Ion Exchange: Mechanism and Factors Affecting Separation

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INTRODUCTION

Ion-exchange chromatography (IEC) is a technique in which ionic solutes bind to charged functional groups on the bonded phase. The power and versatility of IEC as an analytical and preparative technique is due in large part to the ability to drastically change the selectivity through manipulation of the mobile phase. Although it is obvious that the pH determines the charge on the support and the analytes, the nature of the salt is an equally important parameter. The constituent ions of the salt associate with the support functional groups and/or those of the solute, yielding distinct ionic interactions. Mobile-phase additives, temperature, and gradient conditions also contribute to the separation in IEC.

MOBILE PHASE

pH

Adjustment of the pH is a critical factor in IEC because the pH dictates the charge of both the solutes and the ion exchanger, thus controlling their affinity for one another or their ability to release from a bound state. The essential nature of pH in the process necessitates its exact control; therefore, any mobile phase used for IEC should contain an effective buffer (0.02–0.1 M) within its optimum pH range. Some common buffers which cover much of the range of pH used in IEC are phosphate, citrate, acetate, and tris(hydroxymethyl) aminomethane (Tris). The pH should be selected to yield ionization of the functional groups on the support as well as those on the analytes. For molecules with a single charge, the pH should be at least two units from the pK in the direction of ionization. The guideline for zwitterions is that the pH be at least two units from the isoelectric point (pI). A pH near neutrality is often effective for complex mixtures of diverse substances. Even carbohydrates, whose hydroxyl groups do not ionize until the pH is greater than 12, can be separated by IEC when the pH is adjusted to a high enough value with low concentrations of base as the mobile phase.

The choice of pH for IEC of proteins or other macromolecules is not as simplistic as it is for small molecules. Although using the pI as a guide frequently yields an adequate separation, the pI encompasses all the charged groups in the molecule, whereas, because of their defined tertiary structures, only the surface amino acids of proteins are actually involved in the binding. Under denaturing conditions, more amino acids are likely to be exposed to the bonded phase.

Salt Concentration

IEC is a very predictable technique because the mechanism is well defined. The capacity factor (k) for the binding of an ionic solute to an ion-exchange functional group in IEC is directly related to the concentration (c) of salt in the mobile phase:

\[
\log k = \log K_0 + Z_c \log \left( \frac{1}{c} \right)
\]

where \( K_0 \) is the distribution coefficient and \( Z_c \) is an experimentally determined parameter that reflects the apparent number of ionic charges associated with the process of a specific solute with a specific surface. For isocratic separations of simple molecules with up to several charges, the analysis time can be optimized along with resolution by adjustment of the salt concentration. For more complex analytes or mixtures, salt gradients are often necessary to achieve acceptable separations. Generally, a gradient from 0 to 1 M salt in a buffer at a suitable pH will yield a preliminary separation.

An opposite mechanism to ion exchange occurs when the ionic strength is too low. Ion exclusion is a phenomenon in which a charged analyte is repelled by the like charges within a pore. This is very likely to occur if water is used alone as the mobile phase with ion-exchange supports or with other modes of silica-based columns. Adding buffer and salt usually eliminates the problem.

Salt Composition

Elution with increased concentrations of salt is the most common and readily controlled method of achieving displacement of molecules which are strongly bound by an ion exchanger. The salt counterners competitively displace solute ions from the charged sites on the stationary phase. Smaller, more highly charged ions are most effective at this displacement. Specifically, the strength of displacement for cations...


\[ \text{Mg}^{2+} > \text{Ca}^{2+} > \text{NH}_4^+ > \text{Na}^+ > \text{K}^+ \]

is and for anions, it is

\[ \text{SO}_4^{2-} > \text{HPO}_4^{2-} > \text{Cl}^- > \text{CH}_3\text{COO}^- \]

The strength of the ions for displacement is not necessarily related to optimum selectivity or resolution. Selectivity is dictated by the effect of the salt on both the solute and the bonded phase. Besides displacing the solute from the support, either of the ions of the salt can complex with the ion-exchange functional group or the solute, alter the tertiary structure of the solute, or enhance hydrophobic properties. It is this combination of effects which results in selectivity. For example, when a mixture of proteins was run on a polyethyleneimine (PEI) weak anion-exchange column with gradients formed with 1.0 N salt, substitution of sodium acetate for sodium phosphate produced not only longer retention but also much better resolution of the proteins. Sodium phosphate produced narrower peaks with less tailing, but the peaks had only slight differences in retention. In this case, the short retention was proven to be due to a special affinity of phosphate for PEI, which did not occur with anion-exchange supports having quaternary (Q) or diethylaminoethanol (DEAE) functional groups. The salt effects on selectivity encompass anions and cations in both anion-exchange and cation-exchange chromatography, as illustrated in Fig. 1, implying that the selectivity occurs because of ionic interactions with the functional groups of both the support and the solute. In the case of adenosine 5-diphosphate (ADP), divalent ions like calcium can bridge between the oxygens in the phosphate and thus reduce the ionic properties. Phosphate salts reduce the retention of ADP on PEI supports due to the phosphate–PEI affinity discussed earlier. Another example of ion-based selectivity is the excellent resolution obtained for sugars when a calcium salt is used with a cation-exchange resin. This ability to change selectivity so dramatically by varying the salt significantly broadens the utility of IEC.

