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Volume 251

# HPLC of Peptides and Proteins

## *Methods and Protocols*

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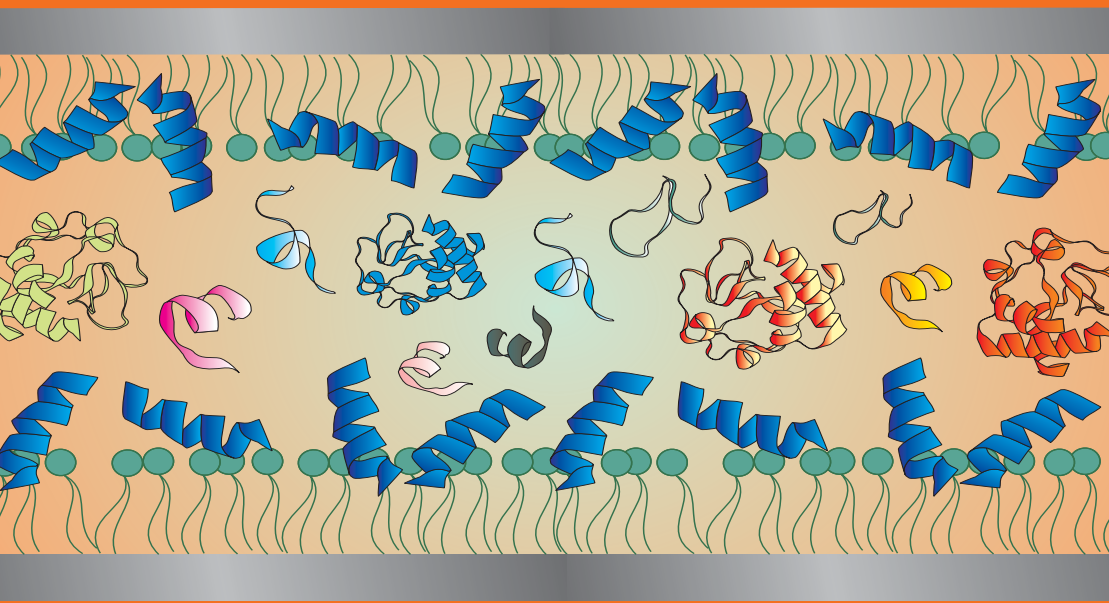

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# Ion-Exchange Chromatography

**Peter Stanton**

## 1. Introduction

Ion-exchange chromatography (IEX) separates biomolecules (proteins, polypeptides, nucleic acids, polynucleotides, charged carbohydrates, and polysaccharides) based on differences in their charge. IEX can be a highly selective chromatographic technique, being able to resolve, for example, proteins which differ by only a single charged group ***(1)***. The process relies upon the formation of ionic bonds between the charged groups on biomolecules (typically, $-NH_3^+$, $=NH_2^+$, $\geq NH^+$, $-COO^-$, $PO_4^-$, $SO_3^{2-}$), and an ion-exchange gel/support carrying the opposite charge. Non-bound biomolecules (i.e., neutral molecules which do not carry any electrical charge, or molecules carrying the same charge as the ion-exchange support) are removed by washing, and bound biomolecules are recovered by elution with a buffer of either higher ionic strength, or altered pH. The advantages of IEX are 1) high resolving power, 2) separations can be fast, 3) in general, recoveries are high, 4) buffer components are nondenaturing and frequently compatible with further downstream chromatographic separation or assay systems, 5) process can be used as a concentration step, to recover proteins from a dilute solution. The disadvantages of IEX are few, but include 1) the sample must be applied to the IEX support under conditions of low ionic strength and controlled pH, which sometimes requires an extra buffer exchange step to be inserted, 2) chromatographic instrumentation should be resistant to salt-induced corrosion, and 3) postchromatographic concentration of dilute solutions of recovered proteins can result in high salt concentrations (>1 *M*), unsuitable, for example, in biological assays unless buffer exchange is carried out. Applications for IEX are numerous, from analytical scale column chromatographic separations in the research laboratory

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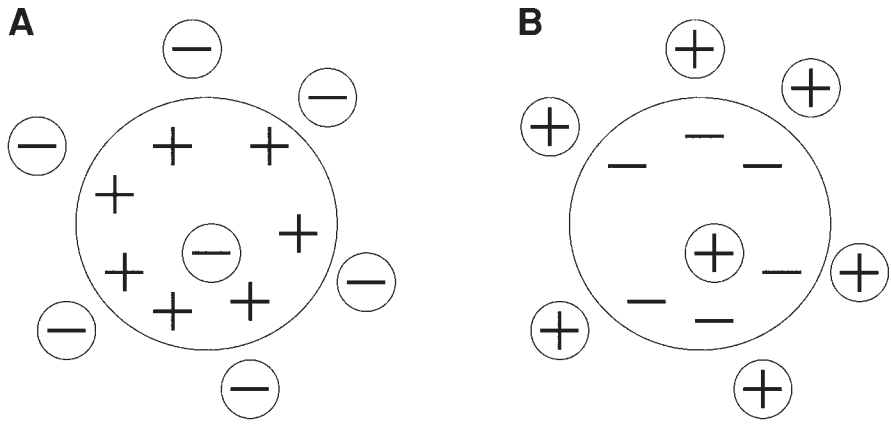


Fig. 1. Types of ion-exchangers. For an anion-exchanger **(A)**, the gel matrix is positively charged, with negatively charged counter-ions (anions) in solution. These anions are reversibly exchanged with other anions in the process of ion-exchange chromatography. For a cation-exchanger **(B)**, the gel matrix is negatively charged, with positively charged counter-ions (cations) in solution.

through to preparative scale column separations at the industrial level. In selected cases, IEX can also be applied successfully in a batch-mode, which has advantages in simplicity and lack of requirement for expensive chromatographic equipment.

There are two basic types of ion-exchangers: 1) anion-exchanger and 2) cation exchanger. The anion exchanger has positively charged groups which have been immobilized onto a chromatographic support, and will therefore bind and exchange negatively charged ions (anions) (*see* **Fig. 1A**), while a cation-exchanger has negatively charged immobilized groups which will bind and exchange positively charged ions (cations) (*see* **Fig. 1B**). In solution, proteins and other biomolecules are ionized, and the extent of ionization is dependent on the pH of the solution. For any given protein, the pH at which the total positive charge is equal to the total negative charge is known as the isoelectric point (p*I*). Hence, when pH = p*I*, the total *net* charge on the protein is zero (*see* **Fig. 2**). At pHs less than the p*I*, the total net charge on the protein will be positive, thus the protein should bind to a cation-exchange column. At pHs greater than the p*I*, the total net charge on the protein will now be negative, and the protein should bind to an anion-exchange column (*see* **Fig. 2**). As a rule of thumb, proteins with p$I < 6$ (i.e., acidic proteins) are chromatographed on an anion-exchange column, while proteins of p$I > 8$ (basic proteins) are chro-

