Chapter 14

# The Role of Ion Exchange Chromatography in Purification and Characterization of Molecules

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/52537

## 1. Introduction

Adsorption chromatography depends upon interactions of different types between solute molecules and ligands immobilized on a chromatography matrix. The first type of interaction to be successfully employed for the separation of macromolecules was that between charged solute molecules and oppositely charged moieties covalently linked to a chromatography matrix. The technique of ion exchange chromatography is based on this interaction.

Ion exchange is probably the most frequently used chromatographic technique for the separation and purification of proteins, polypeptides, nucleic acids, polynucleotides, and other charged biomoleules (1) . The reasons for the success of ion exchange are its widespread applicability, its high resolving power, its high capacity, and the simplicity and controllability of the method.

## 2. The theory of ion exchange

Purification using ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to immobilized ion exchange groups of opposite charge. Most ion exchange experiments are performed in five main stages. These steps are illustrated schematically.

The first stage is equilibration in which the ion exchanger is brought to a starting state, in terms of pH and ionic strength, which allows the binding of the desired solute molecules. The exchanger groups are associated at this time with exchangeable counter-ions (usually simple anions or cations, such as chloride or sodium). The second stage is sample application and adsorption, in which solute molecules carrying the appropriate charge displace counter-ions and bind reversibly to the gel. Unbound substances can be washed out

from the exchanger bed using starting buffer. In the third stage, substances are removed from the column by changing to elution conditions unfavourable for ionic bonding of the solute molecules. This normally involves increasing the ionic strength of the eluting buffer or changing its pH. In Figure 1 desorption is achieved by the introduction of an increasing salt concentration gradient and solute molecules are released from the column in the order of their strengths of binding, the most weakly bound substances being eluted first.