The only restrictions on the choice of salt are those involving analyte solubility or stability. Volatile salts such as ammonium acetate even allow IEC to be interfaced with

![Fig. 1 Anion-exchange chromatography (AEX): SynChropak AX300 (polyethyleneimine, 300 Å, 6 μm); cation-exchange chromatography (CEX): SynChropak CM300 (carboxymethyl, 300 Å, 6 μm); 30 min gradient (0–1N) of sodium or chloride salts in 0.02 M Tris, pH 7. Source: Reprinted with permission of MICRA Scientific.](image-url)
mass spectrometry or evaporative lightscattering detection. It is very important that a given salt be totally stripped from a support before changing to other ions to avoid mixed ion effects. An acid such as trifluoroacetic acid is often effective as a bridge/washing solvent for this purpose.

**SURFACTANTS AND ORGANIC SOLVENTS**

Secondary separation which may be present in IEC is generally size exclusion or hydrophobicity. Size exclusion will occur if macromolecules are larger than the pores in a support. Hydrophobic interactions are most often observed under conditions of high salt for solutes with significant non-polar characteristics, such as certain peptides. The hydrophobicity of an ion-exchange support is due to either the matrix or the cross-linking agents which were employed in the synthesis of the bonded phase. Any hydrophobic interactions are fundamentally undesirable and can be minimized by the addition of 1–10% of an organic solvent, such as methanol, ethanol, or acetonitrile, to the running buffer. The solubility of the salt in the organic mobile phase should always be verified to avoid precipitation.

Non-ionic detergents may also reduce hydrophobic interactions with a column. These detergents, such as CHAPS or urea, can also be added to ion-exchange mobile phases to aid in the solubilization of membrane or other insoluble proteins. Such detergents are easy to equilibrate and remove from ion-exchange columns; however, ionic detergents should be avoided because of their very strong binding to the column or the solutes.

**FLOW RATE AND GRADIENT**

Small molecules can often be effectively separated isocratically by IEC; however, due to multipoint interactions, isocratic IEC of proteins and most biological macromolecules is not usually feasible, yielding no resolution and extreme tailing. Such complex molecules are generally separated by gradient elution.

As a salt gradient proceeds to higher levels in IEC, molecules elute at a specific salt concentration, generally without binding from secondary effects. The relationship of gradient conditions to elution \( k^* \) can be described by

\[
k^* = 0.87t_G \frac{F}{V_M} \left( \log \frac{C_2}{C_1} \right) Z
\]

where \( C_1 \) and \( C_2 \) are the total salt concentrations (salt plus buffer) at the beginning and the end of the gradient, respectively, \( Z \) is the effective charge on the solute molecule, \( F \) is the flow rate; \( V_M \) is the total mobile-phase volume, and \( t_G \) is the gradient time.\(^6\) The \( Z \) number will vary with solute and pH. An initial ion-exchange protocol of a 20–30 min linear gradient from 0–1 M salt in a buffer at a suitable pH will usually yield a separation which can be later optimized, if necessary. For shortest analysis times, a gradient should begin at the highest salt concentration where the analytes are bound and it should end at the lowest ionic strength that causes elution. The pH gradients may also be used to elicit elution during IEC, although this has been a less popular strategy than salt gradients. Ion-exchange columns can be effectively washed with a mobile phase of higher ionic strength than the upper gradient limit or with low pH. For gradients, intermediate flow rates of 1 ml/min for a 4.6 mm inner diameter column are usually satisfactory.

**TEMPERATURE**

The use of elevated temperature in IEC reduces the mobile-phase diffusion coefficient and concomitantly

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Fig. 2 Column: Micropak AA (sulfonated polystyrene); solvent A: 0.2 M sodium citrate, pH 3.25; solvent B: 1 M sodium citrate, pH 7.40. Gradient: 5 min 100% A; 100–75% A in 20 min; 75–70% A in 5 min; 70–35% A in 5 min; 10 min 35%; 35–0% A in 1 min. \( T = 50^\circ C \) for 25 min, then 90° C. Detection after ninhydrin postcolumn reaction.

**Source:** Reprinted from Amino acid analysis with ninhydrin postcolumn derivatization, *LC at Work*, Varian Associates, with permission.
decreases band spreading. Most mobile phases in IEC are composed of water with salts and thus produce efficiencies which are less than those obtained in modes using organic solvents. Because increased temperatures decrease retention, they may permit the use of lower salt concentrations. Elevated temperatures have been especially effective in amino acid analyses by cation-exchange chromatography, as illustrated in Fig. 2.

CONCLUSIONS

The effectiveness of IEC as a method for separating charged species is enhanced by the ability of many operational factors to change the selectivity and resolution. Salt concentration, salt composition, and pH are the most important operational parameters which strengthen the versatility of the technique.

REFERENCES