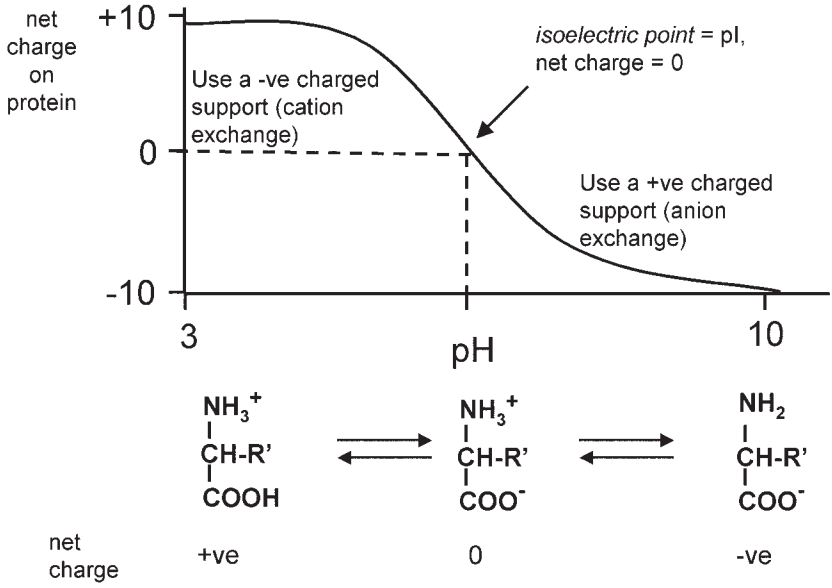


Fig. 2. Generic titration curve for proteins. In solution, charged groups on proteins are ionized, and the extent of ionisation is dependent on solution pH. At a particular pH known as the isoelectric point (p*I*), the total positive charge on the protein is equivalent to the total negative charge, hence the *net* protein charge is zero. At pHs more acidic than the p*I*, the net protein charge is positive as carboxyl and other acidic groups are protonated, and amino groups are ionised. Under these conditions, a negatively charged ion-exchanger (cation exchange) should be used. Conversely, at pHs more basic than the p*I*, the net protein charge is negative, and a positively charged ion-exchanger (anion exchange) should be employed.

matographed on a cation-exchange column, and proteins with p*I* between 6 and 8 can be chromatographed on either type ***(5)***. In the lab, if the p*I* of the protein(s) of interest is unknown, it will be necessary to conduct a pilot experiment to determine the best ion exchanger to use (*see* **Subheading 3.2.**). A list of the p*I*s of a series of standard proteins is shown in **Table 1**.

Despite the fact that the net charge on a protein is zero at its p*I*, it is not unusual that some binding to ion-exchangers will occur. This is owing to 1) the nonuniform distribution of charged groups on the surface of the protein and 2) potential for differences between the pH of the microenvironment inside the pores of the ion-exchange support and the pH of the bulk eluent ***(3)***. Regardless, as the solubility of many proteins is lowest at or near their p*I* ***(4)***, it is suggested to avoid chromatography at this pH value to prevent potential on-column precipitation (*see* **Note 1**).

**Table 1**
**Isoelectric Points and Molecular Weights of Some Standard Proteins[a]**

| Protein | p*I* | M. Wt |
|---|---|---|
| Pepsin | ~1 | 35,500 |
| Ovalbumin | 4.6 | 43,000 |
| Thyroglobulin | 4.6 | 660,000 |
| Albumin (bovine serum) | 4.9 | 68,000 |
| Urease | 5.0 | 480,000 |
| β-Lactoglobulin | 5.2 | 20,000 |
| Insulin | 5.3 | 5,700 |
| Hemoglobin (horse) | 6.8 | 64,000 |
| Myoglobin (horse) | 7.0 | 17,500 |
| Carbonic anhydrase | 7.3 | 29,000 |
| Chymotrypsinogen | 9.5 | 25,700 |
| Cytochrome-*c* | 10.7 | 12,000 |
| Lysozyme (hen egg white) | 11.0 | 14,300 |

[a]Data from **refs. *2*** and ***5***.

The types of charged groups commonly immobilized to chromatographic supports are shown in **Table 2**. These groups can be categorized into *weak* or *strong* ion-exchangers, depending on the pH range over which the exchanger remains charged. Strong ion exchangers, typically containing sulphonic acid groups (cation-exchange) or quaternary amino groups (anion-exchange) (*see* **Table 2**), remain ionized over a wide pH range, whereas weak ion-exchangers are ionized in a narrower pH range. Hence, an advantage for the use of a strong ion-exchanger is that the charge on the exchanger is independent of pH over the pH range 2–12, and therefore, the interaction between the solute and the exchanger follows a simple mechanism ***(1)***. In addition, the sample loading capacity of the matrix is not altered at high or low pH. In contrast, the sample loading capacity of a weak ion-exchanger varies considerably with pH, therefore both the charge on the support and the amount of sample which can be loaded can be less predictable ***(3)***. It is important to realize that the terms "weak" and "strong" *do not* refer to the strength of attraction between the charged exchanger and the solute/molecule of interest.

It is beyond the scope of this chapter to consider the theoretical mechanisms that affect resolution, selectivity, and capacity in ion-exchange chromatography. For detailed considerations of the retention behavior of biomolecules on IEX supports, the reader is referred to reviews elsewhere ***(1,3)***.

**Table 2**
**Types of Functional Groups Immobilized on Ion-Exchange Gels[a]**

| Functional group | Name | Type of exchanger |
|---|---|---|
| $-O-CH_2CH_2N^+H(CH_2CH_3)_2$ | Diethylaminoethyl (DEAE) | Weak anion |
| $-O-CH_2CH_2N^+H(CH_2CH_3)_2-CH_2-CHOH-CH_3$ | Quaternary aminoethyl (QAE) | Strong anion |
| $-O-CH_2OH-CH_2-O-CH_2-CHOH-CH_2-N^+(CH_3)_3$ | Quaternary ammonium (Q) | Strong anion |
| $-O-CH_2-COO^-$ | Carboxymethyl (CM) | Weak cation |
| $-O-CH_2-CHOH-CH_2-O-CH_2-CH_2-CH_2SO_3^-$ | Sulphopropyl (SP) | Strong cation |
| $-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2SO_3^-$ | Methyl sulphonate (S) | Strong cation |

[a]Adapted from **ref. *1***.

## 2. Materials

### *2.1. Gel Media and Selection Criteria*

The selection of appropriate ion-exchange gel media depends on the characteristics of the biomolecule in question (including p*I*, molecular size, and pH stability), as well as on the type of separation being considered (analytical or preparative scale), the column capacity required (*see* **Subheading 2.2.**), the stability of the various supports at the chosen pH, and finally the cost of the support. IEX supports for low-pressure applications are commonly based on polysaccharides (agarose, dextran, cellulose), whereas for HPLC, supports are based on silica particles or polymers (cross-linked polystyrene/divinyl benzene, or methacrylate copolymer) (*see* **Table 3** for a list of some of the commercially available IEX supports).

If the p*I* of the protein is unknown, then one should either set up a pilot experiment to choose the optimal exchanger (*see* **Subheading 3.2.**), or use an anion-exchange support with a running buffer of pH 8.0–8.5 as a starting point. This latter alternative recognizes that the pI of many proteins is less than 7–7.5 ***(3)***, hence, these will bind to an anion-exchanger at pH 8.5. If the pI of the protein is known, choose either an anion-exchange support with running buffers of pH at least one pH unit greater than the pI, or a cation exchanger with running buffers at least one pH unit below the pI ***(1)***. Include at this selection stage any knowledge about the pH stability of the protein of interest, as some proteins readily denature or lose biological activity at extremes of pH.

