Mobile Phases for Ion Exchange Chromatography and Chromatofocusing

Ion exchange and chromatofocusing are effective, widely used techniques for analytical and preparative separations of biomolecules. The interaction on which separations are based is between the charged solute molecules and oppositely charged groups covalently bound to the chromatographic matrix. Since most biomolecules consist of charged subunits (amino acids, nucleotides, and saccharides), ion exchange and chromatofocusing are applicable to a broad spectrum of sample types. This bulletin reviews the basics of ion exchange chromatography and chromatofocusing and explores means of optimizing mobile phase composition and other operating variables.

Key Words:
- ion exchange chromatography
- chromatofocusing biomolecules
- mobile phase

Selectivity and resolution in chromatography are based on media type, pH, buffers, salts, solvents and the sample components themselves. Interactions among these variables are optimized to solve separation problems. Through the use of this selectivity, biomolecules which differ by only one charge unit can be resolved.

Ionizable Groups

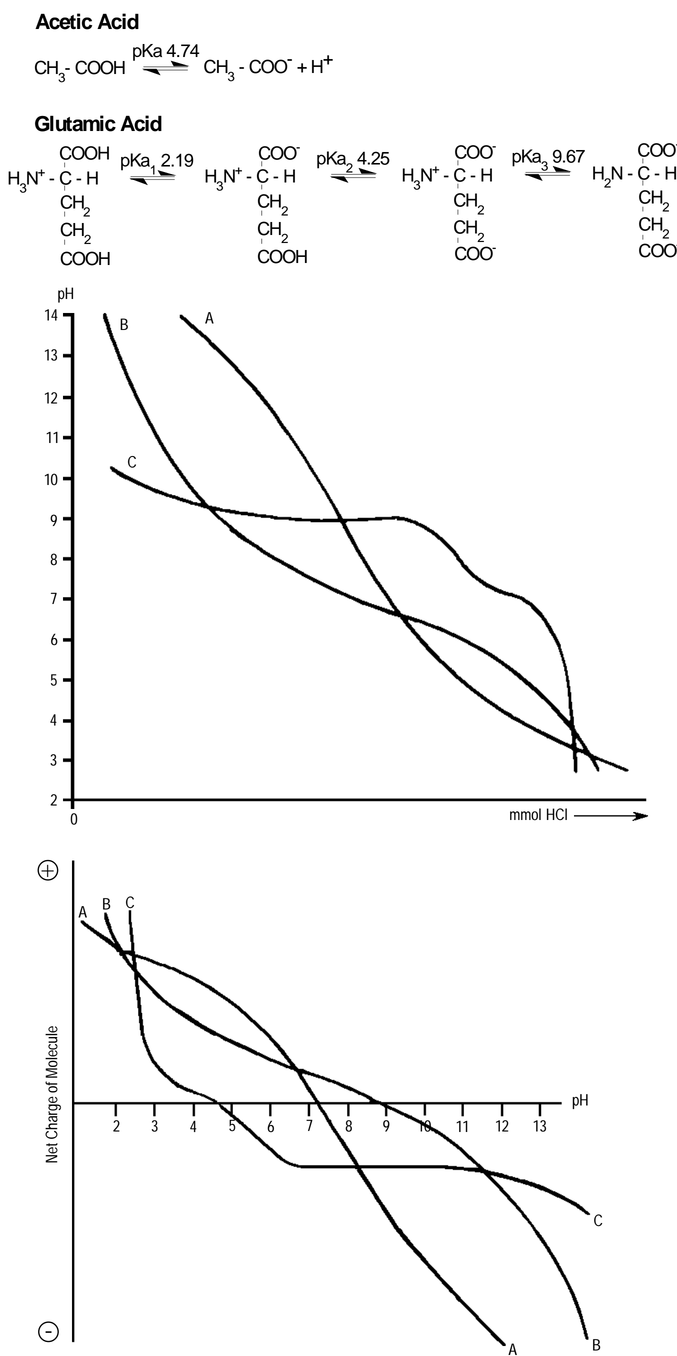
Unlike most small molecules, which have a single pKa value, biomolecules contain multiple charged groups, each of which possesses a different pKa value dependent on both the structure of the functional group and its microenvironment. As a result, the unique titration curves of proteins and other biopolymers change gradually (Figure A). Differences between titration curves indicate the possibilities for resolution by ion exchange or chromatofocusing.

Titration curves also can be used to predict elution order. Curves obtained by electrophoresis give the isoelectric point, pI (no net charge), of each protein and also the net charge at any pH. At pH 4.0 the hypothetical proteins in Figure A, for example, are positively charged and will be retained on a cation exchanger. A salt gradient will elute the proteins in order of increasing net charge, i.e., C, B, A. At pH 9.5, all three proteins are negatively charged and will be retained on an anion exchanger. Again, the proteins will elute in order of increasing net charge (B, C, A) in a salt gradient. In contrast, in chromatofocusing a pH gradient is used to elute the proteins in decreasing order of their isoelectric points, i.e. B, A, C.

Ion Exchange Chromatography

Ion exchange chromatography is based on the reversible adsorption of charged molecules to an immobilized functional group of the opposite charge. Most ion exchange separations are performed in five steps (Figure B). Note that ion exchange is a dynamic

Figure A. pKa for Acetic Acid, an Amino Acid (Glutamic Acid) and Titration Curves for Three Hypothetical Proteins



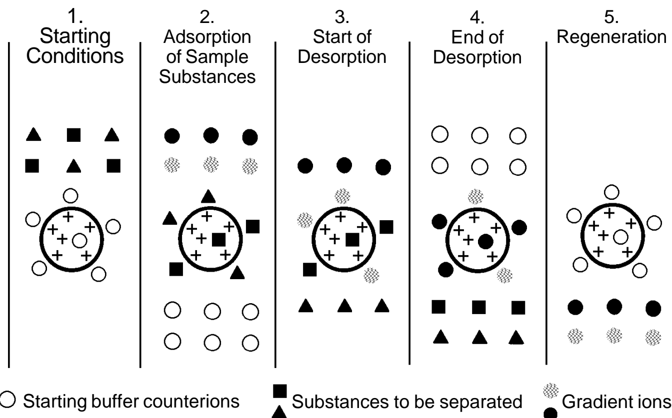
Reproduced with permission of Pharmacia Biotech.

