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Effect of buffer concentration on gradient chromatofocusing performance separating proteins on a high-performance DEAE column

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Abstract

Gradient chromatofocusing is a recently developed chromatographic technique that overcomes the limitations of conventional chromatofocusing. This technique employs a HPLC gradient system and simple low-molecular-mass buffer components to generate linear or other function pH gradients on ion-exchange columns. Results of the present work show a superior separation of β-lactoglobulin A and B in gradient chromatofocusing compared to salt gradient chromatography using the same DEAE column, with an optimized resolution of 2.3 obtained with gradient chromatofocusing compared to 1.1 obtained with NaCl gradients at constant pH. A significant advantage of the gradient chromatofocusing technique over the conventional chromatofocusing technique is its ability to employ a relatively wide range of buffer concentrations in the mobile phase, the effect of which is studied in the present work. Five proteins (conalbumin, ovalbumin, bovine serum albumin, β-lactoglobulin A and B) are chromatographed on a DEAE-polymethacrylate HPLC anion-exchange column using the same approximately linear pH gradient profile but different mobile phase buffer concentrations. Results show a significant effect of buffer concentration on peak width, separation factor and resolution. For example, resolution increases from 1.5 to 2.3 in the separation of β-lactoglobulin A and B when the concentration of each of the components in the 100% elution buffer is increased from 6.25 to 25.0 m*M* (with the same outlet pH gradient). This separation trend is also seen in the chromatography of ovalbumin from a commercial source, noting a progressive increase in resolution of two peaks in the sample (resolution increased from 0.7 to 2.4) when the concentration of each of the components in the 100% elution buffer is increased from 6.25 to 37.5 m*M* (same outlet pH gradient). The gains in the resolution are attributed to an increase in the separation factor, since the peak widths are generally noted to also increase with increased buffer concentration. These results point to a significant interplay between buffer concentration and pH, which is not effectively exploited in either conventional chromatofocusing or in conventional ion-exchange chromatographic procedures employing salt gradient elution at constant pH. Gradient chromatofocusing has the ability of optimizing both parameters, thus providing it with unique capabilities in protein separations.

Keywords: Chromatofocusing; Buffer composition; Gradient elution; pH gradients; Proteins

1. Introduction

Chromatofocusing is a widely used ion-exchange chromatographic technique developed by Sluyterman

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and co-workers [1–5], which employs nearly linear pH gradients to accomplish protein separations. In the usual design of this technique, a weak anion-exchange column is equilibrated with a high-pH mobile phase (application buffer), conditions that favor the retention of negatively charged proteins. A low-pH mobile phase (focusing buffer), usually employing a polymeric ampholyte buffer, is then introduced by a step change. An approximately linear pH gradient is produced over a certain pH range, given that the mobile and stationary phases are buffered within that range. Both a pH gradient in time at the column outlet (outlet pH gradient) and an internal pH gradient in distance within the column (column pH gradient) are generated. The outlet pH gradient affects separation of the peaks based on the protein's charge, while the column pH gradient affects peak width via a focusing effect.

Even though chromatofocusing is a widely used technique in protein separation, it has several significant limitations. Disadvantages include: difficulty in controlling the outlet pH gradient slope, limitation of the mobile phase to low buffer concentration conditions [6], and use of polymeric ampholyte buffers, which are expensive and can contaminate protein fractions (hindering its usefulness in purification procedures) [7]. Recently, a gradient chromatofocusing technique was developed by Liu and Anderson [8,9], which addresses these limitations.

Fig. 1 illustrates the various parameters of the gradient chromatofocusing technique. An HPLC gradient system is used to produce an inlet pH gradient in time (produced by successively increasing the ratio of a low-pH elution buffer to a high-pH application buffer) that is introduced onto a high-performance anion-exchange column. The inlet pH gradient is held up by the column either through the column's buffering action [2] or through the successive displacement on the column of weaker with stronger acidic buffer components [6,10]. This leads to the establishment of a pH gradient in distance in the column (column pH gradient) and a pH gradient in time at the column outlet (outlet pH gradient). The slope of the outlet pH gradient is not easy to control in the conventional technique, but can be readily manipulated in gradient chromatofocusing by changing the slope of the inlet pH gradient. The column pH gradient can be varied in gradient chromatofocusing by changing the relative buffer capacities of the mobile and stationary phase (i.e., changing the concentration of buffers in mobile phase and/or changing the density or composition of the column's ion-exchange groups).

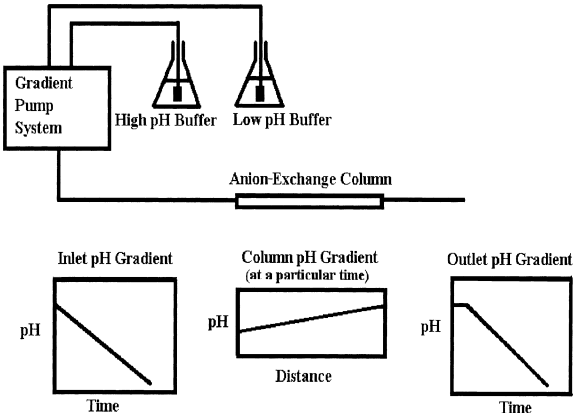

Fig. 1. Schematic of the gradient chromatofocusing technique. An HPLC gradient system produces an inlet pH gradient (pH gradient in time) which enters the anion-exchange column. The inlet pH gradient is transformed into an outlet pH gradient (which is the pH gradient in time at the column outlet) and a column pH gradient (which is the pH gradient in distance within the column). The outlet pH gradient affects separation of the peaks based on the protein's charge, while the column pH gradient affects the peak width via a focusing effect.

Low-molecular-mass buffers are used in gradient chromatofocusing, replacing the polymeric ampholytes used in most of the conventional chromatofocusing techniques. In the present work, the application buffer employs bis-tris-propane (6.80) and piperazine (5.68), and the elution buffer employs acetic acid (4.76), lactic acid (3.81) and chloroacetic acid (2.87) (pK_a in parentheses). The combination of the above application and elution buffers is used in the present work to generate an approximately linear pH gradient from 7.6 to 4.0, as the buffer components have pK_a values which are approximately equally spaced throughout the gradient pH range.

The effect of buffer concentration on the gradient chromatofocusing separation of proteins is studied in the present work. This parameter cannot be used to optimize protein separation in conventional chromatofocusing, since this technique requires a low buffer concentration in order to generate reasonable pH gradient slopes. The present work shows a

general trend of improved resolution with increased buffer concentration, demonstrating the unique capabilities of gradient chromatofocusing compared to conventional chromatofocusing for optimizing protein separations.

