

Guide to Ion-Exchange Chromatography



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Introduction

Ion exchange chromatography is the reversible adsorption of charged molecules to immobilized ion groups on a matrix of an opposite charge. Separation can be selectively achieved by adsorption and release of samples from the matrix. Ion exchange starts with the equilibration of the exchanger using pH, and ionic strength. During equilibration the exchangeable groups are associated with counter ions. Once equilibrium is reached and the sample added the molecules undergo addition and adsorption with an appropriate charge displace the counter ions and bind reversibly to the matrix. The unbound materials will pass through the column with the void volume. In the third stage, substances are removed from the column by increasing the ionic strength of the eluting buffer.

Media

Ion-exchange media is an insoluble matrix with charge groups covalently attached. Negatively charged exchangers bind positively charged ions (cations). Exchangers bind one type of cation but, when presented with a second type of cation, it may displace, and/or exchange with, the first. These resins are called cation-exchange resins. Anion-exchange resins are positively charged and bind and/or exchange negatively charged ions (anions).

Several side-chain groups of the amino acid residues in proteins are ionizable (e.g. lysine or glutamic acid) as are the N-terminal amino and C-terminal carboxyl groups and so proteins are charged molecules. This characteristic can be used to separate different proteins by ion-exchange chromatography.

Types of Exchangers

Two weak exchangers can be used for protein separation are carboxymethyl-cellulose (CM-cellulose) and diethylaminoethyl-cellulose (DEAE-cellulose). These covalently cross-linked agarose bead ion celluloses have been chemically modified. CM-cellulose has a carboxymethyl functional group $-\text{CH}_2\text{OCH}_2\text{COOH}$. At neutral pH the carboxymethyl group is ionized as $-\text{CH}_2\text{OCH}_2\text{COO}^-$ so that CM-cellulose is negatively charged, so it is a weak cation exchanger. DEAE-cellulose contains an diethylaminoethyl group. It is positively charged at neutral pH and so DEAE-cellulose is a weak anion exchanger.

The Sepharose types are particularly useful for the separation of high molecular weight proteins. In practice, since these matrices are very similar to those used for gel filtration some molecular sieving may accompany the ion-exchange process. This may either enhance or reduce the effectiveness of the fractionation compared to using an ion-exchange cellulose.

Introduction (cont.)

Types of Exchangers (cont.)

Two strong exchangers are Q-Sepharose Fast Flow and SP-Sepharose Fast Flow. The charged group of Q-Sepharose is a quarternary amine which carries a non-titratable positive charge. This matrix can be used at alkaline pH values at which the positive charge of the DEAE group would have been titrated. The charged group of S-Sepharose is the sulphonyl group ($-\text{SO}_3^-$).

Method

The fractionation of proteins by ion-exchange chromatography depends upon differences in the charge of different proteins. The charge of a protein depends upon the number and type of ionizable amino acid side chain groups. Lysine residues, have a positively charged side chain group when ionized, whereas glutamic acid residues are negatively charged when ionized. Each ionizable side chain group has a distinct pKa; that is, the pH at which it is half dissociated. Therefore the overall number of charges on a particular protein at a particular pH will depend on the number and type of ionizable amino acid side chain groups it contains. Since, by definition, different proteins have different amino acid compositions, they will tend to have different charges at a given pH and so can be fractionated on this basis.

Each protein has a isoelectric point (pI) where at a certain pH the overall number of negative charges equals the number of positive charges and so it has no net charge. The pI, is the proteins isoionic point. When a protein is at its pI the protein will not bind to the ion-exchange resin. Below this pH the protein will have a net positive charge and will bind to a cation exchanger, and above this pH it will have a net negative charge and bind to an anion exchanger.

In principle, either a cation exchanger or an anion exchanger to bind the protein of interest.

In practice proteins are stable and functionally active within a fairly narrow pH range so the choice of ion exchanger is often dictated by the pH stability of the desired protein. If the protein is stable at pH values below its pI, a cation exchanger should be used if it is stable at pH values above its pI, an anion exchanger would be chosen.

If a protein is stable over a wide pH range, then either type of media should be tried and the selection depends on the best fractionation.

Ion-exchange media, usually have the ions bound to the charged groups on the resin are called 'counter ions'. For CM-cellulose the counter ion is usually Na^+ and for DEAE-cellulose the counter ion is normally Cl^- .

Method (cont.)