795-0002, 794-0668, 0509

ISO 9001 REGISTERED
©1995 Sigma-Aldrich Co.

process and cannot be fully illustrated by a static model. During the equilibration (regeneration) step, the ion exchange support is brought to a starting state, which allows binding of the desired biomolecules through careful selection of pH and ionic strength conditions. Exchangeable counterions and buffer ions are associated with the functional groups. During sample application and adsorption, solute molecules of the appropriate charge displace the counterions and reversibly bind to the support. Unbound molecules are washed from the bed with the starting buffer. Desorption is achieved by increasing the salt concentration (in a gradient) or changing the pH. Sample molecules are released in the order of their strengths of binding, with the most weakly bound substances eluting first. After sample desorption, substances remaining on the support are removed and the bed is re-equilibrated for the next purification. Interactions with the ion exchanger can be controlled by varying conditions such as ionic strength and pH. Different ionic species can also play a significant role in the resolution of a sample, and this fact should be taken into account when optimizing conditions.

Figure B. Principles of Ion Exchange Chromatography (Salt Gradient Elution)



Reproduced with permission of Pharmacia Biotech. 794-0669

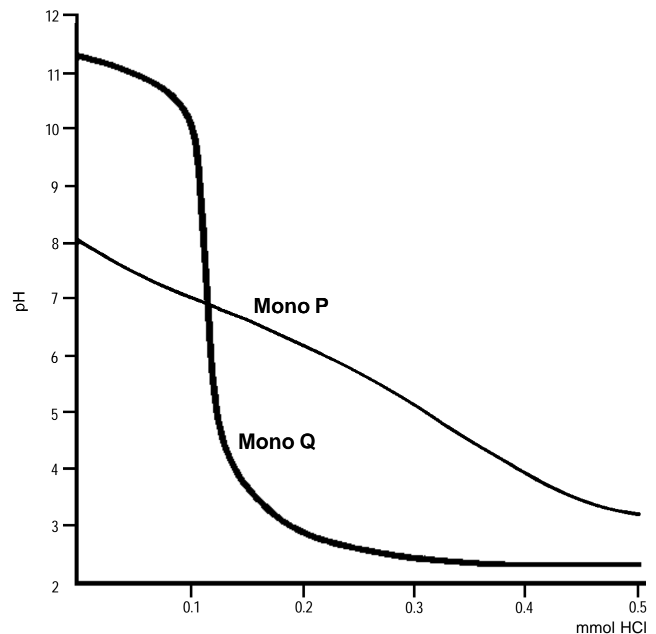
Chromatofocusing

A strong ion exchanger does not have the ability to pick up or lose protons with changing pH, i.e. it has no buffering capacity, and thus its capacity does not vary with a change in pH. A weak ion exchanger's ionic capacity changes with pH and, as a result, a weak exchanger exhibits buffering capacity (Figure C).

In chromatofocusing, biomolecules are separated according to their isoelectric points on a weak anion exchange column. A mixture of ampholytes/polybuffers with different pKa values is used to generate a pH gradient in the column. Ampholytes (e.g., any amino acid) have both positive and negative charges and contribute very little to ionic strength. As a result, they can be used at relatively high concentration to control the pH on the column very closely.

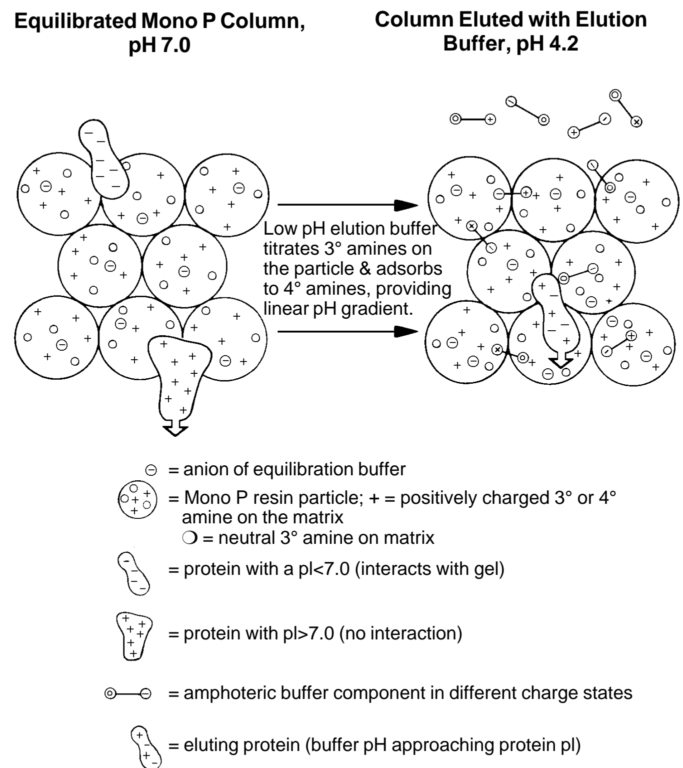
The mechanism of chromatofocusing is based on the buffering action of the charged groups on the column and the fact that biomolecules have a net negative charge at a pH above their pI. The ion exchange column is equilibrated with the ampholyte buffer at a pH above the pI of all proteins in the sample, then the sample is applied. The ampholyte buffer is then adjusted to a lower pH. A pH