As a starting point, choose either a strong anion or strong cation exchanger (*see* **Table 2**), as these supports give a more consistent performance and loading capacity over a wide pH range. After the initial experiments are completed, chromatography on the weak ion-exchangers can be investigated, as in some

cases, these can yield improved resolution although this is sample dependent and must be empirically established.

IEX supports differ in their mechanical stability. Exchangers based on "soft" supports (agarose, dextran, or cellulose, *see* **Table 3**) are suitable for low pressure, open column chromatography of proteins, but these supports are limited to lower linear flow rates, and can swell and shrink to differing extents depending on the pH and ionic strength of the eluent ***(1)***. Alternatively, high-performance media with small, controlled bead sizes (5–10 µm) manufactured from silica, or rigid crosslinked polystyrene/divinyl benzene derivatives (for example, Mono-Q, Mono-S, **Table 3**), do not swell or shrink and are stable at greater pressures making them suitable for HPLC ***(5)***. Silica-based HPLC packings have a disadvantage in that they must be used in the pH range 2–8, as more basic pHs cause the packing to hydrolyze and disintegrate. The mechanical stability of these supports makes them an attractive choice for analytical-scale ion-exchange chromatography, however, commercial cost can become an issue for preparative scale purification. This problem has been addressed by the availability of similar monodisperse packings with particle sizes of 15, 25, or 30 µm (e.g., Source 15S, MacroPrep 25S) suitable for larger scale applications ***(1)***.

Pore-size varies between the various IEX supports (*see* **Table 3**). In general, supports with smaller pore sizes (~125 Å) are best suited for low molecular weight solutes such as nucleotides and small peptides, whereas larger pore sizes (300 Å or greater) will work better for larger peptides, proteins, and nucleic acids ***(3)***. Detailed information about the exclusion limits of particular IEX supports should be checked in the product literature. As examples, Sephadex ion exchangers are based on either the Sephadex G-50 or Sephadex G-25 particles, of which the G-50 support is more porous and better suited for the chromatography of proteins of molecular weights greater than 30,000 ***(1)***. Similarly, the TSK DEAE-2SW prepacked HPLC column (*see* **Table 3**) has a pore size of 125 Å and an exclusion limit of 10,000 Daltons, whereas the TSK DEAE-5PW HPLC column has a pore size of 1000 Å and an exclusion limit of 1,000,000 Daltons.

Invariably, the choice of IEX support will involve some level of compromise between the main factors that influence resolution (particle type and size, porosity) and capacity and cost. Small particle sizes (10 µm) give higher resolution, but also give higher backpressure, which limits flow rates and requires specialist pumps and associated hardware. Larger particles (25–50 µm) allow faster flow rates and lower backpressures, but do not have the same resolution as smaller particles. Note also that the capacity of ion-exchange supports tends to increase with particle size (*see* **Subheading 2.2.**), therefore choose a support that has the smallest particle size (*see* **Table 3**) consistent with the scale of the purification.

### 2.2. Capacity of IEX Supports

The capacity of an ion-exchange support for a particular sample is dependent on a number of variables, including charge and molecular size of the sample, experimental conditions including buffer pH and ionic strength, and flow rate. Product information from the manufacturers can include various measures of capacity, which are 1) total ionic capacity, 2) available capacity, and 3) dynamic capacity ***(4)***. The total ionic capacity of an exchanger refers to the total amount of immobilized charged groups on the support. In practice, most ion-exchangers are porous (*see* **Table 3**), and the ability of large sample ions such as proteins to access all immobilized charged groups is limited by their molecular size and shape. Hence, *available capacity* refers to the amount of a specific protein that will bind to the ion-exchanger under defined conditions of pH, ionic strength, and sample concentration. This parameter is determined in a static or batch type of experiment, and does not take into account the effects of flow rate and column dimensions that contribute to capacity when the ion-exchanger is used in a column chromatography format, which is known as *dynamic capacity*. The dynamic capacity of an ion-exchanger is normally less than the available capacity under the same experimental conditions ***(4)***.

In practice, the available or dynamic capacities of ion-exchangers (*see* **Table 3**) will be of most use in deciding how much of the support is needed for a particular experiment. Where possible, consult the product literature and use the reported capacity as determined with a protein of similar molecular weight to that being purified, and under similar conditions of pH and ionic strength. It is generally recommended not to exceed ~10–20% of the stated capacity if maximum resolution is to be maintained ***(1)***. If column capacity is expected to be an important parameter in the success of your purification scheme, (e.g., for increased sample throughput, shorter overall purification times, and better economy) consideration should be given to pilot experiments aimed at determining ion-exchange capacity with your target protein.

### 2.3. Choice of Appropriate Buffers

As the mechanism of ion-exchange chromatography involves binding of a charged sample to the ion-exchange support, it is preferable that the buffering ions not compete in this process ***(1,4)***. Therefore, the buffering ions should be of the same charge as the ion-exchanger, meaning that anionic buffers (e.g., phosphate, acetate, citrate) should be used for cation-exchange, and cationic buffers (e.g., piperazine, Tris, bis-Tris) for anion-exchange. A list of commonly used buffers and recommended concentrations is given in **Table 4**. For the same reason, any buffer additives (e.g., detergents, protease inhibitors) should also be either noncharged, or carry the same charge as the ion-exchanger. Keep