After choice of the appropriate resin, it is mixed with buffer to form a slurry which is poured into a suitable chromatography column. The pH of this starting buffer is crucial since it will determine the charge on the proteins to be separated. The starting buffer pH should be at least one pH unit above or below the pI of the protein to be bound to the resin to ensure adequate binding. However, bear in mind that CM-cellulose and DEAE-cellulose are examples of weak ion exchangers. A weak ion exchanger is one which is ionized over only a limited pH range. Thus DEAE-cellulose begins to lose its charge above pH 9 whilst CM-cellulose begins to lose its charge below about pH 5. The term 'weak' does not refer to the strength of binding of ions to the resin nor to the physical strength of the resin itself. With these points in mind then, the effective starting pH range when using DEAE-cellulose or CM-cellulose is only about pH 5 - 9. In addition to correct choice of the pH of the starting buffer, one should take care that its ionic strength is reasonably low since the affinity of proteins for ion-exchange resins decreases as ionic strength increases. Indeed this property is used in one method of eluting the bound proteins.

Protocol Samples

The SpinColumns are supplied dry and need to be rehydrated, the bed of ion-exchange resin with starting buffer allow 10-15 minutes for rehydration. After rehydration add a 2ml collection tube to the bottom of the SpinColumn and centrifuge for 1 minutes at 1000rpm. After centrifugation the protein mixture is applied. Proteins which are oppositely charged to the media at the starting pH will bind to it, so displacing the counter ions. Proteins with the same charge as the resin or with no net charge will not bind and flow straight through the column. The different proteins bound to the column, one of which will be the protein of interest, will have different affinities for the ion exchanger due to differences in their net charge. These affinities can be altered by varying the ionic strength of the column buffer or alternating the pH of the elution buffer.

Example #1

A mixture of proteins are bound to the anion exchanger. At low ionic strength, competition between the buffer ions and proteins for charged groups on the ion exchanger is minimal and so the proteins bind strongly. By increasing the ionic strength, you increase the competition between the proteins are bound and so reduces the interaction between the ion exchanger and proteins, causing the proteins to elute. One can elute the bound proteins by increasing ionic strength irrespective of whether an anion or cation exchanger was used.

This is usually accomplished by incorporating a linear concentration gradient of NaCl in the column buffer while keeping the pH constant.

Example #2

A mixture of proteins are bound to the anion exchanger, DEAE-cellulose. As the pH is lowered, $-\text{COO}^-$ groups on the protein begin to become protonated and so lose their charge.

As the overall negative charge of the protein decreases its affinity for the resin decreases.

Different proteins will elute from the resin at different pH values as their number of negative charges decreases to a critical value. Therefore one can resolve the proteins bound to the column by slowly reducing the pH using a buffer pH gradient and collecting fractions, each of which will contain different proteins eluted at different pH values. Conversely, when using a cation exchanger, the pH gradient would be arranged to increase to elute the bound proteins.

By using either a continuous salt (ionic strength) gradient or a continuous pH gradient will result in a high degree of protein fractionation based on protein charge.

Factors Effecting Selectivity

Protein Ionization

Factors determining the pH value for elution:

1. When a protein is in a solution with a pH value above its pI its net charge is negative and binding charge will be positive anion exchange.
2. When a protein is in a solution with a pH value at its pI its net charge is zero and will not bind to the column.
3. When a protein is in a solution with a pH value below its pI its net charge is positive and binding charge will be negative cation exchange.
4. Small effects:
 - a. Van der Waals
 - b. Non-polar interactions.

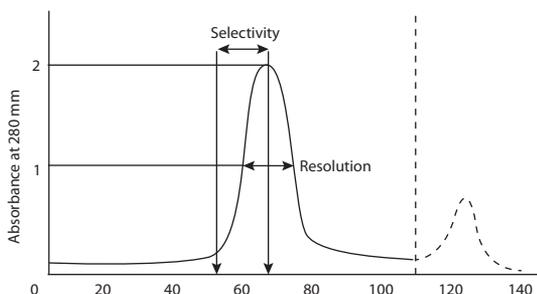
Samples and Sample Buffer

1. Samples should be in the conditions as the start buffer.
2. The temperature of the sample, buffer, and column should be the same to reduce bubbles.
3. When the sample is added to the column. The column should be washed 2 column volumes (CV) so that all non-binding proteins have passed through the column.
4. Proteins are eluted by increasing the ionic strength of the elution buffer.
5. Occasionally increasing the pH of the elution buffer can be used if it has no net effect on the protein.

Note: Buffer volumes used for a column are referred to as column volumes or CV (i.e. 5 CV = 5mls for a 1 ml column).

Resolution

Resolution is the degree of separation between the peaks (maximum volume of eluted samples) from the column (selectivity of the medium). The ability of the column to produce symmetrical peaks and mass of the sample applied.



Factors Effecting Selectivity (cont.)