Figure C. Titration Curves for a Strong and a Weak Anion Exchanger



Reproduced with permission of Pharmacia Biotech. 794-0670, 0671

Figure D. Mechanics of Chromatofocusing



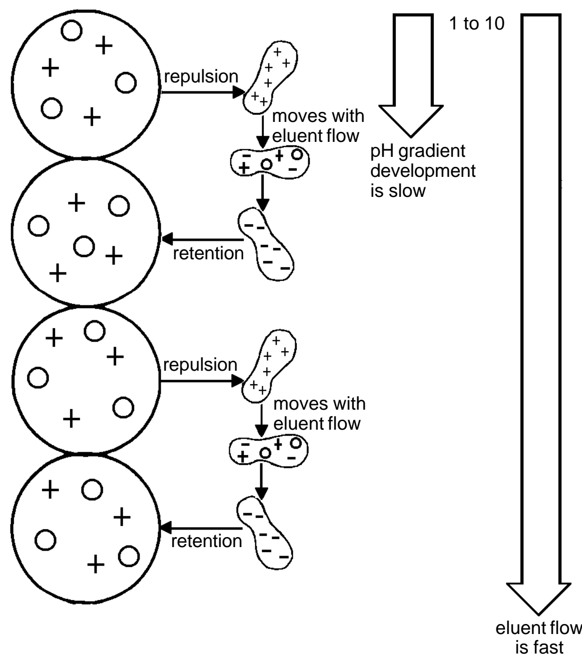
Reproduced with permission of Pharmacia Biotech. 794-0513

gradient develops on the column, causing a steady drop in the pH of the mobile phase eluting from the column. Figure D demonstrates sample application on a Mono P® column.

Titration of the sample molecules results in focused zones of molecules with different pIs. Biomolecules of identical pI focus together because the mobile phase flow exceeds the rate of pH reduction down the column. The individual biomolecules continuously change charged states as the pH gradient develops. The biomolecules at the rear of the sample zone are the first to be titrated by the low pH buffer (Figure E). As these molecules become positively charged, they are repelled from the matrix and are carried rapidly, by the mobile phase flow, to the front of the sample zone. When they reach the front of the sample zone, they encounter a higher pH. This titrates the molecules through neutrality back to their negatively charged forms. The molecules are again retained and fall back to the rear of the sample zone. The exchange of biomolecules between the front and rear of the sample zone results in a continual narrowing of the zone. This process continues until each molecule encounters a pH equivalent to its pI and thus doesn't titrate back to the negative state, but remains with the pH gradient at that value until it elutes from the column.

Under ideal chromatofocusing conditions it is possible to resolve biomolecules differing in pI by as little as 0.02 units. Wide intervals of ampholyte pH can be used for sample screening, while narrower intervals (0.5 to 2 pH units) can be used to obtain very high resolution.

Figure E. Focusing Effect During Chromatofocusing



Molecules at the rear of the sample zone migrate more rapidly than those at the front producing narrow bands of molecules with different isoelectric points.

Reproduced with permission of Pharmacia Biotech. 794-0673

Table 1. Buffers Recommended for Cation Exchange Chromatography

pKa (25°C)	pH Interval	Substance	Conc. (mM)	ΔpKa/ΔT(°C)	Counterion	Cat. No.	Qty.
2.00	1.5-2.5	Maleic acid	20		Na^+	M0375A	500g
2.88	2.38-3.38	Malonic acid	20		Na^+/Li^+	M1750A	25g
3.13	2.63-3.63	Citric acid	20	-0.0024	Na^+	C1909A	500g
3.81	3.6-4.3	Lactic acid	50		Na^+	L1750A	1g
3.75	3.8-4.3	Formic acid	50	+0.0002	Na^+/Li^+	25,136-4A	5g
4.21	4.3-4.8	Butanedioic acid	50	-0.0018	Na^+	—	—
4.76	4.8-5.2	Acetic acid	50	+0.0002	Na^+/Li^+	24,285-3A	100g
5.68	5.0-6.0	Malonic acid	50		Na^+/Li^+	M1750B	100g
6.15	5.7-6.7	MES	50	-0.0110	Na^+/Li^+	M8250A	10g
7.20	6.7-7.6	Phosphate	50	-0.0028	Na^+	21,988-6A	100g
						22,352-2A	25g
7.55	7.6-8.2	HEPES	50	-0.0140	Na^+/Li^+	H3375A	10g
8.35	8.2-8.7	BICINE	50	-0.0180	Na^+	B3876A	25g

Reproduced with permission of Pharmacia Biotech.

Table 2. Buffers Recommended for Anion Exchange Chromatography

pKa (25°C)	pH Interval	Substance	Conc. (mM)	ΔpKa/ΔT(°C)	Counterion	Cat. No.	Qty.
4.75	4.5-5.0	N-methylpiperazine	20	-0.015	Cl^-	—	—
5.68	5.0-6.0	Piperazine	20	-0.015	$Cl^-/HCOO^-$	P3896A	100g
5.96	5.5-6.0	L-histidine	20		Cl^-	H8000A	5g
6.46	5.8-6.4	bis-Tris	20	-0.017	Cl^-	B9754A	25g
6.80	6.4-7.3	bis-Tris propane	20		Cl^-	B6755A	10g
7.76	7.3-7.7	Triethanolamine	20	-0.020	Cl^-/CH_3COO^-	T5,830-0A	25g
8.06	7.6-8.0	Tris	20	-0.028	Cl^-	T3253A	100g
8.52	8.0-8.5	N-methyldiethanolamine	50	-0.028	$SO_4^{2-}/Cl^-/CH_3COO^-$	—	—
8.88	8.4-8.8	Diethanolamine	20/50*	-0.025	Cl^-	D8,330-3A	5g
8.64	8.5-9.0	1,3-Diaminopropane	20	-0.031	Cl^-	23,998-4	50g
9.50	9.0-9.5	Ethanolamine	20	-0.029	Cl^-	11,016-7A	25mL
9.73	9.5-9.8	Piperazine	20	-0.026	Cl^-	P3896A	100g
10.47	9.8-10.3	1,3-Diaminopropane	20	-0.026	Cl^-	23,998-4	50g
11.12	10.6-11.6	Piperidine	20	-0.031	Cl^-	—	—

*At pH 8.4 and pH 8.8, respectively.