## Table 3
## List of Some of the Commercially Available Ion-Exchangers[a,b]

| Product by IEX type[c] | Functional group | Matrix type | Particle size (μm) | Pore size (Å) | Total ionic capacity (μmoles/mL gel) | Available capacity[d] (mg/mL gel) | Dynamic capacity[d] (mg/mL gel) | pH range[e] | Form[f] | Company[g] |
|---|---|---|---|---|---|---|---|---|---|---|
| SCX | | | | | | | | | | |
| Mono S | Sulphonic acid | Crosslinked PS/DVB[i] | 10 | – | 140–180 | – | 75 (human IgG) | 3–11 | pp | AB |
| Mini S | Sulphonic acid | Crosslinked PS/DVB | 3 | – | 16–30 | – | 1.3[j] (ribonuclease) | 3–11 | pp | AB |
| Source 15S | Sulphonic acid | Crosslinked PS/DVB | 15 | – | na | – | 80 (lysozyme) | 2–12 | pp,b | AB |
| Source 30S | Sulphonic acid | Crosslinked PS/DVB | 30 | – | na | – | 80 (lysozyme) | 2–12 | pp,b | AB |
| SP Sepharose HP | Sulphopropyl | Crosslinked 6% agarose | 24–44 | – | 140–200 | – | 55 (RNase) | 4–13 | pp,b | AB |
| SP Sepharose FF | Sulphopropyl | Crosslinked 6% agarose | 45–165 | – | 180–250 | – | 50 (human IgG) | 4–13 | pp,b | AB |
| SP Sephadex | Sulphopropyl | Crosslinked dextran | 40–125 | – | 90–300 | – | 1.6–8.0 (human IgG) | na | b | AB |
| TSK SP-5PW | Sulphopropyl | hydrophilic resin | 10 | 1000 | na | 40 (hemoglobin) | – | 2–12[h] | pp | S, A |
| TSK SP-NPR | Sulphopropyl | Hydrophilic resin | 2.5 | – | | 5 (hemoglobin) | | 2–12[h] | pp | S, A |
| Macro-Prep 25S | Sulphonic acid | Hydrophilic resin | 25 | 725 | 110±30 | – | – | 1–14 | b | BR |
| Macro-Prep High S | Sulphonic acid | Hydrophilic resin | 50 | 1000 | 160±40 | – | 60 (human IgG) | 1–14 | b | BR |
| PL-SCX 1000 Å | Sulphonic acid | Macroporous PS/DVB | 8,10 | 1000 | na | – | – | 1–14 | pp | P |
| PL-SCX 4000 Å | Sulphonic acid | Macroporous PS/DVB | 8,10 | 4000 | na | – | – | 1–14 | pp | P |
| SynChropak SCX | Sulphonic acid | Silica | 6 | 300 | na | – | – | 2–8 | pp | A |
| WCX | | | | | | | | | | |
| CM Sepharose FF | Carboxymethyl | Crosslinked 6% agarose | 45–165 | – | 90–130 | – | 50 (RNase) | 6–10 | pp,b | AB |
| CM Sephadex | Carboxymethyl | Crosslinked dextran | 40–125 | – | 170–550 | – | 1.6–7.0 (human IgG) | 6–10 | b | AB |
| TSK CM-5PW | Carboxymethyl | Hydrophilic resin | 10 | 1000 | na | 45 (hemoglobin) | – | 2–12[h] | pp | S, A |
| TSK CM-2SW | Carboxymethyl | Silica | 5 | 125 | na | na | – | 2–7.58 | pp | S, A |
| TSK CM-3SW | Carboxymethyl | Silica | 10 | 250 | na | 110 (haemoglobin) | – | 2–7.58 | pp | S, A |
| Macro-Prep CM | Carboxymethyl | Hydrophilic resin | 50 | 1000 | 210±40 | – | – | 1–14[h] | b | BR |
| SynChropak WCX | Carboxymethyl | Silica | 6 | 300 | na | – | – | 2-8 | pp | A |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| SAX | | | | | | | | | | |
| Mono Q | Quaternary amine | Crosslinked PS/DVB | 10 | – | 270–370 | – | 65 (BSA) | 3–11 | pp | AB |
| Mini Q | Quaternary amine | Crosslinked PS/DVB | 3 | – | 60–90 | – | 1.4[j] (α-amylase) | 3–11 | pp | AB |
| Source 15Q | Quaternary amine | Crosslinked PS/DVB | 15 | – | na | – | 45 (BSA) | 2–12 | pp,b | AB |
| Source 30Q | Quaternary amine | Crosslinked PS/DVB | 30 | – | na | – | 45 (BSA) | 2–12 | pp,b | AB |
| Q Sepharose HP | Quaternary amine | Crosslinked 6% agarose | 24–44 | – | 140–200 | – | 70 (BSA) | 2–12 | pp,b | AB |
| Q Sepharose FF | Quaternary amine | Crosslinked 6% agarose | 45–165 | – | 180–250 | – | 120 (HSA) | 2–12 | pp,b | AB |
| QAE Sephadex | Diethyl-(2-hydroxy propyl)aminoethyl | Crosslinked dextran | 40–125 | – | 100–500 | – | 10–80 (HSA) | na | b | AB |
| Macro-Prep 25 Q | Quaternary amine | Hydrophilic resin | 25 | 725 | 220±40 | – | | 1–14 | b | BR |
| Macro-Prep High Q | Quaternary amine | Hydrophilic resin | 50 | 1000 | 400±75 | – | 40 (BSA) | 1–14 | b | BR |
| PL-SAX 1000 Å | Quaternary amine | Macroporous PS/DVB | 8,10 | 1000 | na | – | | 1–14 | pp | P |
| PL-SAX 4000 Å | Quaternary amine | Macroporous PS/DVB | 8,10 | 4000 | na | – | | 1–14 | pp | P |
| SynChropak SAX | quaternary amine | silica | 6 | 300 | na | – | | 2–8 | pp | A |
| WAX | | | | | | | | | | |
| DEAE Sepharose FF | Diethylaminoethyl | Crosslinked 6% agarose | 45–165 | – | 110–160 | – | 110 (HSA) | 2–9 | pp,b | AB |
| DEAE Sephacel | Diethylaminoethyl | Bead-formed cellulose | – | – | 100–140 | – | 160 (HSA) | 2–9 | b | AB |
| DEAE Sephadex | Diethylaminoethyl | Crosslinked dextran | 40–125 | – | 175–500 | – | 30–110 (HSA) | 2–9 | b | AB |
| TSK DEAE-5PW | Diethylaminoethyl | Hydrophilic resin | 10 | 1000 | na | 30 (BSA) | – | 2–12[h] | pp | S, A |
| TSK DEAE-NPR | Diethylaminoethyl | Hydrophilic resin | 2.5 | – | na | 5 (BSA) | – | 2–12[h] | pp | S, A |
| TSK DEAE-2SW | Diethylaminoethyl | Silica | 5 | 125 | na | na | – | 2–7.5[h] | pp | S, A |
| TSK DEAE-3SW | Diethylaminoethyl | Silica | 10 | 250 | na | 120 (BSA) | – | 2–7.5[h] | pp | S, A |
| Macro-Prep DEAE | diethylaminoethyl | hydrophilic resin | 50 | 1000 | 175±75 | – | – | 1–14[h] | pp | BR |
| SynChropak WAX | polyethyleneimine | silica | 6 | 300 | na | – | – | 2–8 | pp | A |

[a]Details as provided by the manufacturer.

[b]For a complete listing of commercially available ion-exchangers, *see* **ref. *3*** and ***5***.

[c]SCX, strong cation-exchange; WCX, weak cation-exchange; SAX, strong anion exchange; WAX, weak anion exchange.

[d]Capacity determined with the protein shown in parentheses.

[e]Refers to the pH range over which the ion-exchange groups remain charged, and a high sample capacity is maintained.

[f]Available as either pre-packed columns (pp), or bulk ion-exchanger (b).

[g]Further information available at: Amersham Biosciences (AB) http://www.chromatography.amershambiosciences.com, Bio-Rad (BR) http://www.bio-rad.com/, Sigma-Aldrich (SA) http://www.sigmaaldrich.com, Supelco (S) http://www.sigmaaldrich.com, Agilent (A) http://www.chem.agilent.com, Polymerlabs (P) http://polymerlabs.com.

[h]Refers to the stability of the matrix, as given by the manufacturer. For details of the working pH range of this support, see other exchangers with the same functional group.

[i]PS/DVB: polystyrene/divinyl benzene.

[j]mg/column.