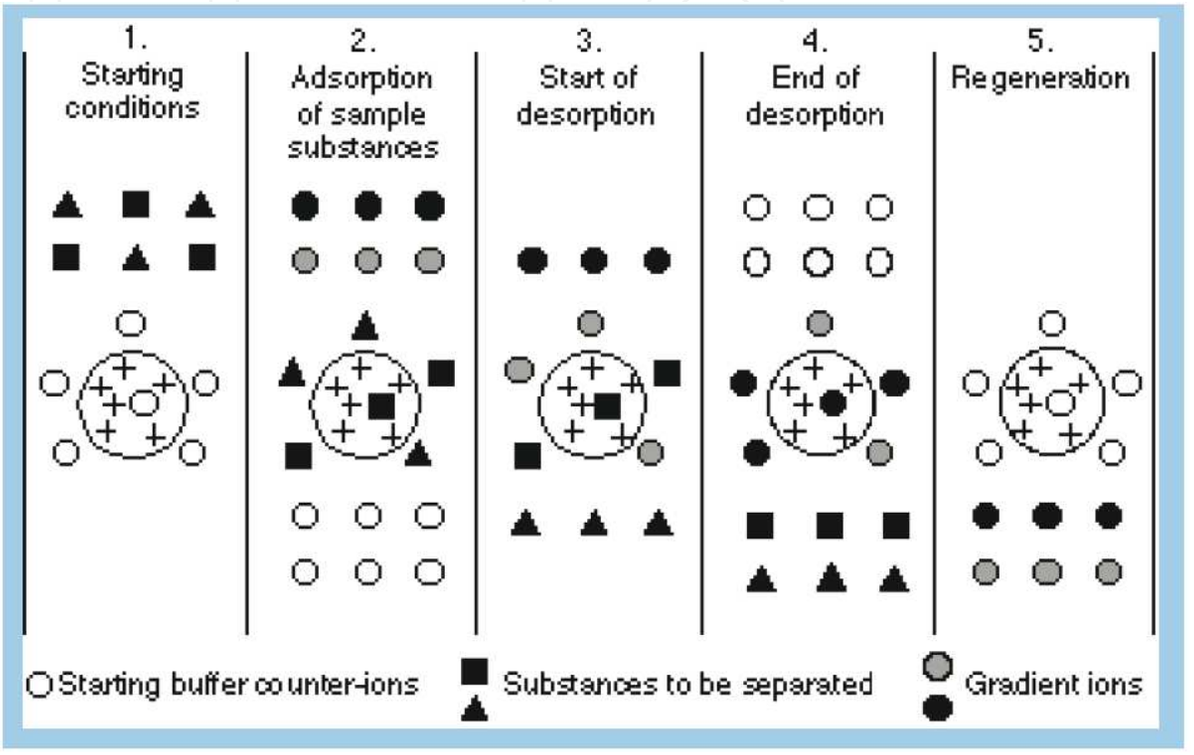


**Figure 1.** The principle of ion exchange chromatography (salt gradient elution).

The fourth and fifth stages are the removal from the column of substances not eluted under the previous experimental conditions and re-equilibration at the starting conditions for the next purification. Separation is obtained since different substances have different degrees of interaction with the ion exchanger due to differences in their charges, charge densities and distribution of charge on their surfaces. These interactions can be controlled by varying conditions such as ionic strength and pH. The differences in charge properties of biological compounds are often considerable, and since ion exchange chromatography is capable of separating species with very minor differences in properties, e.g. two proteins differing by only one charged amino acid, it is a very powerful separation technique. In ion exchange chromatography one can choose whether to bind the substances of interest and allow the contaminants to pass through the column, or to bind the contaminants and allow the substance of interest to pass through. Generally, the first method is more useful since it allows a greater degree of fractionation and concentrates the substances of interest. In addition to the ion exchange effect, other types of binding may occur. These effects are small and are mainly due to van der Waals forces and non-polar interactions. Ion exchange separations may be carried out in a column, by a batch procedure or by expanded bed

adsorption. All three methodologies are performed in the stages of equilibration, sample adsorption etc. described previously.

## 3. The matrix

An ion exchanger consists of an insoluble matrix to which charged groups have been covalently bound. The charged groups are associated with mobile counter ions. These counter-ions can be reversibly exchanged with other ions of the same charge without altering the matrix. It is possible to have both positively and negatively charged exchangers (Fig. 2). Positively charged exchangers have negatively charged counter-ions (anions) available for exchange and are called anion exchangers. Negatively charged exchangers have positively charged counter-ions (cations) and are termed cation exchangers. The matrix may be based on inorganic compounds, synthetic resins or polysaccharides. The characteristics of the matrix determine its chromatographic properties such as efficiency, capacity and recovery as well as its chemical stability, mechanical strength and flow properties.