Reproduced with permission of Pharmacia Biotech.

Important Factors in Optimizing an Ion Exchange or Chromatofocusing Separation

pH

During ion exchange chromatography and chromatofocusing, control of the pH depends on buffer substances, salts, sample components, organic solvents, other additives, and temperature. Each of these variables must be considered individually and in relation to each other for optimum separation of biomolecules.

Titration curves provide an indication of the pH at which charge differences among sample components are the greatest. It is useful to have an idea of the pKa values of the sample components, because changes in pH have the largest effect on analyte retention when the pH is near the pKa of the analyte. Changes in pH during an ion exchange experiment can make a great difference in the chromatogram. Many times, a sharp peak actually will consist of two or three components which have been compressed into one peak due to a sudden change in pH.

Buffers

In all biomolecular work, the correct choice of buffer can be crucial to success. In ion exchange chromatography, maintenance of pH is of primary concern, since ionic interactions vary with pH. The buffer components themselves are ionic and may be taking part in the ion exchange process. The buffer pKa value should be within ± 0.5 pH units of the desired working pH. If conditions are used which cause the pH to change during an experiment, adjust the pH of the buffer to the appropriate side of its pKa to minimize the change.

Since buffers can be prepared in different ways, detailed care in their preparation is essential for reproducible chromatography. A buffer consists of the salt of a weak acid/base and the corresponding acid/base, mixed to attain the desired pH. After adding sodium chloride or another nonbuffering salt to the buffer, it might be necessary to adjust the pH of the buffer. If so, use an acid or base containing the counterion being used for elution (i.e., HCl/Cl^- or $LiOH/Li^+$). Likewise, use buffering ions that have the same charge as the ion exchange functional group: a positively charged buffering species with an anion exchanger and a negatively charged buffering species with a cation exchanger. This prevents fluctuations in pH and conductivity.

To promote binding of the biomolecule, the buffer ion concentration normally should be low to prevent aggregation and preserve activity, but the minimum should be 10mM. In Tables 1-4, concentrations between 20 and 50mM are recommended, depending on the pH and the buffer. The concentration of the initial buffer for chromatofocusing is especially important, since microenvironmental pH differences can be as much as 1 pH unit.

In ion exchange, the mobile phase is chosen to provide adequate solubility for the various salts and buffers, to control sample retention through solvent strength, and to provide separation selectivity. The buffers in Tables 1-3 describe optimized systems for most of the pH intervals listed. A blank run should always be performed to determine the UV adsorption of the buffer. High quality buffers and salts will minimize UV baseline rise due to batch-to-batch variations.

Buffers for chromatofocusing are most often used at a dilution of 1 in 10. For narrow pH intervals, dilutions up to 1 in 20 can provide better resolution. Resolution can sometimes be improved by adding small amounts of salt (10mM). If a collected fraction is analyzed by reversed phase chromatography, the ampholyte buffer may interact with pairing ions. If the pairing ion is very hydrophobic, the ampholyte will be retained on the column and will have an absorbance maximum below 280nm.

Additional Reading:

Ion Exchange Chromatography: Principles and Methods (3rd Edition)

Pharmacia, 1991, 119 pp.

This handbook serves as an introduction to the principles of ion exchange chromatography and as a practical guide to the use of media developed by Pharmacia. The handbook presents the examples of the different types of biomolecules which have been separated using ion exchange chromatography and discusses the many ways the technique can be used.

23581

Protein Purifier

Protein Purifier is a computer software-based learning aid which simulates the experience of working through a protein purification problem using a range of separation and analytical techniques. The program gives practical insight into strategies commonly employed in purification schemes — without wasting time and materials. It comes complete with 1 program diskette and an instruction manual sealed in a protective folder.

System Requirements: IBM PC, AT, XT or compatible with color graphics monitor.

Diskette, 3 1/2" **23586**

Protein Biotechnology: Isolation, Characterization and Stabilization

F. Franks, Ed., The Humana Press, Inc., 1993, 608 pp.

This comprehensive volume gives state-of-the-art techniques for isolating, purifying, characterizing, and analyzing proteins as commercial products, both *in vitro* and *in vivo*. The book also highlights leading-edge analytical methods used throughout biotechnology today.

26579

Guide to Protein Purification: Methods in Enzymology, Vol. 182

M.P. Deutscher, Ed., Academic Press, 1990, 894 pp.

Designed to serve the needs of the student, experienced researcher, and newcomer to the field, this comprehensive manual presents up-to-date procedures necessary for purifying, characterizing, and handling proteins and enzymes.