**Table 4**
**Buffers for Ion-Exchange Chromatography[a]**

| $pK_a$ (25°C) | pH range | Buffer | Concentration (m*M*) | Counter-Ion |
|---|---|---|---|---|
| *Cation exchange* | | | | |
| 2.00 | 1.5–2.5 | Maleic acid | 20 | $Na^+$ |
| 2.88 | 2.4–3.4 | Malonic acid | 20 | $Na^+/Li^+$ |
| 3.13 | 2.6–3.6 | Citric acid | 20 | $Na^+$ |
| 3.81 | 3.6–4.3 | Lactic acid | 50 | $Na^+$ |
| 3.75 | 3.8–4.3 | Formic acid | 50 | $Na^+/Li^+$ |
| 4.21 | 4.3–4.8 | Butanedioic acid | 50 | $Na^+$ |
| 4.76 | 4.8–5.2 | Acetic acid | 50 | $Na^+/Li^+$ |
| 5.68 | 5.0–6.0 | Malonic acid | 50 | $Na^+/Li^+$ |
| 7.20 | 6.7–7.6 | Phosphate | 50 | $Na^+$ |
| 7.55 | 7.6–8.2 | HEPES | 50 | $Na^+/Li^+$ |
| 8.35 | 8.2–8.7 | BICINE | 50 | $Na^+$ |
| *Anion exchange* | | | | |
| 4.75 | 4.5–5.0 | *N*-methylpiperazine | 20 | $Cl^-$ |
| 5.68 | 5.0–6.0 | Piperazine | 20 | $Cl^-/HCOO^-$ |
| 5.96 | 5.5–6.0 | *L*-histidine | 20 | $Cl^-$ |
| 6.46 | 5.8–6.4 | *bis*-Tris | 20 | $Cl^-$ |
| 6.80 | 6.4–7.3 | *bis*-Tris propane | 20 | $Cl^-$ |
| 7.76 | 7.3–7.7 | Triethanolamine | 20 | $Cl^-/CH_3COO^-$ |
| 8.06 | 7.6–8.5 | Tris-Cl | 20 | $Cl^-$ |
| 8.52 | 8.0–8.5 | *N*-methyldiethanolamine | 50 | $SO_2^-/Cl^-/CH_3COO^-$ |
| 8.88 | 8.4–8.8 | Diethanolamine | 20 | $Cl^-$ |
| 8.64 | 8.5–9.0 | 1,3-Diaminopropane | 20 | $Cl^-$ |
| 9.50 | 9.0–9.5 | Ethanolamine | 20 | $Cl^-$ |
| 9.73 | 9.5–9.8 | Piperazine | 20 | $Cl^-$ |
| 10.47 | 9.8–10.3 | 1,3-Diaminopropane | 20 | $Cl^-$ |
| 11.12 | 10.6–11.6 | Piperidine | 20 | $Cl^-$ |

[a]Adapted from **refs. *1*** and ***4***.

in mind that buffer $pK_a$'s vary with temperature, and that the $pK_a$ data listed in **Table 4** are for 25°C. The extent to which these $pK_a$'s change with temperature is given elsewhere ***(1,4)***.

In most cases, cation-exchangers are supplied with $Na^+$ as the counter-ion, whereas $Cl^-$ is commonly used as the counter-ion for anion-exchangers. If required, the counter-ion can be changed by washing the IEX support in an

excess concentration (0.5–1.0 *M*) of a salt of the new counter-ion (*see* **Subheading 3.1.**).

### 2.4. Preparation of Buffers

Buffers should be prepared using high quality deionized or distilled water, and filtered and degassed as appropriate if HPLC columns are being used. In order to ensure run-to-run reproducibility, it is essential that buffer pH be accurately set using a quality pH electrode which is routinely calibrated using two pH standards. Monitor and record the volume of acid or base used to set the pH for any particular buffer, and as a quality control step make sure that a similar volume is always added. In some cases, buffers may include a proportion of an organic solvent (e.g., 10–20% (v/v) acetonitrile) to limit hydrophobic interactions with the exchanger, and the solvent should be added after the pH of the buffer has been set.

### 2.5. Batch vs Column Purification

IEX separations can be carried out using either a batch technique, or with the exchanger packed into a chromatographic column (or with prepacked columns supplied by the manufacturer). Each approach has its advantages. Batch processing can readily be applied to selectively and rapidly separate proteins into two groups, consisting of a group bound to the exchanger and subsequently eluted with elution buffer, and the nonbound group. If the desired protein is present exclusively in either group, a useful purification can be achieved using a minimum of specialist equipment. In contrast, much greater resolution between proteins can be gained using column chromatography, hence, this is the recommended approach if IEX is to be used as part of a purification strategy.

### 2.6. Mode of Elution

Elution of bound components from an ion-exchange support is normally achieved by increasing the salt concentration of the eluent, although changing the buffer pH can also be used. The salt concentration can be increased in either a step-wise fashion (i.e., discontinuous elution) as normally used in batchwise ion-exchange chromatography, or by the application of a linear gradient (continuous elution), which is the method of choice for column chromatography as higher resolution can be more readily achieved.

The number of increments to include in a stepwise elution system must be determined empirically, although a minimum of three steps is commonly employed. These will include an initial step to remove all weakly bound molecules, followed by elution with a higher salt concentration to desorb the more strongly bound molecule of interest, and a final wash with the maximum salt

concentration to strip the column of remaining bound substances. Additional steps can be included to suit the particular application.

Elution with linear gradients will require a suitable pumping system and a gradient controller (*see* **Subheading 2.7.**). Nonlinear gradients can also be employed if available, however, it is suggested that these be included into a separation only after initial experiments with linear gradients have indicated their suitability. Use of a linear gradient will require a decision about the gradient steepness parameter, which is a measure of the rate of change of counter ion on the column during a separation. The steepness of the gradient is dictated by the column volume, flow rate, the initial and final salt concentrations of salt in the eluent, and the gradient time ***(3)***. Shallow gradients will result in optimal resolution, but this occurs at the expense of separation time and increased band-broadening ***(3)***. In contrast, fast gradients yield short separation times and minimal band-broadening, but at the expense of resolution. Where available, use the manufacturers recommendations for the gradient steepness parameter as a starting point, and vary this according to the desired resolution. For example, a rate of change of 17.5 m*M*/mL of $Cl^-$ as the counter-ion is suggested to give optimal resolution for analytical separations on the strong anion exchanger Mono Q (5 cm length, 0.5 cm internal diameter) ***(1)***, but a slower rate (8.75 m*M* $Cl^-$/min) may give better results. If no data is available about the gradient steepness for an ion-exchanger, set up an experiment with a gradient from 0.05 *M* to 1 *M* of the counter-ion over 10–20 column volumes as a starting point ***(3)***, and compare the resolution achieved with similar data obtained at faster and slower rates of change.

The concentration of salt needed to elute all bound substances from an ion-exchange column is dependent on the type of exchanger, the pH of the buffer, and the p*I*s of the molecules loaded onto the gel. Hence, no general recommendation can be given, although the normal maximum salt concentration needed is in the range 0.5 *M*–1 *M*. This concentration can readily be adjusted after the results of the first chromatographic experiment become available. A series of empirical tests can also be undertaken to determine the maximum salt concentration needed (*see* **Subheading 3.2.**).