Selectivity

Good selectivity is equal to the degree of separation between peaks. This is important in determining resolution and depends on:

1. Nature of functional groups
2. Number of functional groups
3. Experimental conditions:
 - a. pH
 - b. Ionic strength
 - c. Elution conditions

Selectivity of pH

Optimum selectivity equals maximum separation between titration curves of individual proteins (i.e. net charge difference).

The order in which proteins are eluted can not always be predicted with absolute certainty since a titration curve reflects the total net charge of a protein. Ion exchange depends on the net charge of the surface of the protein.

Selectivity and Elution

1. **Linear Gradient or Step Elution Specific:** Proteins are eluted by increasing the ionic strength of a buffer. The UV and conductivity will show the elution peak and changes in salt concentration.
2. **Gradient Elution:** When starting with an unknown sample and for high resolution separation analysis.
3. **Step Elution:** When the ion exchange separation has been optimized using gradient elution. This speeds up the separation time.
4. **Group Elution:** Group elution is also a Step elution. Step elution can be used to remove contaminants by choosing conditions that maximize binding of the contaminants and allow target proteins to pass through the column.

Particle size

As particle size increases the resolution decreases. Selection of particle size depends on Purification requirements:

1. Speed
2. Resolution
3. Purity
4. Viscosity

Note: High viscosity reduces resolution and increases time. A simple solution is dilution.

Components of Ion Exchange Media

Matrix

1. **High porosity**
 - a. Large surface area covered by charged groups
 - b. Advantage with large molecules
 - c. Non-porous are preferable for high resolution separation when diffusion effect must be avoided.
2. **Inert matrix**
 - a. Immunizes non-specific binding
3. **High physical stability**
 - a. Insures stable constant packing
 - i. No change do to extreme changes in ionic strength
 - ii. No change do to pH
4. **High physical stability and uniformity of particle size**
 - a. High flow rate
 - b. Improved throughput
5. **High chemical stability**
 - a. Cleaning using stringent solutions.
6. **Ion Exchange polymer on agarose based matrices**
 - a. High binding capacity
 - b. Physical and chemical stability
 - c. Large selection of particle sizes

Functional Groups

Type of IX Exchange	Functional Group	Common Name	pH Range	Ionic Capacity
Weak Cation Exchange	Carboxymethyl	CM Cellulose Sepharose	5-9	.09 - .13mmol (Cl-)/ml
Strong Cation Exchange	Sulfopropyl	SP Fast Flow Sepharose	6-10	.18 - .25mmol (Cl-)/ml
Weak Anion Exchange	Diethylaminoethyl	DEAE Cellulose sepharose	5-9	.11 - .16mmol (Cl-)/ml
Strong Anion Exchange	Quaternary Ammonium	Q sepharose	2-9	.18 - .25mmol (Cl-)/ml

Components of Ion Exchange Media (cont.)

Weak and strong ion exchanger refers to the extent that the ionization state of the functional groups varies with pH.

Strong ion exchange shows no variation in ion exchange capacity with changes in pH. These exchanges show no variation with pH changes so have no buffering capacity and remain fully charged over a broad pH range.

Advantages of Working with Strong Ion Exchange Media

1. Development and optimization separation are fast since charge characteristics do not change or are less variable than weak ion exchange media.
2. The sample interaction is simple so there are no intermediate forms of charge interactions.
3. Sample loading (binding) capacity is maintained at high or low pH.

Note: Most proteins have an isoelectric point (pI) of 5.5 to 7.5 and can be separated on either strong or weak ion exchange media.

Weak Ion Exchange i.e. DEAE, ANX, CM

The advantage of weak ion exchange is that they offer a different selectivity than strong ion exchange media.

The disadvantage of weak ion exchange media is that they can take up or lose protons with changes in pH. So their ion exchange capacity varies with pH.

1. **Binding capacity and recovery:** The capacity of an ion exchange medium is its ability to take up counter ions (protein or other charged materials).
2. Total ionic capacity equals the number of charged functional groups per ml of medium. The end line is the actual amount of protein which can bind to an ion exchange medium under defined conditions; this is the available capacity for a specific protein.

If defined conditions include flow rate the amount bound is referred to as the "Dynamic capacity" of the medium.

3. The available and dynamic capacity depends on the properties of the protein, the ion exchange medium and experimental conditions.
 - a. Capacity of ion exchange medium is dependent on molecular size of the protein, the charge /pH relationship (net charge and density of charge at the surface) at a chosen pH.

Experimental Conditions of Ion Exchange Chromatography

Final resolution

1. Particle size
2. Porosity
3. Column packing

Separation influence

1. Net surface charge of a protein at a pH
2. The pH of the buffer
3. Ionic strength of the buffer
4. Elution conditions.