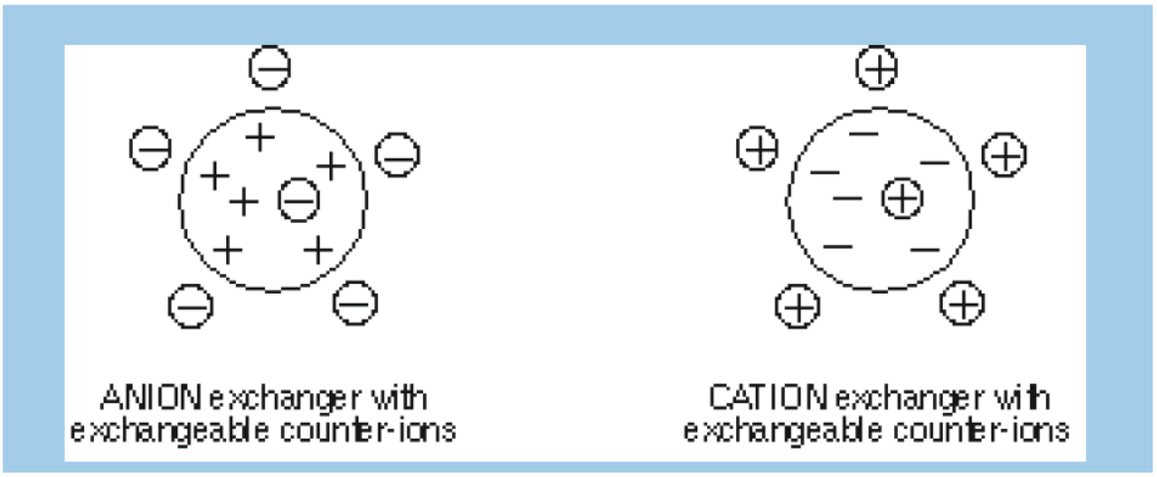


**Figure 2.** Ion exchanger types.

The nature of the matrix will also affect its behaviour towards biological substances and the maintenance of biological activity. The first ion exchangers were synthetic resins designed for applications such as demineralisation, water treatment, and recovery of ions from wastes. Such ion exchangers consist of hydrophobic polymer matrices highly substituted with ionic groups, and have very high capacities for small ions. Due to their low permeability these matrices have low capacities for proteins and other macromolecules. In addition, the extremely high charge density gives very strong binding and the hydrophobic matrix tends to denature labile biological materials. Thus despite their excellent flow properties and capacities for small ions, these types of ion exchanger are unsuitable for use with biological samples. The first ion exchangers designed for use with biological substances were the cellulose ion exchangers (2) . Because of the hydrophilic nature of cellulose, these exchangers had little tendency to denature proteins. Unfortunately, many cellulose ion exchangers had low capacities (otherwise the cellulose became soluble in water) and had

poor flow properties due to their irregular shape. Ion exchangers based on dextran (Sephadex), followed by those based on agarose (Sepharose CL-6B) and cross-linked cellulose (DEAE Sephacel) were the first ion exchange matrices to combine a spherical form with high porosity, leading to improved flow properties and high capacities for macromolecules.

The presence of charged groups is a fundamental property of an ion exchanger. The type of group determines the type and strength of the ion exchanger; their total number and availability determines the capacity. There is a variety of groups which have been chosen for use in ion exchangers (3); some of these are shown in Table 1

| **Anion exchangers** | **Functional group** |
|---|---|
| Diethylaminoethyl (DEAE) | -O-CH2-CH2-N+H(CH2CH3)2 |
| Quaternary aminoethyl (QAE) | -O-CH2-CH2-N+(C2H5)2-CH2-CHOH-CH3 |
| Quaternary ammonium (Q) | -O-CH2-CHOH-CH2-O-CH2-CHOH-CH2-N+(CH3)3 |
| **Cation exchangers** | **Functional group** |
| Carboxymethyl (CM) - | O-CH2-COO |
| Sulphopropyl (SP) | -O-CH2-CHOH-CH2-O-CH2-CH2-CH2SO3 |
| Methyl sulphonate (S) | -O-CH2-CHOH-CH2-O-CH2-CHOH-CH2SO3 |

**Table 1.** Functional groups used on ion exchangers.

Sulphonic and quaternary amino groups are used to form strong ion exchangers; the other groups form weak ion exchangers. The terms strong and weak refer to the extent of variation of ionization with pH and not the strength of binding. Strong ion exchangers are completely ionized over a wide pH range. Whereas with weak ion exchangers,

the degree of dissociation and thus exchange capacity varies much more markedly with pH. Some properties of strong ion exchangers are:

- Sample loading capacity does not decrease at high or low pH values due to loss of charge from the ion exchanger.
- A very simple mechanism of interaction exists between the ion exchanger and the solute.
- Ion exchange experiments are more controllable since the charge characteristics of the media do not change with changes in pH.

## 4. Resolution in ion exchange chromatography

The result of an ion exchange experiment, as with any other chromatographic separation, is often expressed as the resolution between the peaks of interest. The resolution is defined as the distance between peak maxima compared with average base width of the two peaks. Elution volumes and peak widths should be measured with the same units to give a dimensionless value to the resolution (4, 5). The resolution (Rs) is determined from the chromatogram as shown in Figure 3.