2. Experimental

2.1. Materials

Conalbumin (from chicken egg white, Catalog No. C-0755), bovine serum albumin (BSA) (Catalog No. A-7906), ovalbumin (from chicken egg, Catalog No. A-2512), β-lactoglobulin A (from bovine milk, Catalog No. L-7880), β-lactoglobulin B (from bovine milk, Catalog No. L-8005), bis-tris-propane (>99%, Catalog No. B-9410), piperazine (Catalog No. P-3896), Na_2HPO_4 (>99%, Catalog No. S-7907), and NaCl (>99%, Catalog No. S-7653) were from Sigma (St. Louis, MO, USA). Glacial acetic acid (99.7%, Catalog No. 3-9507) was from J.T. Baker (Phillipsburg, NJ, USA), lactic acid (75%, Catalog No. 02634) was from Merck (Whitehouse Station, NJ, USA), and chloroacetic acid (>99%, Catalog No. 40,292-3) was from Aldrich (Milwaukee, WI, USA). H_3PO_4 (Catalog No. A242-212) and HCl (Catalog No. A144-212) were from Fisher Scientific (Pittsburgh, PA, USA). Bulk anion-exchange material (Protein-Pak DEAE 8HR, 8 μm diameter, 1000-Å pore diameter, DEAE-functionalized polymethacrylate) was from Waters (Milford, MA, USA). The protein sample solutions were prepared with a 20.0 m*M* NaH_2PO_4–Na_2HPO_4, pH 7.00 buffer solution (prepared by adding concentrated H_3PO_4 to 20.0 m*M* Na_2HPO_4) and aliquots stored frozen at −20°C until use. All solutions were aqueous, prepared with HPLC grade water.

Mobile phase buffer A (high-pH application buffer) consisted of a bis-tris-propane and piperazine solution, with the pH adjusted to 7.60 using concentrated HCl. Mobile phase buffer B (low-pH elution buffer) consisted of an acetic, lactic and chloroacetic acid solution (no pH adjustment). Seven different concentrations of application and elution buffers were used as specified in Table 1.

Anion-exchange HPLC experiments employing NaCl gradients were also done to compare with gradient chromatofocusing results. For these experiments, the application buffer (buffer A) was 9.60 m*M* bis-tris-propane, and 16.0 m*M* piperazine, pH 7.60 (adjusted with concentrated HCl), while the elution buffer (buffer B) was 9.60 m*M* bis-tris-propane, 16.0 m*M* piperazine, 1.00 *M* NaCl, pH 7.60 (adjusted with concentrated HCl).

2.2. Chromatographic design and procedure

The HPLC system consisted of a System Gold 127 solvent module gradient system from Beckman Instruments (Fullerton, CA, USA), a SP4270 integrator from Thermo Separation Products (San Jose, CA, USA), a variable-wavelength detector set at 280 nm from Dionex (Sunnyvale, CA, USA) and an Epson Equity I+ personal computer. A Rheodyne Model 7125 injection valve from Rainin (Woburn, MA, USA) was used, employing a 500-μl injection

Table 1
Concentration of the components used in buffers A and B

System No.	Concentration (m*M*)				
	Buffer A		Buffer B		
	Bis-tris-propane	Piperazine	Acetic acid	Lactic acid	Chloroacetic acid
1	9.60	16.0	6.25	6.25	6.25
2	12.0	20.0	12.5	12.5	12.5
3	15.0	25.0	25.0	25.0	25.0
4	22.5	37.5	37.5	37.5	37.5
5	27.0	45.0	45.0	45.0	45.0
6	30.0	50.0	50.0	50.0	50.0
7	45.0	75.0	75.0	75.0	75.0

loop. The column (50×4.1 mm I.D.) was packed at 500 p.s.i. using a HPLC packer from Alltech (Deerfield, IL, USA) (1 p.s.i.=6894.76 Pa). The volume from the gradient valve to the column was approximately 2.8 ml (including the injection loop). The dead volume of the column was 0.51 ml, determined by injecting acetone. The flow-rate of the mobile phase was 1.0 ml/min. The column was equilibrated with the application buffer prior to the start of each run (usually 30 min) until the pH of the column eluant was the same as that of the applied application buffer.

2.3. Other procedures

The pH of 2.0-min fractions of the inlet and outlet mobile phase (Figs. 2 and 3, Table 3) was determined using a model 915 pH meter and a standard Ag/AgCl Micro Probe Combination pH electrode from Fisher (Pittsburgh, PA, USA). The inlet fractions were collected immediately before the column, while the outlet fractions were collected immediately after the detector. These pH gradients were determined with the injection loop in line, in order to duplicate the exact conditions of the chromatographic runs.

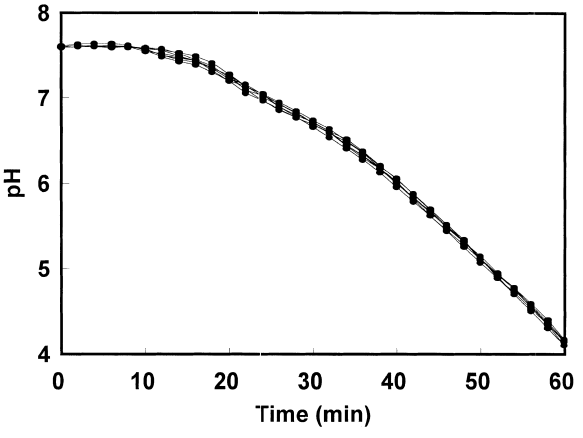

Fig. 2. The outlet pH gradient profile is produced by seven different mobile phase systems described in Sections 2.1 and 2.2, and Tables 1 and 2. Coefficients for the fitted equations for each pH gradient are given in Table 3.

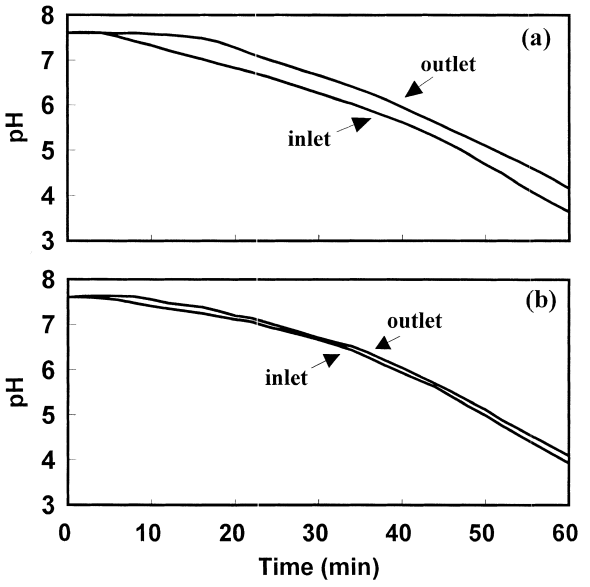

Fig. 3. The outlet and inlet pH gradients of (a) mobile phase system 1 (lowest buffer concentration) and (b) mobile phase system 7 (highest buffer concentration) described in Sections 2.1 and 2.2, and Tables 1 and 2. Coefficients for the fitted equation of each plot are given in Table 3.

2.4. Equations

The resolution was calculated using Eq. (1) [11]:

$$R_s = \frac{t_{R2} - t_{R1}}{2(\sigma_2 + \sigma_1)} = \frac{2.354(t_{R2} - t_{R1})}{2(w_{h2} + w_{h1})} \quad (1)$$

where R_s is the resolution; t_{R2} and t_{R1} are the retention times of peak 2 (late eluting peak) and peak 1 (early eluting peak), respectively; σ_2 and σ_1 are the standard deviations of peak 2 and peak 1, respectively; and, w_{h2} and w_{h1} are the width of peak 2 and peak 1 at half height, respectively.

