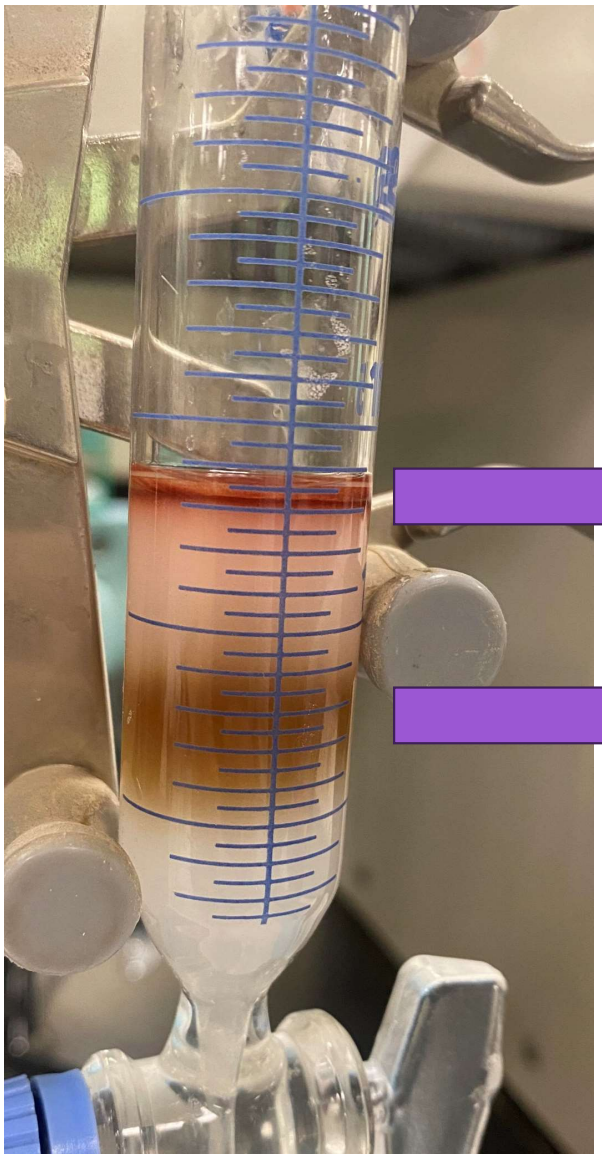
METHOD:

- The gel material that will be used in the experiment is called ***Sephadex C-25*** (cation exchange) and it will separate molecules based on their charges.
- So, those proteins which are ***positively*** charged at pH=8 will attach to the negatively charged beads (cation exchange), while ***negatively*** charged proteins at pH=8 will elute first.

➢ In your experiment:

You will separate a mixture of two proteins, myoglobin and cytochrome C (pI=7.2 and 10.2 respectively) dissolved in phosphate buffer (pH=8) using cation exchange chromatography column Sephadex C-25. Separate the mixture by using the buffer (pH=8) and 1M NaCl.

Cation exchange



Cytochrome C (+)

Myoglobin (-)

:RESULTS

Fraction number	*Absorbance at 410 nm*

- Plot the absorbance at 410nm against fraction number.
- Identify each peak.

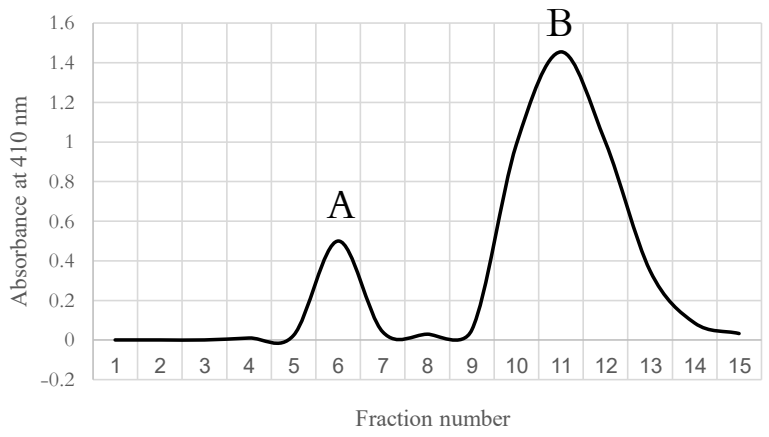


Figure 1. Cation exchange chromatography of separation of A and B at pH 8.