As mentioned earlier (*see* **Subheading 1.** and **Fig. 1**), the charge on a protein is pH dependent. Hence, once bound to the IEX support, alteration of the pH of the eluent can be used to change the charge on the protein and bring it closer to its p*I*, causing elution of the protein from the ion-exchanger. However, it is difficult to produce a linear pH gradient at a constant salt concentration, and for this reason, pH gradients are not as common as salt gradients as a means to elute proteins in ion-exchange chromatography ***(4)***. In contrast, steps in pH are easier to control and reproduce, and for anion-exchangers this requires a drop in eluent pH, while an increase in pH is needed for cation exchangers

(*see* **Fig. 1**). An in-line pH electrode can be included in the flow path immediately postcolumn to monitor eluent pH, or alternatively this can be measured in collected fractions manually. A combination of a change in pH with an increase in salt concentration can also be considered as a means of elution in ion-exchange chromatography ***(4)***.

### *2.7. Recommended Hardware Configuration*

A typical system for low-pressure ion-exchange chromatography for protein purification will require a pumping system capable of generating gradients, plus an injector (or a mechanism to introduce the sample into the buffer flow), the chromatographic column, an in-line UV (280 nm) detector and chart recorder, and a fraction collector. Access to a stand-alone conductivity meter to monitor the conductivity of the column eluent is also very useful. A "bare-bones" system without the UV detector and chart recorder can also be used, although this has the disadvantage that UV measurements on eluted fractions must be done separately. For low-pressure systems a peristaltic pump and a simple gradient formerly made up of two interconnected vessels of identical shape can be used to generate a linear gradient. If using this approach, make sure that the gradient maker is level, and place the two buffers (the eluting buffer containing NaCl, and the initial equilibration buffer) into the two vessels, with the initial buffer vessel placed over a magnetic stirrer and connected by a second stopcock to the pump. Start the mixer, and open the stopcock between the vessels before starting the pump (*see* **Note 2**). Nonlinear gradients can also be generated by this approach by altering the shape of the nonmixing vessel.

IEX chromatography hardware for FPLC and HPLC should be matched to the expected flow rates and back-pressures of the columns to be used. Several systems for low-pressure and medium pressure ion-exchange chromatography [e.g., ÄKTA-FPLC (Amersham BioSciences), BioLogic HR system (Bio-Rad)] are available from the major manufacturers which are specifically designed for use with halide-containing buffers (*see* **Note 3**).

If columns are to be packed with bulk ion-exchanger, this should be carried out as specified by the manufacturer, and may require initial swelling of the gel in some cases, or use of a preswollen support. In either case, the support has to be preequilibrated before packing into the column (*see* **Subheading 3.1.**). Alternatively, prepacked columns may be used (*see* **Table 3**). As IEX is an adsorptive technique, columns are normally short, with a bed height of 5–15 cm ***(1)***. Scaling-up of IEX columns is readily achieved by increasing the column diameter ***(1)***.

## 3. Method

The following procedures describe ion-exchange chromatography on an anion-exchange support, using as an example the strong anion-exchanger

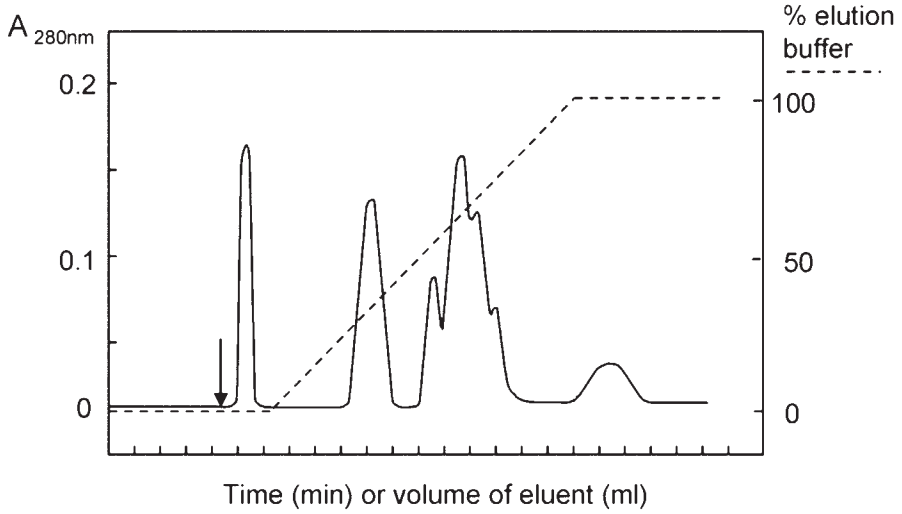


Fig. 3. Example of a typical ion-exchange column chromatogram. Initially, the ion-exchange column is equilibrated in the starting buffer (low ionic strength) until a stable baseline of UV absorbance at 280 nm (filled line) is achieved. The pH and conductivity of the column eluent at this point should be the same as the starting buffer, and confirms adequate equilibration. The sample is then injected (indicated by arrow), and the column is washed with starting buffer until all nonbound proteins are eluted. A linear gradient of elution buffer (high ionic strength) (dashed line) is then initiated to elute bound proteins, after which the column is washed with 100% elution buffer to remove strongly bound material. At the completion of the run, the column is stripped with stripping solution (1–2 *M* NaCl) (not shown) before reequilibration in the starting buffer. (*See* **Subheading 3.** for further details.)

Mono Q (HR5/5) prepacked column. Details specific for this column will be added to the method in italics. A linear gradient will be used to elute bound proteins, although conditions for the use of a step-elution system can readily be substituted for the gradient as will be indicated. An example chromatogram for this application is shown in **Fig. 3**.

### 3.1. Prepare the Gel/Column

1. Most IEX supports are available as preswollen gels or prepacked columns, but if working with one of the dry powder supports, it must first be preswollen at the pH to be used in the experiment according to the manufacturers instructions (normally 2–3 h at 90°C, or 48 h at room temperature) (*see* **Note 4**).
2. Preswollen gels are supplied in a ready-to-use form. Mix the gel to a slurry, and remove the required amount into a vacuum flask and allow to settle. For both types of gels, swirl-mix the gel approximately five times with an excess of the starting

buffer (~5 volumes) at the correct pH, and after settling discard the supernatant. To test whether the gel is fully equilibrated, monitor the pH and conductivity of the buffer removed from the gel, which should be identical to the starting buffer.

3. If the counter-ion on the IEX support is to be changed from that which the gel was supplied with (which is normally $Cl^-$ for anion exchangers, and $Na^+$ for cation exchangers), wash the gel first with a 0.5 *M*–1.0 *M* solution of the salt of the new counter-ion, and then equilibrate into the starting buffer as above.
4. Pack the column according to the manufacturer's instructions (*see* also **Subheadings 3.1.–3.3.**, Chapter 5 (Gel filtration) for notes regarding the packing procedure).
5. If a prepacked column is being used, this should be placed in the liquid chromatography system, and the pressure limit of the pump set at the recommended value (5 MPa for Mono Q). The column is then equilibrated with ~5 column volumes (~5 mL, as the column volume of the Mono Q HR5/5 is 1 mL) of the starting buffer (20 m*M* piperazine, pH 9.6) at the recommended flow rate (1 mL/min), and the pH and conductivity checked (as detailed in **Subheading 3.1.2.**) (*see* **Fig. 3**).
6. It is good practice to pre-cycle the column with 5 column volumes each of 1) starting buffer (20 m*M* piperazine, pH 9.6), 2) eluting buffer (20 m*M* piperazine, 300 m*M* NaCl, pH 9.6), and 3) column stripping buffer (1 *M* NaCl), before actual use. Re-equilibrate the column with 5–10 column volumes of starting buffer and ensure that both the pH and conductivity of the eluent are the same as the starting buffer.