The apparent plate number was calculated using Eq. (2) [11]:

$$N = 5.54 \cdot \left(\frac{t_R}{w_h}\right)^2 \quad (2)$$

where N is the apparent plate number, and t_R and w_h are specified previously.

The separation factor for two peaks was calculated using Eq. (3) [12]:

$$\alpha = \left(\frac{k'_2}{k'_1}\right) \quad (3)$$

where α is the separation factor, and k' is the capacity factor for proteins with the lower (subscript 1) and higher (subscript 2) retention times.

Fitted equations for the outlet and inlet pH gradients given in Table 3 were determined with the regression function of Quattro Pro 5.00 (Borland International, Scotts Valley, CA, USA).

3. Results

3.1. Production of the same outlet pH gradient for seven different buffer concentration systems

Similar outlet pH gradient profiles (Fig. 2) are produced for the seven different buffer concentration systems described in Table 1 by adjusting the rate of mixing of the application (buffer A) and elution (buffer B) mobile phases with the HPLC gradient system (Table 2). Table 2 shows that the two lowest buffer concentration mobile phases (systems 1 and 2) require a steeper gradient in buffer B than mobile phase systems 3–7. This is because buffer A has a proportionally higher buffer concentration relative to buffer B (for systems 1 and 2 compared to systems 3–7) making it less responsive to pH adjustment (see Table 1). Use of a lower concentration of buffer components in buffer A, lower than that given in Table 1 for systems 1 and 2, yield outlet pH gradients that have a precipitous drop in the pH at the beginning of the gradient (after the plateau region). This irregularity is thought to result from the buffering action of the column at the high pH range of the gradient (suggested by the fact that the corresponding inlet pH gradient for these low concentrations of buffer A show a smooth profile without the initial pH drop). Raising the concentration of buffer A to that given in Table 1 for systems 1 and 2 overcomes this buffering effect of the column, allowing for the production of the smooth gradients plotted in Fig. 2. Mobile phase systems 3–7 employ almost identical gradient mixing rates because there is a constant concentration ratio of the buffer A to buffer B components in going from one mobile phase system to another (see Table 1).

The outlet pH gradient plots in Fig. 2 appear to be linear after the plateau region (after the first 20 min). However, detailed slope analysis shows these plots to be convex. For example, the slope (pH/min) for the system 1 plot gets steeper with time, varying from -0.053 (20 min) to -0.118 (60 min). Fitted equation parameters for each outlet and inlet pH gradient are given in Table 3.

3.2. Column pH gradient

Fig. 3 shows the inlet and outlet pH gradients for the lowest concentration buffer system (Fig. 3a, system 1) and the highest concentration buffer system (Fig. 3b, system 7). As mentioned in the introduction and diagrammed in Fig. 1, there are two types of gradients in a chromatofocusing technique: a pH gradient in time at the column outlet (outlet pH

Table 2
Gradient programs used to produce pH gradients of Table 3 and Fig. 2

System 1: 100% A (0% B) to 20% A (80% B) in 60 min.
System 2: 100% A (0% B) to 78% A (22% B) in the first 28 min, 78% A (22% B) to 32% A (68% B) in the following 32 min.
System 3: 100% A (0% B) to 94% A (6% B) in the first 16 min, 94% A (6% B) to 84% A (16% B) in the following 14 min, and 84% A (16% B) to 50.5% A (49.5% B) in the last 30 min.
System 4: 100% A (0% B) to 94% A (6% B) in the first 16 min, 94% A (6% B) to 85% A (15% B) in the following 14 min, and 85% A (15% B) to 50.5% A (49.5% B) in the last 30 min.
Systems 5–7: 100% A (0% B) to 94% A (6% B) in the first 16 min, 94% A (6% B) to 85% A (15% B) in the following 14 min, and 85% A (15% B) to 51% A (49% B) in the last 30 min.

Table 3
The coefficients of the fitted equation ($pH = a + bt + ct^2 + dt^3 + et^4 + ft^5 + gt^6 + ht^7$) for the outlet and inlet pH gradients of the mobile phase systems[a]

	a	$b\times10^2$	$c\times10^3$	$d\times10^4$	$e\times10^5$	$f\times10^7$	$g\times10^9$	$h\times10^{11}$
Outlet pH of system 1	7.61650	−3.80501	11.1224	−11.2870	4.81269	−10.6100	11.7981	−5.23542
Inlet pH of system 1	7.60783	3.37561	−12.2923	9.06469	−3.66024	8.18114	−9.57449	4.55021
Outlet pH of system 2	7.61694	−3.80082	14.1779	−17.0021	8.39647	−20.7036	25.0161	−11.8091
Inlet pH of system 2	7.58882	3.32424	−12.3035	8.46762	−2.87601	5.06332	−4.57790	1.71686
Outlet pH of system 3	7.58647	0.228752	2.35785	−4.58794	2.45834	−6.43674	8.18561	−4.03316
Inlet pH of system 3	7.59940	1.66368	−8.53944	6.95579	−3.02833	6.97057	−8.17736	3.85035
Outlet pH of system 4	7.58795	1.54652	−0.417425	−3.54638	2.53466	−7.46580	10.0231	−5.06786
Inlet pH of system 4	7.59420	1.63775	−7.79452	6.05784	−2.56300	5.83291	−6.89456	3.31315
Outlet pH of system 5	7.59011	0.885814	0.721010	−3.90782	2.46941	−6.95090	9.13922	−4.57671
Inlet pH of system 5	7.59209	1.69498	−7.08540	4.82064	−1.74741	3.37512	−3.45213	1.49202
Outlet pH of system 6	7.58803	1.72436	−1.69671	−1.29934	1.17667	−3.70362	5.10030	−2.60504
Inlet pH of system 6	7.60798	0.873644	−3.98167	1.67085	−0.256724	−0.325333	1.17231	−0.788877
Outlet pH of system 7	7.59850	3.64316	−6.89935	3.70828	−1.18391	2.14170	−2.15634	0.948300
Inlet pH of system 7	7.60932	1.34161	−6.77587	5.30147	−2.18068	4.66637	−5.12632	2.29852

[a] See Tables 1 and 2, t is in min.

gradient) causing differential elution of proteins, and a pH gradient in distance within the column (column pH gradient) affecting the focusing of the protein bands. In Fig. 3a or b the upper plot is the outlet pH gradient, with a slope in units of pH/min, while the vertical difference of the outlet from the inlet gradient plots at a particular time divided by the column length is taken to be the value of the column pH gradient slope in units of pH/cm [9]. Both the column pH gradient and the delay in time of the outlet pH gradient with respect to the inlet pH gradient result from either the buffering action of the stationary phase on the inlet pH gradient [2] or from mechanisms imposed on the inlet pH gradient based on the adsorption and desorption of buffer components on the column [6,10,13–16]. The lowest concentration buffer system in Fig. 3a gives the largest vertical difference and hence steepest column pH gradient. The average column pH gradient slope (defined in Fig. 4) has been calculated for each mobile phase system and is given in Fig. 4.