Capture

1. Intermediate purification
2. Final purification

Sample preparation

1. The buffer of the sample should be the same as the starting buffer.
2. For small sample volume diluting the sample in the start buffer in order to lower ionic strength and adjust pH
3. If the pI of the protein is known the pH of the starting buffer should be 0.5 to 1 pH unit away from the pI.

Description	Ultra-Micro 1	Micro	Macro
Bed volume	37.96 μl	66.42 μl	191.45 μl
Sample volume	10-25 μl	25-75 μl	75-150 μl
Sample concentration	3-30 μg	5-60 μg	30-300 μg

Method Optimization

1. The pH should .05-1.0 pH units from the proteins pI
2. Use weak or strong ion exchange media very them
3. Use gradient that give pH
4. Flow rate that maintains resolution and minimizes separation time.
5. Maximum sample load that can be applied while maintaining satisfactory resolution.

Method Optimization (cont.)

pH and Ionic Strength

The pH should be as close to the point of release as possible.

Note: If the pH is too low or high elution becomes more difficult and high salt concentrations are needed.

Note: Avoid extreme changes in pH or other conditions that may cause inactivation or precipitation.

Proteins will begin to dissociate from the ion exchange column media at ~ 0.5 pH units from their pI, at an ionic strength of ~ 0.1 M.

The pH of the starting buffer should be at least 0.5-1 pH units below the pI of the protein when using a cation exchanger CSP, CM.

Note: If the sample components are most stable below pI use cation exchange media.

If the sample components are most stable above pI use anion exchange media.

If stability is high over a wide pH range on both sides of the pI use either the cation or anion exchange media.

Weak Exchangers

1. The sample loading (binding) capacity can vary with increasing pH due to loss of charge from the exchange.
2. Resolution is more readily affected by changes in flow rate or sample load due to the intermediate forms of charge interaction which occur.
3. Predicted results may not correlate with the actual results since the number of charged groups on weak ion exchangers can vary with pH.
4. Larger equilibration times may be required in order to titrate the weak ion exchange functional groups.

Media	pH ranges
DEAE	pH 2-9
ANX	pH 2-9
CM	pH 6-10

Buffer Ions

1. Use a buffer concentration that is sufficient to maintain buffering capacity and constant pH typically 20-50mM
2. Use volatile buffer if the sample is lyophilized
3. Filter the buffer through a 1 μ m filter for particle size above 90 μ m, 0.45 μ m filter for 34 μ m particle and 0.22 μ m for particles below 15 μ m

References

- Himmelhoch, SR (1971) Chromatography of proteins on ion-exchange adsorbents. *Meth. Enzymol.* 22:273-286.
- Peterson, EA, Sober, HA (1956) Chromatography of proteins. 1. Cellulose ion-exchange adsorbents. *J. Am. Chem. Soc.* 78:751-758.
- Scopes, RK (1982) Ion exchangers-principles, properties and uses. In "Protein Purification: Principles and Practice", pp75-101. Springer-Verlag, New York.

Ordering Information

Media Type	Ultra-Micro SpinColumns		Micro SpinColumns		Macro SpinColumns	
	24 Pack	96 Pack	24 Pack	96 Pack	24 Pack	96 Pack
Strong Anion Exchanger	74-7233	74-7213	74-4704	74-4700	74-4204	74-4200
Strong Cation Exchanger	74-7235	74-7215	74-4706	74-4702	74-4206	74-4202
Weak Anion Exchanger	74-7234	74-7214	74-4705	74-4701	74-4205	74-4201
Weak Cation Exchanger	74-7236	74-7216	74-4707	74-4703	74-4207	74-4203
Strong Cation Exchanger SA PolySulfoethyl A	74-4426	74-4425	74-4413	74-4112	74-4153	74-4152
Weak Anion Exchanger PEI PolyWax PL	74-4424	74-4423	74-4411	74-4410	74-4151	74-4150
Weak Cation Exchanger AA Poly Cat A	74-4428	74-4427	74-4415	74-4414	74-4155	74-4154

Media Type	Micro SpinColumns 96-Well	Macro SpinColumns 96-Well
Strong Anion Exchanger	74-5624	74-5664
Strong Cation Exchanger	74-5625	74-5665
Weak Anion Exchanger	74-5626	74-5666
Weak Cation Exchanger	74-5627	74-5667
Strong Cation Exchanger SA PolySulfoethyl A	74-5632	74-5672
Weak Anion Exchanger PEI PolyWax PL	74-5633	74-5673
Weak Cation Exchanger AA Poly Cat A	74-5634	74-5674

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