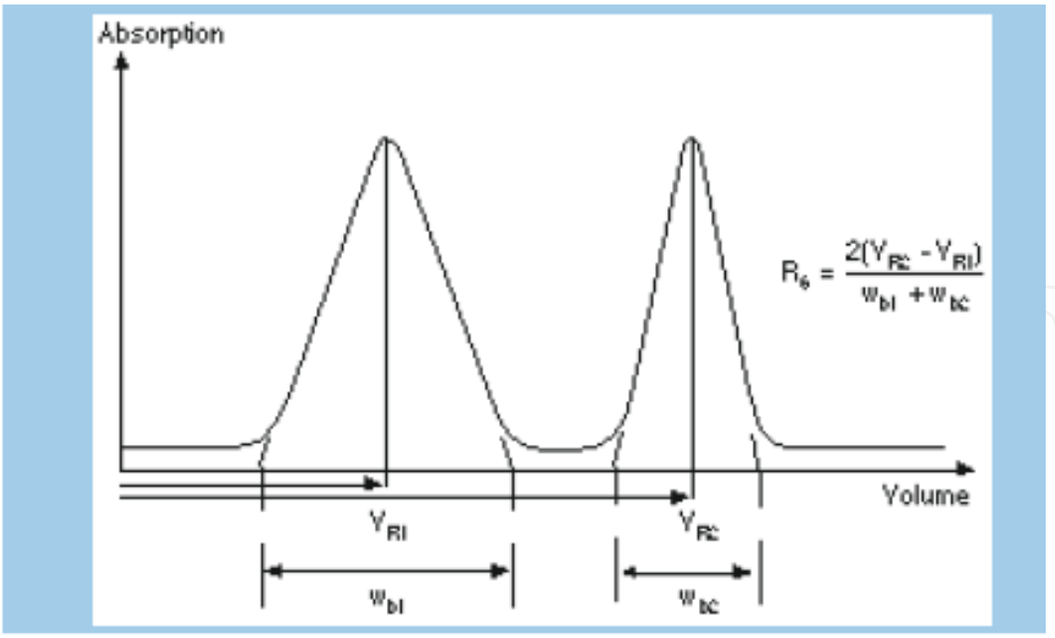


**Figure 3.** Determination of the resolution (Rs) between two peaks.

Rs is a measure of the relative separation between two peaks and can be used to determine if further optimization of the chromatographic procedure is necessary.

### 4.1. Capacity

The capacity of an ion exchanger is a quantitative measure of its ability to take up exchangeable counter-ions and is therefore of major importance. The capacity may be expressed as total ionic capacity, available capacity or dynamic capacity. The total ionic capacity is the number of charged substituent groups per gram dry ion exchanger or per ml swollen gel. Total capacity can be measured by titration with a strong acid or base. The actual amount of protein which can be bound to an ion exchanger, under defined experimental conditions, is referred to as the available capacity for the gel. If the defined conditions include the flow rate at which the gel was operated, the amount bound is referred to as the dynamic capacity for the ion exchanger. Available and dynamic capacities depend upon: The properties of the protein. The properties of the ion exchanger. The chosen experimental conditions. The properties of the protein which determine the available or dynamic capacity on a particular ion exchange matrix are its molecular size and its charge/pH relationship. The capacity of an ion exchanger is thus different for different protein.

## 5. Choice of exchanger group

Substances are bound to ion exchangers when they carry a net charge opposite to that of the ion exchanger. This binding is electrostatic and reversible. In the case of substances which carry only one type of charged group the choice of ion exchanger is clear-cut. Substances which carry both positively and negatively charged groups, however, are termed

amphoteric and the net charge which they carry depends on pH (Fig. 4). Consequently at a certain pH value an amphoteric substance will have zero net charge. This value is termed the isoelectric point (pI) and at this point substances will bind to neither anion or cation exchangers (6). The pH ranges in which the protein is bound to anion or cation exchangers and an arbitrary range of stability are shown in Figure 4.