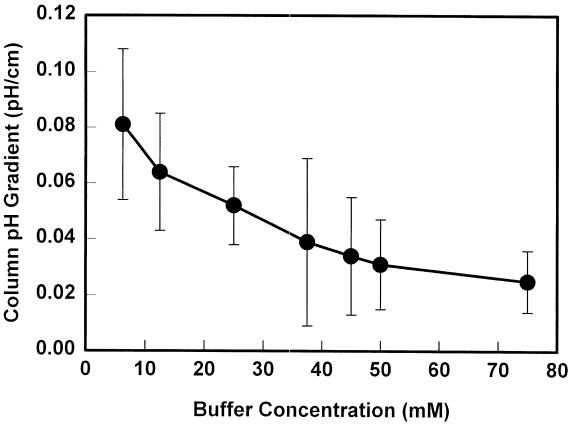

Fig. 4. Plot of the average column pH gradient slope (±2s) versus buffer concentration in gradient chromatofocusing. Plotted points from left to right are for mobile phase systems 1–7 successively, which have the same outlet pH gradients but different buffer concentrations (Fig. 2). The average column pH gradient slope, along with its ±2-s limits, is determined by taking the average and standard deviation of the difference between the corresponding outlet and inlet pH at a particular time t for each 2-min fraction ($t \geq 20$ min) and dividing this by the column length. The chromatographic setup and conditions are described in Sections 2.1 and 2.2, and Tables 1 and 2. Note: buffer concentrations of the various buffer components continually change throughout the run according to the gradient given in Table 2. The buffer concentration plotted is the concentration of each elution buffer component in 100% B as given in Table 1.

3.3. Focusing effect of gradient chromatofocusing demonstrated in the chromatography of β-lactoglobulin A and B

The results of gradient chromatofocusing (Fig. 5) are compared with the results of NaCl gradient elution at constant pH (Fig. 6) in the chromatog-

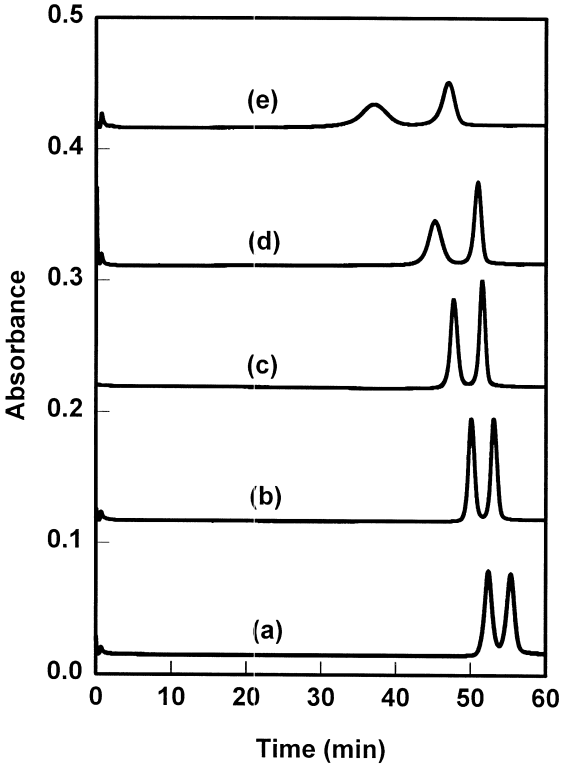

Fig. 5. Chromatograms of a sample containing β-lactoglobulin A (late eluting peak) and B (early eluting peak) using gradient chromatofocusing at different mobile phase buffer concentrations but the same outlet pH gradient profile (see Fig. 2) [(a) has the lowest buffer concentration and (e) has the highest buffer concentration, with the concentration increasing progressively from (a) to (e)]. A sample volume of 40 μl at a concentration of 5 mg/ml each for β-lactoglobulin A and B is injected. The chromatographic setup and conditions are described in Sections 2.1 and 2.2, and Tables 1 and 2. Subscripts A and B given below are for β-lactoglobulin A and B, respectively. (a) Mobile phase system No. 1 ($R_s = 1.53$, $\alpha = 1.06$, $w_{h,A} = 1.18$ min, $w_{h,B} = 1.11$ min, $N_A = 1.22 \cdot 10^4$, $N_B = 1.23 \cdot 10^4$); (b) mobile phase system No. 2 ($R_s = 1.89$, $\alpha = 1.06$, $w_{h,A} = 0.93$ min, $w_{h,B} = 0.93$ min, $N_A = 1.80 \cdot 10^4$, $N_B = 1.61 \cdot 10^4$); (c) mobile phase system No. 3 ($R_s = 2.33$, $\alpha = 1.08$, $w_{h,A} = 0.87$ min, $w_{h,B} = 1.04$ min, $N_A = 1.94 \cdot 10^4$, $N_B = 1.17 \cdot 10^4$); (d) mobile phase system No. 4 ($R_s = 2.17$, $\alpha = 1.13$, $w_{h,A} = 1.07$ min, $w_{h,B} = 2.06$ min, $N_A = 1.26 \cdot 10^4$, $N_B = 2.67 \cdot 10^3$); (e) mobile phase system No. 6 ($R_s = 1.98$, $\alpha = 1.27$, $w_{h,A} = 1.93$ min, $w_{h,B} = 3.97$ min, $N_A = 3.29 \cdot 10^3$, $N_B = 4.83 \cdot 10^2$).