26565

Table 3. Buffer Substances for Chromatofocusing

pH 9-4, broad pH intervals

pH	Start buffer	Eluent* (100mL)	Approximate Volumes (mL) pre-gradient	total eluent
9-7	0.025M Diethanolamine, pH 9.5, HCl	1.0mL Pharmalyte® 8-10.5, 5.2mL Polybuffer® 96, pH 7.0, HCl	7	34
9-6	0.025M Diethanolamine, pH 9.5, HCl or	10mL Polybuffer 96, pH 6.0, HCl	9	34
	0.075M Tris, pH 9.3, CH_3COOH	10mL Polybuffer 96, pH 6.0, CH_3COOH	3	30
8-6	0.025M Triethanolamine, pH 8.3, CH_3COOH	0.21mL Pharmalyte 8-10.5, 9.0mL Polybuffer 96, pH 6.0, CH_3COOH	7	37
8-5	0.025M Triethanolamine, pH 8.3, iminodiacetic acid	3.0mL Polybuffer 96, 7.0mL Polybuffer 74, pH 5.0, iminodiacetic acid	6	47
7-5	0.025M bis-Tris, pH 7.1, HCl	10mL Polybuffer 74, pH 5.0, HCl	3	26
7-4	0.025M bis-Tris, pH 7.1, iminodiacetic acid	10mL Polybuffer 74, pH 4.0, iminodiacetic acid	3	46
6-4	0.025M bis-Tris, pH 6.3, HCl	10mL Polybuffer 74, pH 4.0, HCl	3	39

pH 9-4, narrow pH intervals

pH	Start buffer	Eluent* (100mL)	Approximate Volumes (mL) pre-gradient	total eluent
9-8	0.025M Diethanolamine, pH 9.4, HCl	1.0mL Pharmalyte 8-10.5, 5.2mL Polybuffer 96, pH 8.0, HCl	3	28
8.5-7.5	0.025M Tris, pH 8.8, CH_3COOH	0.11mL Pharmalyte 8-10.5, 9.5mL Polybuffer 96, pH 7.5, CH_3COOH	4	29
8-7	0.025M Triethanolamine pH 8.3, HCl	10mL Polybuffer 96, pH 7.0, HCl	5	29
7.5-6.5	0.025M 2-Methylimidazole, pH 7.6, CH_3COOH	10mL Polybuffer 96, pH 6.5, CH_3COOH	9	27
7-6	0.025M bis-Tris, pH 7.0, CH_3COOH	9.5mL Polybuffer 96, 0.5mL Polybuffer 74, pH 6.0, CH_3COOH	10	28
6.5-5.5	0.025M bis-Tris, pH 6.7, CH_3COOH	4.0mL Polybuffer 96, 6.0mL Polybuffer 74, pH 5.5, CH_3COOH	5	23
6-5	0.025M bis-Tris, pH 6.4, HCl	10mL Polybuffer 74, pH 5.0, HCl	3	25
5.5-4.5	0.025M Piperazine, pH 6.3, HCl or iminodiacetic acid	10mL Polybuffer 74, pH 4.5, HCl or iminodiacetic acid	3	24
5-4	0.025M N-Methylpiperazine pH 5.7, HCl or iminodiacetic acid	10mL Polybuffer 74, pH 4.0, HCl or iminodiacetic acid	7	27

Reproduced with permission of Pharmacia Biotech.

* In the eluent recipes the concentration of Polybuffer and/or Pharmalyte mixtures are diluted to a volume of ca. 95mL. This volume is then titrated to the correct pH with the listed acid (1-2M). When the final pH has been reached, distilled water is added to make a total volume of 100mL.

For shallower gradients, within the same pH interval, increase the total volume. Make sure that the titration is always carried out on a maximum volume before adding the final few milliliters of water. When diluted eluents are used, proteins elute with increased volumes, therefore pre-gradient and total volumes increase also.

Table 4. Volatile Buffer Systems

pH	Substance	Counterion
2.0	Formic acid	H^+
2.3-3.5	Pyridine/formic acid	$HCOO^-$
3.0-5.0	Trimethylamine/formic acid	$HCOO^-$
3.0-6.0	Pyridine/acetic acid	CH_3COO^-
4.0-6.0	Trimethylamine/acetic acid	CH_3COO^-
6.8-8.8	Trimethylamine/HCl	Cl^-
7.0-8.5	Ammonia/formic acid	$HCOO^-$
8.5-10.0	Ammonia/acetic acid	CH_3COO^-
7.0-12.0	Trimethylamine/CO_2	CO_3^{-2}
7.0-12.0	Triethylamine/CO_2	CO_3^{-2}
7.9	Ammonium bicarbonate	HCO_3^-
8.0-9.5	Ammonium carbonate/ammonia	CO_3^{-2}
8.5-10.5	Ethanolamine/HCl	Cl^-
8.9	Ammonium carbonate	CO_3^{-2}

Reproduced with permission of Pharmacia Biotech.

Salts

A nonbuffering salt, such as NaCl, is usually added to the buffer to elute biomolecules from an ion exchange column by increasing salt concentration. During elution, salt counterions displace sample ions from the charged sites on the matrix. The counterions also may affect retention by complexing with the ion exchange groups or the analytes to change their ionic properties. Specific salts may alter protein tertiary structure and thus affect ion exchange interactions. Different salts have different elution strengths when used during ion exchange chromatography. The following anions and cations are listed in order of increasing strength:

Anions: OH^- < acetate < formate < Cl^- < SCN^- < Br^- < CrO_4^{2-} < NO_3^- < I^- < oxalate < SO_4^{2-} < citrate

Cations: Li^+ < H^+ < Na^+ < NH_4^+ < K^+ < Rb^+ < Cs^+ < Ag^+ < Tl^+ < Mg^{2+} < Zn^{2+} < Co^{2+} < Cu^{2+} < Cd^{2+} < Ni^{2+} < Ca^{2+} < Sr^{2+} < Pb^{2+} < Ba^{2+}

In general, divalent ions tend to be stronger displacers than monovalent species and thus produce lower retention. Smaller ions tend to have a higher elution strength than larger ions of the same periodic group.