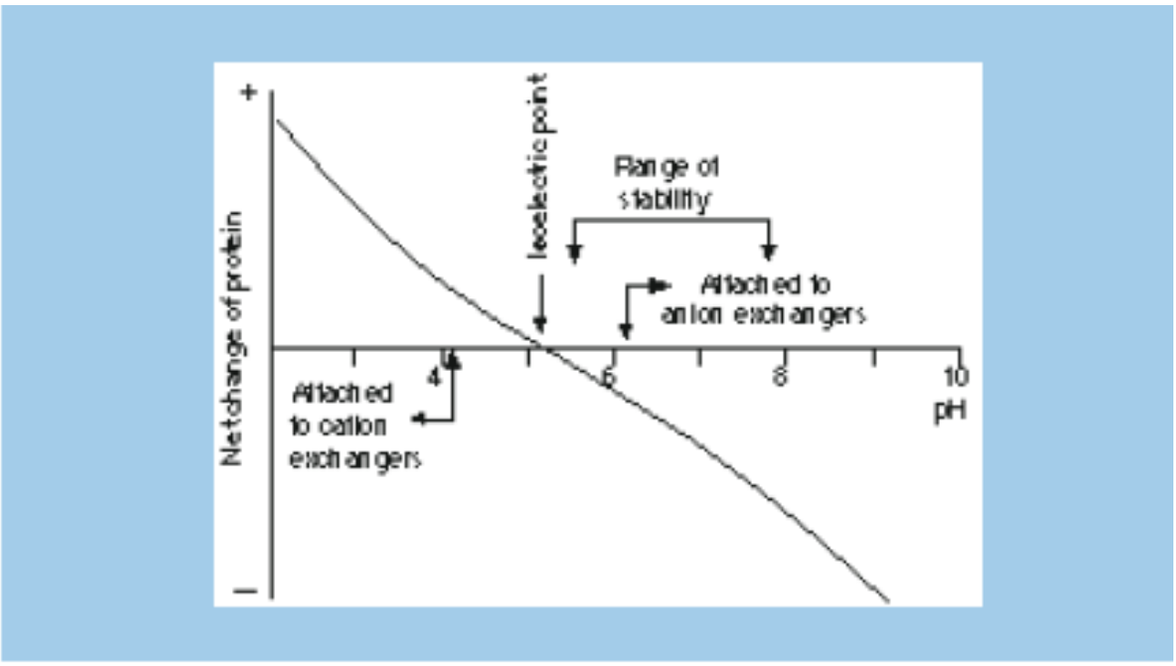


**Figure 4.** The net charge of protein as a function of pH.

The pH of the buffer thus determines the charge on amphoteric molecules during the experiment. In principle therefore, one could use either an anion or a cation exchanger to bind amphoteric samples by selecting the appropriate pH. In practice however, the choice is based on which exchanger type and pH give the best separation of the molecules of interest, within the constraints of their pH stability (7, 8).

Many biological macromolecules become denatured or lose activity outside a certain pH range and thus the choice of ion exchanger may be limited by the stability of the sample. This is illustrated in Figure 4. Below its isoelectric point a protein has a net positive charge and can therefore adsorb to cation exchangers. Above its pI the protein has a net negative charge and can be adsorbed to anion exchangers. However, it is only stable in the range pH 5-8 and so an anion exchanger has to be used.

### 5.1. In summary

1. If the sample components are most stable below their pI's, a cation exchanger should be used.
2. If they are most stable above their pI's, an anion exchanger should be used.
3. If stability is high over a wide pH range on both sides of pI, either type of ion exchanger can be used.

## 6. Determination of starting conditions

### 6.1. The isoelectric point

The starting buffer pH is chosen so that substances to be bound to the exchanger are charged. The starting pH should be at least 1 pH unit above the isoelectric point for anion exchangers or at least 1 pH unit below the isoelectric point for cation exchangers to facilitate adequate binding ( 6). Substances begin to dissociate from ion exchangers about 0.5 pH units from their isoelectric points at ionic strength 0.1 M (15). If the isoelectric point of the sample is unknown, a simple test can be performed to determine which starting pH can be used. range of pH 5-9 for anion and pH 4-8 for cation exchangers, with 0.5 pH unit intervals between tubes.

### 6.2. Choice of buffer

As with the choice of ion exchanger, there are a number of variables which have to be considered. These include:

1. The choice of buffer pH and ionic strength.
2. The choice of buffering substance.

It should be pointed out, however, that in many applications the optimum separation may be achieved by choosing conditions so that major and troublesome contaminants are bound to the exchanger while the substance of interest is eluted during the wash phase (9). This procedure is sometimes referred to as "starting state elution".

*Note:* Concentration of sample does not occur with starting state elution.

The highest ionic strength which permits binding of the selected substances and the lowest ionic strength that causes their elution should normally be used as the starting and final ionic strengths in subsequent column experiments (i.e. the starting and limiting buffers for gradient elution). A third and higher ionic strength buffer is frequently employed as a wash step before column regeneration and re-use.

The required concentration of the start buffer will vary depending on the nature of the buffering substance. In the majority of cases a starting ionic strength of at least 10 mM is required to ensure adequate buffering capacity. Salts also play a role in stabilizing protein structures in solution and so it is important that the ionic strength should not be so low that protein denaturation or precipitation occurs.

### 6.3. Choice of buffer substance

If the buffering ions carry a charge opposite to that of the functional groups of the ion exchanger they will take part in the ion exchange process and cause local disturbances in pH. It is preferable, therefore, to use buffering ions with the same charge sign as the substituent groups on the ion exchanger. There are of course exceptions to this rule as

illustrated by the frequency with which phosphate buffers are cited in the literature in connection with anion exchangers. In those instances when a buffering ion which interacts with the ionic groups on the matrixnis used, extra care must be taken to ensure that the system has come to equilibrium before application of sample.