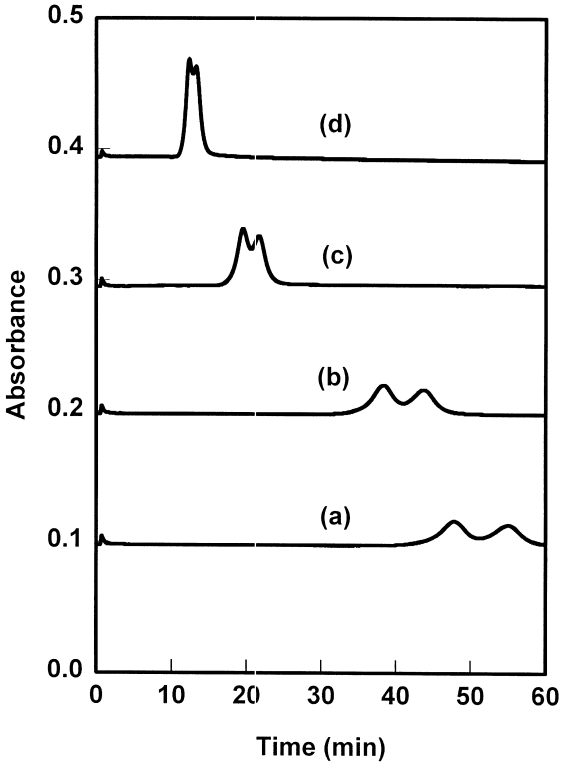

Fig. 6. Chromatograms of a sample containing β-lactoglobulin A (late eluting peak) and B (early eluting peak) using NaCl gradient chromatography. A sample volume of 40 μl at a concentration of 5 mg/ml each for β-lactoglobulin A and B is injected. Chromatographic setup and conditions are described in Sections 2.1 and 2.2. The chromatograms are for four different salt gradients as specified below [(a) has the shallowest salt gradient and (d) has the steepest salt gradient, with the salt gradient slope increasing progressively from (a) to (d)]. Subscripts A and B given below are for β-lactoglobulin A and B, respectively. The parameters below are determined from experiments of individually chromatographed proteins. (a) 100% A (0% B) to 85% A (15% B) in 60 min ($R_s = 1.09$, $\alpha = 1.15$, $w_{h,A} = 3.88$ min, $w_{h,B} = 3.75$ min, $N_A = 1.11 \cdot 10^3$, $N_B = 9.04 \cdot 10^2$); (b) 100% A (0% B) to 80% A (20% B) in 60 min ($R_s = 1.00$, $\alpha = 1.15$, $w_{h,A} = 3.26$ min, $w_{h,B} = 3.20$ min, $N_A = 1.01 \cdot 10^3$, $N_B = 8.07 \cdot 10^2$); (c) 100% A (0% B) to 50% A (50% B) in 60 min ($R_s = 0.69$, $\alpha = 1.10$, $w_{h,A} = 1.79$ min, $w_{h,B} = 1.63$ min, $N_A = 8.34 \cdot 10^2$, $N_B = 8.27 \cdot 10^2$); (d) 100% A (0% B) to 0% A (100% B) in 60 min ($R_s = 0.64$, $\alpha = 1.10$, $w_{h,A} = 1.07$ min, $w_{h,B} = 1.01$ min, $N_A = 8.88 \cdot 10^2$, $N_B = 8.43 \cdot 10^2$).

raphy of β-lactoglobulin A and B. The same column and similar starting mobile phase conditions are used for each set of experiments so that the results can be directly compared. Although plate number analysis (N) does not strictly apply for these experiments (since the protein is not migrating through most of the chromatographic run), the power of gradient chromatofocusing is illustrated by comparing the apparent plate number for each experiment. Apparent plate numbers for gradient chromatofocusing of Fig.

5b ($1.80 \cdot 10^4$ for β-lactoglobulin A and $1.61 \cdot 10^4$ for β-lactoglobulin B) are more than an order of magnitude higher than the NaCl gradient runs of Fig. 6a ($1.11 \cdot 10^3$ for β-lactoglobulin A and $9.04 \cdot 10^2$ for β-lactoglobulin B), comparing results of similar retention times.

Comparison of Figs. 5 and 6 shows the focusing effect of gradient chromatofocusing, as the peak widths for the gradient chromatofocusing peaks in Fig. 5b are four times smaller than the peak widths for NaCl gradient peaks in Fig. 6a, comparing results of similar retention times. Although peak width decreases with increased gradient slope in the NaCl gradient runs, the resolution (R_s) is noted to progressively decrease (going from a to d in Fig. 6). The gradient chromatofocusing runs (Fig. 5), which have the same outlet pH profile but different buffer concentrations (Fig. 2), show significantly better resolution, with the optimized resolution for gradient chromatofocusing being 2.33 compared to the optimized resolution of 1.09 for the NaCl gradient runs.

In chromatofocusing, peak width is predicted to decrease with decreased mobile phase buffer concentration, as an increase in column pH gradient slope is expected with decreased buffer concentration (confirmed in Fig. 4), causing an increased focusing of the peak [2,3,9]. Fig. 5 generally supports this expectation of peak focusing in gradient chromatofocusing, showing the narrowest peak widths at the lower buffer concentrations (although not the lowest, see Section 4.2.3.2 for an explanation). Other reasons for a decrease in peak width with a decrease in buffer concentration are also possible. These are presented in Section 4.2.3.

3.4. Optimization of separation factor in gradient chromatofocusing demonstrated in the chromatography of β-lactoglobulin A and B

Data calculated from Fig. 5 also shows an increase in α with increase in the buffer concentration in gradient chromatofocusing (see Fig. 5 caption). This leads to a general trend of an increase in resolution with an increase in buffer concentration, except at the highest buffer concentrations where the resulting broad peaks have a counter effect on the resolution (see Fig. 5 caption).

3.5. Gradient chromatofocusing of a multiple protein standard

A more complete characterization of gradient chromatofocusing was done by chromatographing a sample containing five proteins: conalbumin (5.9), BSA (4.7, 4.9), ovalbumin (4.7), β-lactoglobulin B (5.23), and β-lactoglobulin A (5.13) (isoelectric points p*I* values in parentheses [17–19]). The chromatograms obtained with the same outlet pH gradient profile but different mobile phase buffer concentrations (Fig. 2) are given in Fig. 7. In general, the narrowest peak widths (and hence most focusing) occur at lower buffer concentrations. However, the poorest resolution of proteins is also noted at these lower buffer concentration conditions. Use of higher buffer concentrations can effectively separate these proteins, with optimal separation seen at 45–50 m*M* concentration of elution buffer components in chromatograms (e) and (f) in Fig. 7. However, peak widths are seen to be wider at these higher buffer concentrations, which is explained in the discussion section (Section 4.2.3).

Proteins were individually chromatographed in gradient chromatofocusing experiments to better characterize the results of Fig. 7, with peak width plotted in Fig. 8, retention time plotted in Fig. 9, resolution plotted in Fig. 10, and the elution pH given in Table 4. Conalbumin is not retained appreciably at any concentration, with retention times for the main peak ranging from 2.1 to 0.7 min for systems 1–6 (lowest to highest buffer concentration) respectively.

Several observations are noted for Fig. 8. In general, the lowest peak widths are obtained at lower buffer concentrations (but not necessarily the lowest). The peak width reaches a maximum for several proteins in Fig. 8, which then decreases with a further increase in buffer concentration. This is expected, since the proteins will have greatly reduced retention at high salt concentrations.

Fig. 9 shows that buffer concentration can also affect selectivity, as the order of elution of the ovalbumin peak 2 is seen to shift with respect to β-lactoglobulin B and BSA as the buffer concentration is increased. The same behavior is noted for ovalbumin peak 1 with respect to BSA. However, the

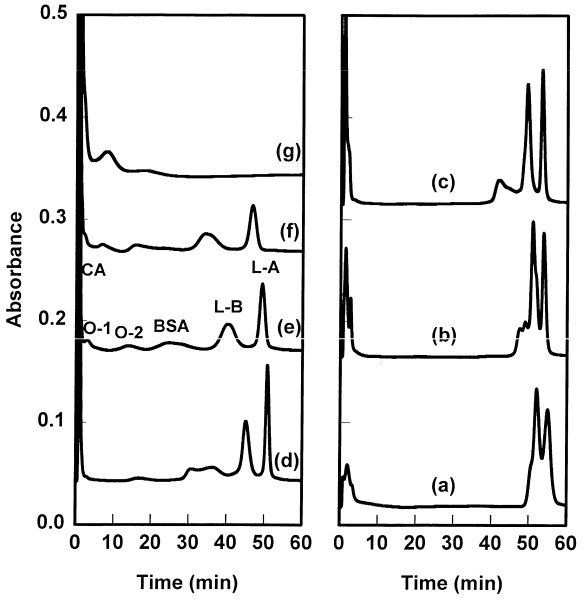

Fig. 7. Gradient chromatofocusing runs of a sample containing conalbumin, ovalbumin, BSA, β-lactoglobulin A and B at different mobile phase buffer concentrations but the same outlet pH gradient profile (see Fig. 2) [(a) has the lowest buffer concentration and (g) has the highest buffer concentration, with the concentration increasing progressively from (a) to (g)]. The injection volume of the sample is 150 μl, with the concentration for each protein being 2 mg/ml. Chromatographic setup and conditions are described in Sections 2.1 and 2.2, and Tables 1 and 2. Chromatograms are those obtained using: (a) mobile phase system 1; (b) mobile phase system 2; (c) mobile phase system 3; (d) mobile phase system 4; (e) mobile phase system 5; (f) mobile phase system 6; (g) mobile phase system 7. Peak identities for chromatogram e are conalbumin (CA), ovalbumin peak 1 (0-1) and 2 (0-2), bovine serum albumin (BSA), β-lactoglobulin B (L-B), and β-lactoglobulin A (L-A).

other proteins maintain elution order throughout the range of buffer concentrations studied.