Table 5 lists anions and cations commonly employed as salts in anion and cation exchange chromatography. Since some salt-buffer combinations form insoluble precipitates, (e.g., phosphate with $LiClO_4$), solubility tests should be performed before samples are separated. The solubility of some biomolecules decreases as the salt concentration decreases, the result of aggregation. To minimize the electrostatic interactions between sample components, a low concentration (10-50mM) of the salt used in the elution buffer can be added to the starting buffer and the sample buffer.

Table 5. Salts and Their Recommended Concentration Change/mL

Anions for gradient elution on strong anion exchange and recommended concentration change/mL of gradient volume.

Anion	Recommended concentration change (mM/mL)	Practical concentration for 100 % Buffer B (mM)*
SO_4^{-2}	7.5	150
ClO_4^-	8.0	160
I^-	10.5	210
Br^-	14.0	280
Cl^-	17.5	350
$CH_3SO_3^-$	21.5	430
$HCOO^-$	30.0	600
CH_3COO^-	35.0	700

Cations for gradient elution on strong cation exchange and recommended concentration change/mL of gradient volume.

Cation	Recommended concentration change (mM/mL)	Practical concentration for 100 % Buffer B (mM)*
K^-	17.5	350
NH_4^+	17.5	350
Na^+	21.5	430
$HN^+(CH_3)_3$	23.0	460
Li^+	25.0	460

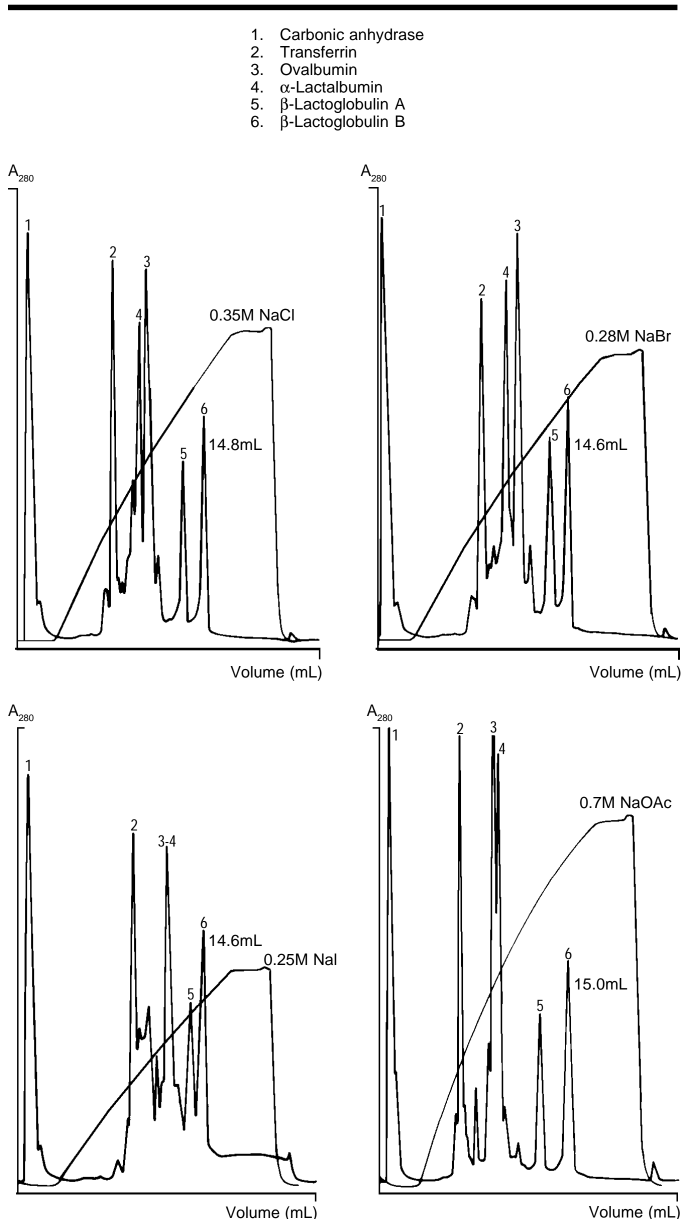
*These concentrations are based on a 20mL gradient. When running reduced gradient volumes the recommended concentration change alters; this can be compromised for speed.

Reproduced with permission of Pharmacia Biotech.

Most sample components elute before the salt concentration is increased to 1 M. If a sample has not eluted from the column at this concentration, it is best to change the pH and/or the counterion. Mixed pH and salt gradients also can be used to elute the sample. Also, a detergent or organic solvent may be added to increase the solubility of the sample in the starting buffer (see Detergents on page 7).

Counterions can affect sample loading and selectivity of a separation. Specific counterions can change the retention of single components or whole groups of compounds. Figure F shows the effect of varying the counterion on separations on a Mono Q® column. Resolution and selectivity vary with different salts. Therefore, it may be advantageous to test a variety of co-ions (ions with the same charge as the support) with a single counterion, and vice versa. Sample loading may be affected through aggregation, inefficient exchange of the counterion already present on the column, or solubility issues.

Figure F. Effect of Varying Counterion on Separations on Mono Q



Reproduced with permission of Pharmacia Biotech.

794-0672

An important quality that can be exploited during ion exchange and chromatofocusing is the chaotropic character of a salt, i.e., the ability of a salt to make a solvent less polar. Salts with high chaotropic character tend to lead to higher recoveries for hydrophobic proteins/peptides. This might be due to a lower salting-out effect on hydrophobic molecules, and thus maximum sample solubility. Ions in order of increasing chaotropic effect are:

Anions: $PO_4^{3-} < SO_4^{2-} < CH_3COO^- < Br^- < NO_3^- < ClO_4^- < I^- < CF_3COO^- < SCN^- < Cl_3COO^-$

Cations: $NH_4^+ < Pb^+ < K^+ < Na^+ < Cs^+ < Li^+ < Mg^{2+} < Ca^{2+} < Ba^{2+}$

Chaotropic salts are useful for ion exchange in organic solvents, because they are more soluble than other salts. Because salts such as $(NH_4)_2SO_4$ or K_3PO_4 have weakly chaotropic character, they must be used in high concentration, and are a poor choice for gradients if analytes show decreased solubility at high salt concentrations.