## 7. Column chromatography

Good results in column chromatography are not solely dependent on the correct choice of gel media. The design of the column and good packing technique are also important in realising the full separation potential of any gel.

The material used in the construction of the column should be chosen to prevent destruction of labile biological substances and minimize non-specific binding to exposed surfaces. The bed support should be designed so it is easily exchangeable to restore column performance whenever contamination and/or blockage in the column occur. Bed supports made from coarse sintered glass or glass wool cannot be recommended because they soon become clogged, are difficult to clean and cause artifacts (10).

The pressure specifications of the column have to match the back-pressure generated in the packed bed when run at optimal flow rate. This is particularly important when using high performance media with small bead size. All are easy to dismantle and reassemble to allow thorough cleaning, which is a particularly important aspect when handling biological samples.

As for most adsorptive, high selectivity techniques, ion exchange chromatography is normally carried out in short columns. A typical ion exchange column is packed to a bed height of 5-15 cm. Once the separation parameters have been determined, scale-up is easily achieved by increasing the column diameter.

## 8. Quantity and preparation of ion exchanger

The amount of ion exchanger required for a given experiment depends on the amount of sample to be chromatographed and on the available or dynamic capacity of the ion exchanger for the sample substances. For the best resolution in ion exchange chromatography, it is not usually advisable to use more than 10-20% of this capacity, although this value can be exceeded if resolution is adequate. Preparation of the ion exchanger Having chosen the appropriate ion exchanger and starting buffer it is essential that the exchanger is brought to equilibrium with start buffer before sample application.

To prepare the gel, the supernatant is decanted and replaced with starting buffer to a ratio of approximately 75% settled gel to 25% buffer. If large amounts of ion exchangers are to be equilibrated with a weak buffer, the ion exchanger should first be equilibrated with a 10 times concentrated buffer solution at the correct pH, and then with a few volumes of starting buffer.

## 9. Sample preparation

The amount of sample which can be applied to a column depends on the dynamic capacity of the ion exchanger and the degree of resolution required. For the best resolution it is not usually advisable to use more than 10-20% of this capacity(11). Information on the available capacities for the different exchangers is given in the relevant product sections.

The ionic composition should be the same as that of the starting buffer. If it is not, it can be changed by gel filtration on Sephadex G-25 using.Desalting Columns, dialysis, diafiltration or possibly by addition of concentrated start buffer.

If the ion exchanger is to be developed with the starting buffer (isocratic elution), the sample volume is important and should be limited to between 1 and 5% of the bed volume. If however, the ion exchanger is to be developed with a gradient, starting conditions are normally chosen so that all important substances are adsorbed at the top of the bed. In this case, the sample mass applied is of far greater importance than the sample volume. This means that large volumes of dilute solutions, such as pooled fractions from a preceding gel filtration step or a cell culture supernatant can be applied directly to the ion exchanger without prior concentration.Ion exchange thus serves as a useful means of concentrating a sample in addition to fractionating it. If contaminants are to be adsorbed, and the component of interest is allowed to pass straight through, then the sample volume is less important than the amount of contaminant which is present. Under these conditions there will be no concentration of the purified component, rather some degree of dilution due to diffusion.

The viscosity may limit the quantity of sample that can be applied to a column. A high sample viscosity causes instability of the zone and an irregular flow pattern. The critical variable is the viscosity of the sample relative to the eluent. This corresponds to a protein concentration of approximately 5%. Approximate relative viscosities can be quickly estimated by comparing emptying times from a pipette.

## 10. Sample load

When the selectivity parameters have been defined to achieve the most optimal balance between resolution, capacity, speed and recovery, in ion exchange chromatography, as for most other adsorption techniques, there are then basically two alternative routes to follow for optimization of sample load and flow rate to achieve highest possible productivity in the system. I. In a typical capture situation the sample will be applied to the column, nonbound substances will be washed out from the column and the compound of interest will be eluted from the column with a simple step elution procedure. The difference in eluting strength, between the different steps will usually be large, i.e. it will be possible to elute one group of compounds while the others are still retained on the column. In this mode, the entire bed volume can be utilized for sample bin ding and the prime consideration when optimising for highest possible productivity is to define the highest possible sample load over the shortest possible sample application time with acceptable loss in yield.