4. Discussion

There are two major parameters of the mobile phase controlling the elution of proteins in gradient chromatofocusing: pH and anion concentration (or ionic strength). This work demonstrates the interplay between these two parameters and their effect on the chromatography.

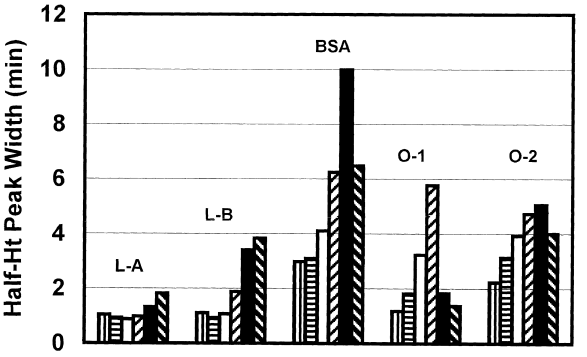

Fig. 8. Bar graph of half-height peak width of various individually chromatographed proteins of Fig. 7. Proteins are chromatographed with the same outlet pH gradient profile but different buffer concentrations (see Fig. 2). Bars are clustered according to the protein (abbreviation key is given in Fig. 7 caption), with each bar indicating the peak width for successively higher mobile phase buffer concentrations (proceeding left to right in the cluster). Bars are the following: vertical stripe, mobile phase system 1 (mp 1) (6.25 m*M*); horizontal stripe, mp 2 (12.5 m*M*); white solid, mp 3 (25.0 m*M*); black diagonal (/) on white background, mp 4 (37.5 m*M*); black solid, mp 5 (45.0 m*M*); and white diagonal (\) on black background, mp 6 (50.0 m*M*). See note on buffer concentration in Fig. 4. The chromatographic setup and conditions are described in Sections 2.1 and 2.2, and Tables 1 and 2.

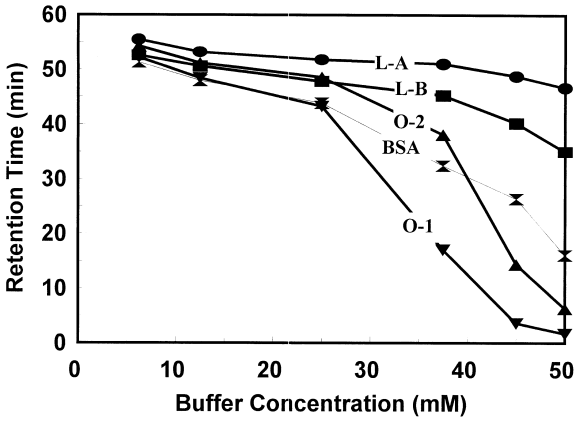

Fig. 9. Plot of the retention time of various individually chromatographed proteins versus buffer concentration of Fig. 7. Proteins are chromatographed with the same outlet pH gradient profile but different buffer concentrations (see Fig. 2). Plotted points proceeding from left to right are for mobile phase systems 1–6 successfully. The chromatographic setup and conditions are described in Sections 2.1 and 2.2, and Tables 1 and 2. See note on buffer concentration in Fig. 4. Abbreviations for proteins are given in Fig. 7 caption.

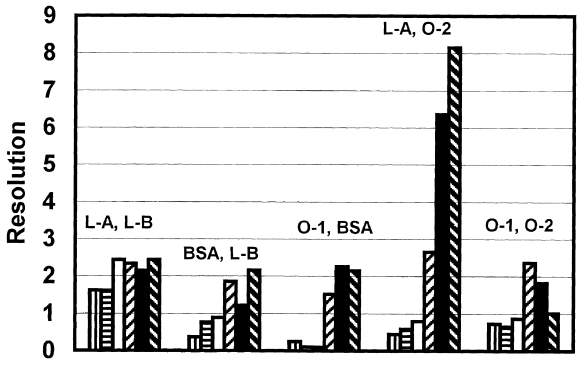

Fig. 10. Bar graph of resolution (calculated by using the data of individually chromatographed proteins) for different protein pairs of Fig. 7. Proteins are chromatographed with the same outlet pH gradient profile but different buffer concentrations (see Fig. 2). Bars are clustered according to the protein pair (abbreviation key is given in Fig. 7 caption), with each bar indicating the resolution for successively higher mobile phase buffer concentrations (proceeding left to right in the cluster). Bars are the following: vertical stripe, mobile phase system 1 (mp 1) (6.25 m*M*); horizontal stripe, mp 2 (12.5 m*M*); white solid, mp 3 (25.0 m*M*); black diagonal (/) on white background, mp 4 (37.5 m*M*); black solid, mp 5 (45.0 m*M*); and white diagonal (\) on black background, mp 6 (50.0 m*M*). See note on buffer concentration in Fig. 4. The chromatographic setup and conditions are described in Sections 2.1 and 2.2, and Tables 1 and 2.

4.1. Effects of mobile phase pH

The mobile phase pH affects the following in ion-exchange chromatography of proteins: (1) the net charge and the distribution of charges on the protein molecule, by changing the protonation state of the protein's acidic and basic groups; (2) the anionic makeup of the mobile phase, due to the pH dependent distribution of buffer species and their conjugate forms; and (3) the charge status of the ion-exchanger. This last effect should not be a factor in the present work, since the DEAE ligands remain positively charged throughout the 7.6 to 4.0 pH range employed (pK_a of DEAE is approximately 9.8 [20]). The second pH effect, the distribution of different buffer species forms, impacts the anion concentration of the mobile phase. This anion concentration effect will be discussed in Section 4.2.

Concerning the first effect, a simple model for elution in chromatofocusing depicts the protein eluting at that point in the gradient where the mobile phase pH equals the protein's p*I*. At this point the net charge on the protein changes from negative to neutral, leading to its elution from the anion-exchanger. Experimental results, however, do not fit this simple model. Elution of proteins at pH values higher than their p*I* values is commonly observed in chromatofocusing [3,21]. In the present work, conalbumin, BSA, and ovalbumin elute at pH values above their p*I* values at all the buffer concentrations studied, while β-lactoglobulin A and B elute either above or below their p*I* values, depending on the buffer concentration (see Table 4). Conalbumin (p*I* = 5.9) is not appreciably retained on the column at any buffer concentration, even though it is negatively charged in the application buffer. In addition, the order of elution does not fit this net-charge theory. Counter to expectations, BSA and ovalbumin (low-p*I* proteins) elute before the lactoglobulins (high-p*I* proteins) in most cases (see Fig. 9).