## 3.2. Pilot Study to Choose Optimal pH

1. A pilot test to determine the optimal pH to use for ion-exchange chromatography relies on binding of the molecule(s) of interest to an aliquot of the ion-exchanger, and its subsequent disappearance from solution ***(4)***. The basis of this approach is to set up a series of aliquots (~1 mL) of the ion-exchanger in disposable tubes at pH intervals from 0.5 to 1 pH unit. For an anion-exchanger, start at pH 5.0 using buffers as described in **Table 4**, and prepare aliquots of gel at pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. Wash the gel aliquots in respective buffers five times to ensure adequate preequilibration (*see* **Subheading 3.1.5.**).
2. Prepare aliquots of the sample molecule in the same buffers. It is important that the buffer pH of the sample is the same as the aliquot of the ion-exchanger to which it is to be added. Similarly, the sample should not contain any salts, as these will interfere with binding. If excess salts are present, these should be removed by either dialysis or gel filtration chromatography.
3. Add the sample aliquot to the ion-exchange gel of the same pH, mix gently on a rotating wheel for 15 min, then allow the gel to settle. Remove the supernatant, and assay it for the presence of the sample of interest. This can be done either as a general test monitoring changes in absorbance, or with a specific assay. The disappearance, or marked decrease of the sample in solution indicates the pH at which the sample binds to the ion-exchanger, and this pH should be used for ion exchange chromatography. Avoid choosing a pH which is more than ~0.5–1.0 pH unit higher

than this optimal pH, as the sample will adsorb more strongly to the ion-exchange column at higher pHs, and hence will require a higher salt concentration to elute it (*see* **Note 5**).

4. This type of pilot test is more appropriate for setting up ion-exchange chromatography using preswollen gels. For prepacked FPLC or HPLC columns operated on chromatography systems with automatic valves controlling buffer selection and sample injection, the effect of different pHs on sample retention can be rapidly evaluated in an automated format.
5. Once the optimal pH for binding has been established, a similar strategy using aliquots of ion-exchanger at this pH can be used to determine the maximum salt concentration needed to cause elution of the sample from the gel. In this case, set up buffers and gel with NaCl increasing in increments of 0.1 *M* from 0 to 1.0 *M* NaCl ***(1,4)***. The salt concentration at which all the sample is present in the supernatant indicates the concentration needed in the elution buffer.

### 3.3. Prepare the Sample for Loading

1. For successful ion-exchange chromatography, the sample should be at the same pH as the starting buffer, and contain either the same (or less) salt. The options for samples containing higher salt concentrations include buffer exchange by dialysis or gel filtration chromatography, or in some cases simple dilution with the starting buffer can be sufficient (*see* **Note 6**).
2. Remove any particulates from the sample by either filtration through a 0.22-µm filter (*see* **Note 7**), or by high-speed centrifugation (15,000*g*, 10 min). Set aside an aliquot of the sample as starting material, which should be assayed in conjunction with the collected fractions in order to determine the yield of sample from the column.

### 3.4. Prepare the Column for Loading and Injection

1. Establish a flat UV baseline at 280 nm by washing the column with starting buffer (*see* **Fig. 3**). Check the flow rate (1 m /min), chart speed (typically, 0.25 cm/min), and maximum absorbance setting on the detector (0.01–2.0 AUFS).
2. Calculate the gradient time required using the recommended rate of change of counter-ion from the product guidelines. (For the Mono Q column operating at 1 mL/min, this value is 17.5 m*M* $Cl^-$/min. Hence, with a starting buffer of 20 m*M* piperazine, pH 9.6, and an elution buffer of 20 m*M* piperazine, 300 m*M* NaCl, pH 9.6, the rate of change value to enter into the gradient controller will be 300/17.5 = 17.1 min).
3. If a step elution system is to be used, choose the steps (e.g., increments of 20%, 40%, 60%, and 80% elution buffer), and program the gradient controller accordingly.
4. Inject the protein sample, start the fraction collector (1 min fractions), and continue washing the column until the UV trace returns to baseline (*see* **Fig. 3**).
5. Start the gradient (*see* **Note 8**). At completion of the gradient, continue to wash the column with five column volumes of the elution buffer (5 mL), then apply five

column volumes of stripping buffer (1 *M* NaCl) to elute any strongly bound material.
6. Reequilibrate the column with 5–10 column volumes of starting buffer.
7. Assay collected fractions (and the starting material aliquot) for the presence of the sample of interest. Determine % yield of activity, and pool appropriate fractions ready for the next purification step.

## 3.5. Column Cleaning and Storage

1. After the final chromatographic run, wash the column extensively and store it in the recommended storage solvent (*see* **Note 9**).
2. If an increased column backpressure has occurred, or if visual fouling of the top of the column is evident, clean the ion-exchanger according to the manufacturers guidelines. A typical cleaning process involves treating the column with 1) ~2 column volumes of 0.1–0.2 *M* NaOH (*not recommended* for silica-based columns, 2) extensive washing with water, 3) ~2 column volumes of 70% ethanol, 4) extensive washing with water. In some cases, it is possible to invert the column prior to these washing steps to assist in removing particulate matter caught in the top of the column, although for pre-packed columns this step should only be used as a last resort.

## 3.6. Troubleshooting

### 3.6.1. Sample Elutes With the Starting Buffer

There are several potential reasons for this problem: 1) the pH of the starting buffer is incorrect, 2) the starting buffer has too much salt in it, 3) the sample is not at the correct pH or has too much salt present, 4) the column is not fully equilibrated in the starting buffer. To fix these, firstly check your information about the p*I* of the sample of interest (*see* **Subheading 1.** and **Fig. 1**). If no data is available, do the pilot experiment outlined in **Subheading 3.2.** to choose the optimal pH. To make an anion-exchange column bind more proteins, increase the pH of the starting buffer. Similarly, for a cation exchange column decrease the pH of the starting buffer. For both types of support, remove any salt in the starting buffer.

Ensure that the sample is fully equilibrated in the start buffer before loading (*see* **Subheading 3.1.1.**), and also that the column has been properly preequilibrated (**Subheading 3.1.6.**).

### 3.6.2. Sample Does Not Elute From the Column

This problem indicates that 1) the salt concentration of the elution buffer is too low, or 2) the pH of the eluent is wrong, 3) the sample has irreversibly bound to the column, or 4) the sample precipitated or was lost prior to loading. To fix these, increase the salt concentration of the elution buffer to a maximum of 1 *M*, and/or change the pH of the elution buffer (decrease the pH for an anion-

exchanger, or increase the pH for a cation-exchanger). If the recovered amount of protein is lower than expected after these steps, binding to the column by mechanism(s) other than ion-exchange may be occurring. Hydrophobic interactions can be limited by the addition of 10% ethylene glycol ***(1)*** or acetonitrile to the buffers, but be alert to the fact that some salts precipitate at high organic solvent concentration. Addition of a nonionic detergent (e.g., 0.1% Triton X-100) will limit nonspecific sticking to surfaces. Be sure that the sample was actually loaded onto the column.