## 11. Flow rate

The maximum flow rate that can be applied in any particular ion exchange chromatography step will differ between different parts of the chromatographic cycle. Since low molecular weight substances show high diffusion rates, i.e. are transported rapidly between the mobile phase and stationary phase, the flow rate during equilibration, washing and regeneration procedures is limited primarily by the rigidity of the chromatography media and by system constraints regarding pressure specification. Larger molecules, i.e. the substances to be separated during the

Chromatographic run, show a lower diffusion rate which will limit the flow rate that can be applied during sample adsorption and desorption. In a typical capture situation, the flow rate during sample application has to be controlled so that the residence time in the column allows for a complete binding without leakage in the flow through fraction. Maximum flow rate is defined by running the frontal analysis test (break-through) referred to above at a number of different flow rates. Optimal conditions will depend on the requirements for speed and capacity in the system. If speed, i.e. sample application time, is critical due to proteolysis or other detrimental effects in the feed material, a higher flow rate may have to be used on the expense of the binding capacity in terms of amount of sample that can be applied per volume of media. If speed is not a big issue, binding capacity can be increased on the expense of flow rate which will reduce the scale of work in the final production process (12).

## 12. Elution

If starting conditions are chosen such that only unwanted substances in the sample are adsorbed, then no change in elution conditions is required since the substance of interest passes straight through the column. Similarly no changes are required if sample components are differentially retarded and separated under starting conditions. This procedure is termed isocratic elution, and the column is said to be developed under starting conditions. Isocratic elution can be useful since no gradient apparatus is required for the run and, if all retarded substances elute, regeneration is not required. Normally, however, separation and elution are achieved by selectively decreasing the affinity of the solute molecules for the charged groups on the gel by continuously changing either buffer pH or ionic strength or possibly both. This procedure is termed gradient elution.

## 13. Change of pH and ionic strength

The net charge on a molecule depends on pH. Thus altering the pH towards the isoelectric point of a substance causes it to lose its net charge, desorb, and elute from the ion exchanger (13).

At low ionic strengths, competition for charged groups on the ion exchanger is at a minimum and substances are bound strongly. Increasing the ionic strength increases competition and reduces the interaction between the ion exchanger and the sample substances, resulting in their elution.

## 14. Regeneration

After each cycle, bound substances must be washed out from the column to restore the original function of the media. Ion exchange adsorbents can normally be regenerated after each run by washing with a salt solution until an ionic strength of about 2 M has been reached. This should remove any substances bound by ionic forces. The salt should contain the counter-ion to the ion exchanger to facilitate equilibration. To prevent a slow build up of contaminants on the column over time, more rigorous cleaning protocols may have to be applied on a regular basis

## 15. Applications

Ion exchange has proven to be one of the major methods of fractionation of labile biological substances. In the development of modern high performance media for purification, ion exchange chromatography has played a major role in the separation and purification of biomolecules and contributed sgnificantly to our understanding of biological processes. Analytical and preparative applications from the research laboratory. Ion exchange chromatography has played a role in the purificationof thousands of enzymes, and using modern matrices with optimized separation conditions gives extremely high recoveries. Normally the isoforms of an enzyme have approximately the same molecular weight. This makes their separation impossible by gel filtration. However, the small differences in charge properties resulting from altered amino acid composition enable the separation of isoenzymes using ion exchange chromatography. Ion exchange is frequently used for the purification of immunoglobulins.

Ion exchange chromatography also has many important applications in the field of industrial and pilot scale preparations. Many blood products such as albumin and IgG as well as the products of recombinant DNA technology, such as growth factors and pharmaceutically important enzymes are purified using this technique. Analytical applications of ion exchange chromatography are to be found in diverse areas such as quality control of purified products or process monitoring in biotechnology.

Other areas of application include food research where FPLC ion exchange can be used in the and in clinical research where ion exchange chromatography has been used in studies such as the relationship between post-partum depression and β-endorphin secretion and the correlation of a chromatogram of the urine from patients exhibiting tubular proteinuria, due to acute pyelonephritis, severe burns or renal transplants, shows distinct peaks corresponding to β2-microglobulin, retinol binding protein and α1-acid glycoprotein.

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