Thus, chromatofocusing behavior of proteins is complex. Ionic strength effects have been postulated

Table 4
Elution pH of individually chromatographed proteins of Fig. 7

Mobile phase system[a]	Conalbumin (5.9[b])	BSA (4.7, 4.9[b])	Ovalbumin (4.7[b])		β-Lactoglobulin B (5.23[b])	β-Lactoglobulin A (5.13[b])
			Peak 1	Peak 2		
1 (6.25 m*M*)	7.60	4.99	4.91	4.73	4.90	4.63
2 (12.5 m*M*)	7.60	5.25	5.20	4.93	5.03	4.75
3 (25.0 m*M*)	7.60	5.69	5.74	5.26	5.34	5.00
4 (37.5 m*M*)	7.60	6.59	7.31	6.21	5.61	5.06
5 (45.0 m*M*)	7.60	6.86	7.60	7.38	6.01	5.23
6 (50.0 m*M*)	7.60	7.35	7.60	7.60	6.28	5.42

[a] Concentrations in parentheses are those given in Table 1 for each component in 100% buffer B.
[b] The p*I* values for these proteins are given in the parentheses [17–19].

as a cause for proteins eluting above their p*I* [21]. Also, chromatofocusing, unlike isoelectric focusing, is a surface dependent process, in which a portion of the protein molecule binds to the packing material through individual charge sites or via specific surfaces of the protein [19,22–27]. Thus, the charge of a particular region is more important than the charge on the entire protein molecule in ion-exchange chromatography. Hydrophobic effects may also play a role [19,28,29]. These additional factors are likely reasons for the non-ideal elution behavior of the proteins observed in chromatofocusing.

4.2. Effects of mobile phase buffer concentration

Mobile phase buffer concentration effects are seen in the present work, significantly affecting peak width, resolution, and separation factor.

4.2.1. Anion displacement mechanism

Anion concentration, which causes elution by a displacement mechanism, is the other important mobile phase parameter affecting anion-exchange chromatographic behavior. This mechanism has been proposed for the technique referred to as ampholyte displacement chromatography, which was initially reported [30] prior to the first work using the term chromatofocusing [1]. Ampholyte displacement chromatography is essentially a chromatofocusing technique, although some have differentiated it from chromatofocusing on the basis of mechanism [10,21]; with chromatofocusing depicting the mechanism as a buffering action of the mobile and stationary phases, and ampholyte displacement chromatography depicting the mechanism as a process involving adsorption and desorption of the mobile phase buffer components. However, there does not appear to be a clear distinction between these two techniques. For example, Mono P columns are commonly used with polybuffers in chromatofocusing techniques to generate pH gradients down to pH 4.0. However, titration of these mono P columns shows little buffering capacity at pH values below 9.0 (unpublished data). Thus, the displacement mechanism appears to be applicable in many of the reported chromatofocusing techniques.

The displacement mechanism [6,10] of ampholyte displacement chromatography proposes the establishment of a pH gradient within the column via a gradient distribution of different acidic ampholytes within the anion-exchange column, with the proportion of stronger to weaker acidic components steadily decreasing down the length of the column. In this mechanism, more acidic components successively move down the column replacing less acidic components and proteins, continuously lowering the pH of the column. The rate of migration of the protein down the column is equal to the rate of the migration of the buffer component with the same p*I* and charge characteristics.

Elements of an anion displacement mechanism are most likely operative in gradient chromatofocusing, requiring further investigation. A displacement mechanism for chromatofocusing has been derived and experimentally verified which refutes the pure buffering action mechanism of chromatofocusing [13,14]. The authors of this work introduce a new term, chromatophoresis, reflecting their newly proposed frontal development mechanism of chromatofocusing. Another model for chromatofocusing based on adsorption equilibrium principles has been reported [15,16]. Even Sluyterman and Elgersma, who derived equations based on a buffering mechanism, state that the true mechanism of pH gradient generation is displacement (noting that the derived equations are independent of mechanism) [2]. Although different terminologies have been used in the literature to distinguish mechanistic differences, it is proposed here that all reports incorporating pH gradients on ion-exchange columns demonstrating focused bands should be classified under the general term of chromatofocusing, regardless of mechanism.

4.2.2. Effects of buffer concentration on separation factor

The present work shows that the buffer concentration affects the separation factor (α). Examination of Fig. 7 shows poor separation of the proteins in gradient chromatofocusing at low and intermediate buffer concentrations. However, use of a high buffer concentration leads to greater than baseline separation of the peaks, with the protein peaks being evenly distributed throughout the chromatogram in Fig. 7e. This capability of being able to progressively separate closely eluting peaks with increasing buffer concentration is also illustrated in Fig. 5 in the

separation of β-lactoglobulin A and B, where α increases from 1.06 to 1.27 in going from the lowest to the highest concentration of mobile phase buffer (same outlet pH gradient profile). Following this trend, chromatography of commercial ovalbumin shows a progressive increase in the separation of two peaks in the sample as the concentration of each of the components in the 100% elution buffer increases from 6.25 m*M* to 50.0 m*M*, with α progressively increasing from 1.04 to 4.85. These two peaks are possibly the monomer and dimer of ovalbumin which have been reported in a cation-exchange chromatography separation [31].

The mechanism of this improved separation effect with increasing buffer concentration requires further study. Several of the explanations offered for the peak widening effect at higher buffer concentrations (see Section 4.2.3.1) may also explain the noted increase in separation, including: (1) hydrophobic retention (a greater degree of hydrophobic character in one protein compared to another might cause a relative increase in retention of the hydrophobic protein at higher buffer concentrations due to a hydrophobic effect); (2) the possibility that there is a greater difference in the number of negative charges on different proteins (that interact with the ion-exchanger) at increased buffer concentration due to their elution at a higher pH (see Table 4) (this effect will be greatly magnified because the retention of a protein is proportional to the power of the number of interaction sites [22,23]); and (3) the convex nature of the outlet pH gradient.

4.2.3. Effects of buffer concentration on peak width

4.2.3.1. General trends of the peak width/buffer concentration relationship

A general trend of increased peak widths with increased buffer concentration is seen in Figs. 5 and 7 for gradient chromatofocusing, with the values plotted in Fig. 8 (exceptions to this trend are discussed later in Section 4.2.3.2). Several factors may contribute to this peak broadening as discussed below. Further studies are required to establish the extent that these factors contribute to the broadening and whether there are other causes.

One possible explanation for the widening of peaks at higher buffer concentrations is the inverse relationship between column pH gradient slope and buffer concentration (Fig. 4). Thus, there is an expected decrease in focusing of the band (i.e. wider peaks) at higher buffer concentrations. This hypothesis is supported by theory derived for conventional chromatofocusing. An expression has been derived by Sluyterman and Elgersma [2] (Eq. (25) in this Reference) that shows an inverse relationship of peak width to two terms inversely affected by buffer concentration [3,9]: the magnitude of the column pH gradient (dpH/dx) and a term proportional to the Donnan potential (φ). Accordingly, the narrowest peak widths are expected at the lowest buffer concentrations in chromatofocusing experiments.