### *3.6.3. Elution of Peaks Requires a Very High Salt Concentration*

The pH of the eluent is either too high (anion exchanger) or too low (cation exchanger). Adjust the pH of the buffer system to be closer to the p*I* of the sample of interest.

### *3.6.4. Sample Elution Pattern Is Not Reproducible*

The most obvious cause is that the column has not been fully reequilibrated into the starting buffer (*see* **Subheading 3.1.1.**). Also check that the stripping buffer (normally 1–2 *M* NaCl) contains the same ionic species as the counterion on the column ($Na^+$ for cation exchangers, $Cl^-$ for anion exchangers). If the reproducibility problem occurred between runs carried out with different buffers, check the buffer preparation and calibration of the pH meter.

Ensure that the sample has not changed between the chromatographic runs, by either inappropriate storage, proteolysis, or precipitation. If there is a possibility that the sample has precipitated on the column, clean and regenerate the column (**Subheading 3.5.**) and repeat the chromatography.

### *3.6.5. Resolution of Sample Is Poor*

There are several reasons why resolution can be poor. These are 1) the gradient slope is too steep, 2) there are large precolumn dead volumes in the flow path, 3) the column is poorly packed, 4) the sample has precipitated on the column, 5) the column is contaminated, and 6) the column has been overloaded.

Test the effect of decreasing the gradient slope (by a factor of 2) on sample resolution, or of inserting a step mid-way through the gradient. Make sure that there are no dead volumes prior to or after the column, particularly if using a Superloop. Check the packing of the column by loading a coloured marker protein (myoglobin, haemoglobin, cytochrome-*c*, *see* **Table 1**) and observing the band during chromatography. If it is suspected that components of the sample have precipitated, or that the column is contaminated, clean the column and modify the eluents to prevent reoccurrence (**Subheading 4.2.**). Finally, check the effect that loading less sample has on resolution.

### *3.6.6. Recovery of Sample Activity Is Poor*

If the recovery of sample activity is low compared to the activity present in the starting material aliquot, which was set aside immediately before injection, first check the amount of protein that has eluted from the column. If the peak heights are generally lower than expected, indicating that the recovery of total protein is also low, the problem could be loss of protein on the column (*see* **Note 2**). If total protein recovery appears acceptable, but sample activity is low, check the stability of the sample in the ion-exchange buffers, and make sure that the added salt in the eluted sample is not interfering in the sample assay. Alternatively, the sample may have been separated from an essential cofactor or similar on the column, which can be checked by pooling all the recovered fractions and reassaying for activity.

### *3.6.7. Backpressure of Column Has Increased*

Increasing column backpressure is caused by the introduction of particulate matter (either in the buffers, or in the sample), or precipitation of sample components in the column inlet filter or on top of the column itself. Of these, the main culprit is normally the sample. Ensure that the sample has been properly filtered before loading. If the sample is turbid, either dilute it prior to filtering, or try to improve sample solubility by changing the pH of the buffer, or through the addition of a nonionic detergent or organic solvent (*see* **Note 2**). These additives should also be added to the column eluents.

For laboratory-packed columns, change the filter on top of the column, and if possible make the buffer filtration more stringent. Apply column cleaning procedures as recommended by the manufacturer of the ion-exchanger (*see* **Subheading 3.5.**).

### *3.6.8. Column Has Run Dry*

For laboratory-packed columns this is a terminal problem, requiring unpacking and repacking of the column. For prepacked columns, try introducing a 50 : 50 solvent/water mix (methanol, acetonitrile, or dimethyl sulfoxide (DMSO), depending on the stability of the exchanger to solvents) at a very slow flow rate overnight to exclude all the air bubbles from the column, after which the column should be washed with 5–10 column volumes of water, and then reequilibrated into the ion-exchange eluents. Test the resolution of the column as per the manufacturers instructions to see whether column performance has been altered.

### *3.6.9. Peaks Observed in the Blank Chromatogram*

The presence of unexplained peaks in the chromatogram indicates 1) impurities in the buffer components, or 2) incomplete elution of a previously loaded

sample, which is "ghosting" in each subsequent elution profile. Ghosting can be readily checked, as the peak height should decrease with each subsequent blank run. Clean the column (*see* **Subheading 3.5.**), and then improve the washing conditions at the end of each run to remove all bound material. The presence of buffer impurities indicates that higher quality reagents should be used, or if this is not possible, a guard or precolumn can be placed before the main column.

## 4. Notes

1. The solubility of many proteins frequently is lowest at (or near) their isoelectric points. Therefore, avoid use of pH conditions near the p*I* of the protein, which can lead to isoelectric precipitation on the column, resulting in blockage of the column and/or poor recoveries. Test the effects of particular pH and salt concentrations on protein stability in solution prior to final selection of ion-exchange support.
2. Ensure that the level of the two buffers remains the same when the mixer is switched on. If the mixer is rotating too quickly, the level of the buffer in the mixing chamber will increase and cause backflow into the second vessel. A simple gradient maker with an overhead stirrer which works very well is available from Amersham-BioSciences.
3. Halide-containing buffers will attack and corrode stainless steel high-pressure liquid chromatography (HPLC) systems, particularly frits, piston seals, and check valves. Leaching of metal ions can also interfere with some metal-sensitive proteins. Therefore, long-term use of these systems for ion-exchange chromatography is not recommended. If a stainless steel HPLC is used for IEX, wash the system with distilled or deionized $H_2O$ as soon as possible after completion of the experiment.
4. Do not use a magnetic stirrer with any ion-exchange supports, as this can break gel particles. Avoid direct application of heat via a hotplate, as this can melt the gel media—use a waterbath instead.
5. The highest pH at which the *least* amount of sample is bound in this pilot test can be used as the pH of the elution buffer if a pH step elution system is to be used.
6. As ion-exchange chromatography is an adsorption technique, it is possible to load large volumes of dilute sample solution onto the column, and then elute the sample with elution buffer. For pre-packed columns on FPLC or HPLC systems, large sample volumes can be loaded using either a large loading loop (e.g., Superloop from Amersham Biosciences), or pumped directly onto the column using a third sample-loading pump.
7. A 0.22-µm filter is suitable for prepacked columns and ion exchangers with particle sizes up to 25 µm. For preparative ion-exchangers with particle sizes >25 µm, filters 0.45–1 µm can be substituted.
8. If a large-volume sample loading loop (Superloop) has been used, it is essential to isolate this from the flow-path before the gradient is initiated. If this is not done, the volume of starting buffer in the loop will significantly delay and dilute the gradient onto the column.

9. Storage solvents can include 20% ethanol, 0.01 *M* NaOH (*not* for silica columns) or equilibration buffer containing recommended bacteriostats (anion exchangers: 0.002% chlorhexidine or 0.001% phenyl mercuric salts, cation exchangers: 0.005% merthiolate) ***(1,4)***. Consult the manufacturers recommendations for each type of ion exchanger.

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## Web Sites:

Amersham Biosciences-Chromatography: http://www.chromatography.amershambiosciences.com.
Bio-Rad Laboratories: http://www.bio-rad.com/.
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Agilent: http://www.chem.agilent.com.
Supelco: http://www.sigmaaldrich.com.
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