Detergents

Frequently, additives such as detergents are used to increase protein solubility. The detergent must be neutral or of the same charge as the ion exchange support, and should be free of salts and other contaminants. In order to obtain reproducible separations, the column must be equilibrated with the detergent and/or mobile phase.

Which detergent to use depends on the type of biomolecule to be isolated. Different peak profiles are possible, due to differences in solubilizing properties. Test runs are required to find the detergent that best solubilizes the protein and yields optimal resolution.

Use the minimum concentration required to keep the sample in solution. To avoid background UV absorption, ion exchange is often performed well below the detergent concentration at which micelles begin to form. Blank salt gradients are run and the UV absorption monitored to determine micelle formation (indicated by a sudden increase in UV absorption). The detergent concentration and/or salt gradient can be adjusted to minimize light scattering due to micelle formation.

Chromatofocusing is compatible with detergent inclusion. Due to the low ionic strength, there is no interference with detection as a result of micelle formation. Blank runs should be performed to determine the UV profile of the detergents, however, and whether there is any effect on the pH gradient. Urea, detergents and glycerol have been especially useful for problematic hydrophobic proteins.

Zwitterions

During ion exchange, zwitterions can be used to decrease protein aggregation. Zwitterions cause samples to elute more closely to their true isoelectric points, possibly due to minimization of microenvironmental pH effects. The solubilities of samples at low salt concentrations can be improved by adding betaine or taurine to the buffers. Betaine is effective from pH 2-12 at concentrations up to 10% (w:v) at 25°C. Taurine is best for pH 4-6 at concentrations up to 4% (w:v) at 25°C and should not be used above pH 8.5. Betaine and taurine improve chromatofocusing resolution due to the solubilizing and stabilizing properties of the zwitterions.

Organic Solvents

When hydrophobic interactions between the matrix and the analytes are important, it is evident that organic solvents will decrease that binding. Ion exchange and chromatofocusing can be performed in organic solvents, but the solubility of the salt, sample and buffer all must be considered. Many biomolecules, salts and buffers can precipitate at high organic concentrations, while the biological activity and/or of some proteins can be irreversibly lost in the presence of even low concentrations of organic solvents.

Organic modifiers decrease the dielectric constant and thus increase the ionic forces. The actual pKa of a buffering substance increases, which may result in the improper selection of a buffer for the desired working pH. The pH gradient should be monitored when using organic solvents with buffers. If the pH is unstable over the gradient, replace the buffer with one which can provide the buffering capacity needed. Adjust the pH of the buffer after adding the organic solvent.

Temperature

The pKa of a buffering substance varies with the temperature (see Tables 1 and 2). This effect should be considered during optimization, since the change in pKa can be significant. Tris (tris[hydroxymethyl]aminomethane) is notorious for its fluctuations in buffering capacity with temperature. Buffers should always be used at the temperature at which they are prepared.

Decreased temperatures (<10°C) can minimize aggregation and preserve protein activity/structure, and can be used to suppress hydrophobic interactions when detergents or organic solvents are unacceptable. On the other hand, higher temperature can increase resolution of biomolecules restricted from moving into the pores of the support due to molecular weight or size.

Selectivity is the key to high resolution and is determined by a large number of variables. With ion exchange and chromatofocusing, this selectivity is based on the media, pH, buffers, salts, solvents, and biomolecules. Optimization of these variables allows for innumerable possibilities for solving separation problems.

Ordering Information:

Ion Exchange and Chromatofocusing Columns

Product	Particle Size	Dimensions	Cat. No.
Pharmacia Biotech FPLC® Columns			
Mono Q HR5/5	10µm	5cm x 5mm	**54807**
Mono S® HR5/5	10µm	5cm x 5mm	**54808**
Mono P HR5/5	10µm	5cm x 5mm	**54809**
TSK-GEL® HPLC Columns, Guard Columns, and Packings			
Polymer-Based			
DEAE-NPR	2.5µm	3.5cm x 4.6mm	**813075**
DEAE-NPR guard column	5µm	0.5cm x 4.6mm	**817088**
DEAE-5PW	10µm	7.5cm x 7.5mm	**807164**
DEAE-5PW guard column kit	20µm		**807210**
DEAE-5PW packing (5mL)	20µm		**807207**
CM-5PW	10µm	7.5cm x 7.5mm	**813068**
CM-5PW guard column kit	20µm		**813069**
CM-5PW packing (5mL)	20µm		**813070**
SP-NPR	2.5µm	3.5cm x 4.6mm	**813076**
SP-5PW	10µm	7.5cm x 7.5mm	**807161**
SP-5PW guard column kit	20µm		**807211**
SP-5PW packing (5mL)	20µm		**807208**
Silica-Based			
DEAE-2SW	5µm	25cm x 4.6mm	**807168**
DEAE-3SW	10µm	7.5cm x 7.5mm	**807163**
DEAE-SW guard column kit	10µm		**807648**
DEAE-SW packing (5mL)	10µm		**807647**
CM-2SW	5µm	25cm x 4.6mm	**807167**
CM-3SW	10µm	7.5cm x 7.5mm	**807162**
CM-SW guard column kit	10um		**807650**
CM-SW packing (5mL)	10µm		**807649**
MCI GEL® ProtEx® PEEK HPLC Columns			
SP	5µm	5cm x 4.6mm	**54740-U**
Q	5µm	5cm x 4.6mm	**54742**

Salts and Buffers

Product	Quantity*	Cat. No.
Acetic Acid	500g	**24,285-3B**
Ammonium Hydroxide	500mL	**22,122-8B**
Ammonium Carbonate	500g	**20,786-1B**
BICINE	100g	**B3876B**
Bis-Tris	100g	**B9754B**
Bis-Tris Propane	100g	**B6755C**
Citric Acid	500g	**C1909A**
1,3-Diaminopropane	50g	**23,998-4**
Diethanolamine	100g	**D8,330-3B**
Ethanolamine	1L	**11,016-7B**
Formic Acid	500g	**25,136-4C**
HEPES	100g	**H3375D**
L-Histidine	100g	**H8000D**
Lactic Acid	10g	**L1750C**
Maleic Acid	500g	**M0375A**
Malonic Acid	100g	**M1750B**
MES, Free Acid	100g	**M8250C**
Sodium Phosphate, Di	500g	**21,988-6B**
Sodium Phosphate, Mono	500g	**22,352-2B**
Potassium Phosphate, Di	500g	**23,450-8C**
Potassium Phosphate, Mono	500g	**22,130-9B**
Piperazine	100g	**P3896A**
Triethanolamine	1kg	**T5,830-0B**
Tris	100g	**T1503B**
Tris-HCl	100g	**T3253A**

*See the current Supelco catalog for additional quantities.

Contact our Technical Service Department (phone 800-359-3041 or 814-359-3041, FAX 800-359-3044 or 814-359-5468) for expert answers to your questions.

Trademarks

FPLC, Mono P, Mono Q, Mono S, Pharmalyte, Polybuffer — Pharmacia Biotech AB
MCI GEL, ProtEx — Mitsubishi Chemical Co.
SigmaChrom — Sigma-Aldrich Co.
TSK-GEL — Tosoh Corp.

For more information, or current prices, contact your nearest Supelco subsidiary listed below. To obtain further contact information, visit our website (www.sigma-aldrich.com), see the Supelco catalog, or contact Supelco, Bellefonte, PA 16823-0048 USA.

ARGENTINA · Sigma-Aldrich de Argentina, S.A. · Buenos Aires 1119 **AUSTRALIA** · Sigma-Aldrich Pty. Ltd. · Castle Hill NSW 2154 **AUSTRIA** · Sigma-Aldrich Handels GmbH · A-1110 Wien **BELGIUM** · Sigma-Aldrich N.V./S.A. · B-2880 Bornem **BRAZIL** · Sigma-Aldrich Quimica Brasil Ltda. · 01239-010 São Paulo, SP **CANADA** · Sigma-Aldrich Canada, Ltd. · 2149 Winston Park Dr., Oakville, ON L6H 6J8 **CZECH REPUBLIC** · Sigma-Aldrich s.r.o. · 186 00 Praha 8 **DENMARK** · Sigma-Aldrich Denmark A/S · DK-2665 Vallensbaek Strand **FINLAND** · Sigma-Aldrich Finland/YA-Kemia Oy · FIN-00700 Helsinki **FRANCE** · Sigma-Aldrich Chimie · 38297 Saint-Quentin-Fallavier Cedex **GERMANY** · Sigma-Aldrich Chemie GmbH · D-82041 Deisenhofen **GREECE** · Sigma-Aldrich (o.m.) Ltd. · Ilioupoli 16346, Athens **HUNGARY** · Sigma-Aldrich Kft. · H-1067 Budapest **INDIA** · Sigma-Aldrich Co. · Bangalore 560 048 **IRELAND** · Sigma-Aldrich Ireland Ltd. · Dublin 24 **ISRAEL** · Sigma Israel Chemicals Ltd. · Rehovot 76100 **ITALY** · Sigma-Aldrich s.r.l. · 20151 Milano **JAPAN** · Sigma-Aldrich Japan K.K. · Chuo-ku, Tokyo 103 **KOREA** · Sigma-Aldrich Korea · Seoul **MALAYSIA** · Sigma-Aldrich (M) Sdn. Bhd. · Selangor **MEXICO** · Sigma-Aldrich Química S.A. de C.V. · 50200 Toluca **NETHERLANDS** · Sigma-Aldrich Chemie BV · 3330 AA Zwijndrecht **NORWAY** · Sigma-Aldrich Norway · Torshov · N-0401 Oslo **POLAND** · Sigma-Aldrich Sp. z o.o. · 61-663 Poznañ **PORTUGAL** · Sigma-Aldrich Quimica, S.A. · Sintra 2710 **RUSSIA** · Sigma-Aldrich Russia · Moscow 103062 **SINGAPORE** · Sigma-Aldrich Pte. Ltd. **SOUTH AFRICA** · Sigma-Aldrich (pty) Ltd. · Jet Park 1459 **SPAIN** · Sigma-Aldrich Quimica, S.A. · 28100 Alcobendas, Madrid **SWEDEN** · Sigma-Aldrich Sweden AB · 135 70 Stockholm **SWITZERLAND** · Supelco · CH-9471 Buchs **UNITED KINGDOM** · Sigma-Aldrich Company Ltd. · Poole, Dorset BH12 4QH **UNITED STATES** · Supelco · Supelco Park · Bellefonte, PA 16823-0048 · Phone 800-247-6628 or 814-359-3441 · Fax 800-447-3044 or 814-359-3044 · email:supelco@sial.com H

Supelco is a member of the Sigma-Aldrich family. Supelco products are sold through Sigma-Aldrich, Inc. Sigma-Aldrich warrants that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product for a particular use. Additional terms and conditions may apply. Please see the reverse side of the invoice or packing slip. AKY