Another possible explanation for peak broadening is the fact that the proteins have a greater negative charge (up to a threshold pH level) in gradient chromatofocusing as the buffer concentration is increased, since the proteins elute at a higher pH at higher buffer concentrations (see Table 4). Supporting this hypothesis is a report of the anion-exchange chromatography of albumin showing an increase in peak width with increased pH [26]. This peak broadening at high pH is attributed to an increase in the number of negatively charged sites interacting with the ion-exchanger (resulting from the increased pH). Another study did a more rigorous analysis of the pH effect on the peak width [19]. Two proteins were chromatographed isocratically on an anion-exchange column, determining the peak widths at different pH values over a range of different capacity factors. This study reveals a less clear trend, showing both increases and decreases in peak width as pH is increased.

There may be other explanations. Increased salt concentration in the mobile phase can lead to an increase in the hydrophobic interaction of proteins with ion-exchange packing materials [19,28,29]. This hydrophobic effect has been shown to increase peak broadening of proteins in weak cation-exchange chromatography [28]. The extent of the hydrophobic effect depends on the protein, the type of salt in the mobile phase, and the packing material employed [19,28,29]. The particular combination of buffers, proteins, and ion-exchanger used in the present work has not been extensively characterized according to hydrophobic properties. All proteins in the present work (including conalbumin) show a decreased retention time with increased buffer concentration

throughout the concentration range studied, arguing against there being a significant hydrophobic effect. However, further characterization studies are needed to make a more definitive assessment.

A final consideration in explaining the trend of increased peak broadening with increased buffer concentration is the linearity of the outlet pH gradient. Peak broadness in time is related to the slope of the outlet pH gradient, since the peak elutes within a finite range of pH. This finite pH range is expected to elute over a greater time period as the outlet pH gradient slope decreases, which results in a broader peak. Detailed analysis shows the outlet pH gradient in the present work to have a convex character (Section 3.1), with the slope (pH/min) decreasing with decreased time of the gradient. In the present work, proteins chromatographed at higher buffer concentrations elute at decreased times in the gradient (Fig. 9), which is expected to lead to broader peaks (compared to peaks eluting at increased times in the gradient) due to the shallower outlet pH gradient slopes at these decreased times. However, this can account for only a fraction of the peak broadening observed. Closer examination of the peak widths in Fig. 5 and the range of outlet pH gradient slopes calculated from the derivatives of equations in Table 3 bears this out. For example, the upper and lower extremes of the peak widths for β-lactoglobulin B in Fig. 5 differ by a factor of 4.3, while the outlet pH gradient slopes for these extremes (calculated for the retention times of these peaks) differs by only 30%.

4.2.3.2. Exceptions to general trend

A more detailed analysis shows deviation from the general trend of increased peak width with increased buffer concentration. Fig. 8 shows the lowest buffer concentration giving the narrowest peak widths for ovalbumin and BSA. However, the peak width versus buffer concentration plot for these proteins (Fig. 8) shows a maximum at a threshold buffer concentration, beyond which the peak width decreases with increasing buffer concentration. This decrease in peak width at the highest buffer concentrations results presumably from the high anion concentration (high ionic strength) present in the mobile phase.

Deviations are also noted for β-lactoglobulin A and B, which show the narrowest peak widths at intermediate buffer concentrations (see Fig. 8). However, there is only a 15% decrease noted in comparing the peak widths of the lowest buffer concentration with the peak widths for the optimal intermediate buffer concentrations (Fig. 8). This moderate effect may be artifactual. For example, it is possible that an error in producing the exact pH profile for all the different buffer concentrations can account for this variation (see, for example, the overlap in ranges of the column pH gradient slope for different buffer concentrations in Fig. 4 and the noted differences in outlet pH gradient profiles for different buffer concentrations in Fig. 2).

4.2.4. Effects of buffer concentration on resolution

Countering the dramatic improvement in α with increased buffer concentration discussed in Section 4.2.2 is the just discussed increase in peak widths. This causes the resolution to plateau and even decrease at higher buffer concentrations for several of the protein pairs plotted in Fig. 10. However, as a general trend, resolution is seen to increase with increased mobile phase buffer concentration (Fig. 10). This is different from conventional chromatofocusing which shows the best resolution at the lowest mobile phase buffer concentrations [3,6,32]. However, one reason for this is that a decrease in buffer concentration causes a decrease in the outlet pH gradient slope in the conventional technique, which in and of itself causes an increase in resolution [6]. The advantage of gradient chromatofocusing is that low concentration buffers are not required in order to generate outlet pH gradients with shallow slopes. Thus, the ability to study the independent effect of buffer concentration in gradient chromatofocusing is possible in the present work, showing the aforementioned general trend of increased resolution at higher buffer concentrations.

4.2.5. Preliminary data supporting an anion displacement/ionic strength effect of buffer components

Changing the buffer concentration in the mobile phase, superimposed on the pH gradient profile, adds another dimension of separation in the gradient chromatofocusing technique. The mechanism of this concentration effect appears to be either a displace-

ment or an ionic strength effect, as opposed to a buffer effect. This is supported by the results of gradient chromatofocusing experiments employing various concentrations of non-buffering salts in low concentration application and elution buffers used to generate the pH gradient. These experiments show similar results of increased resolution with increased salt concentration. This work will be reported in a future publication.

5. Conclusion

The present work demonstrates the potential of gradient chromatofocusing for optimizing protein separation. It accomplishes this through its ability to manipulate both the pH and buffer concentration of the mobile phase, a capability that neither conventional chromatofocusing (is limited to low buffer concentrations in the mobile phase) nor conventional salt gradient ion-exchange chromatography (operates at constant pH) has. Further studies are warranted to better understand how these effects work in conjunction with one another.

The effect of buffer concentration in gradient chromatofocusing is reported in the present work. The chromatography of proteins at higher buffer concentrations magnifies the retention differences of the proteins leading to an increase in α. Increased buffer concentration also leads to a general trend of increased peak width. Resolution is seen to increase with increased buffer concentration, except at the highest buffer concentrations where it plateaus or declines because of the large peak widths. Further work needs to be done to understand the mechanism of these buffer concentration effects.

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References

[1] L.A.AE. Sluyterman, J. Wijdenes, in: B.J. Radola, D. Graesslin (Eds.), Electrofocusing and Isotachophoresis (Proceedings of the International Symposium, August 2–4, 1976), Walter de Gruyter, Berlin, 1977, pp. 463–466.
[2] L.A.AE. Sluyterman, O. Elgersma, J. Chromatogr. 150 (1978) 17.
[3] L.A.AE. Sluyterman, J. Wijdenes, J. Chromatogr. 150 (1978) 31.
[4] L.A.AE. Sluyterman, J. Wijdenes, J. Chromatogr. 206 (1981) 429.
[5] L.A.AE. Sluyterman, J. Wijdenes, J. Chromatogr. 206 (1981) 441.
[6] M.T.W. Hearn, D.J. Lyttle, J. Chromatogr. 218 (1981) 483.
[7] J.H. Scott, K.L. Kelner, H.B. Pollard, Anal. Biochem. 149 (1985) 163.
[8] Y. Liu, D.J. Anderson, J. Chromatogr. A 762 (1997) 207.
[9] Y. Liu, D.J. Anderson, J. Chromatogr. A 762 (1997) 47.
[10] J.P. Emond, M. Pagé, J. Chromatogr. 200 (1980) 57.
[11] P.R. Haddad, P.E. Jackson, Ion Chromatography — Principles and Applications, Elsevier, Amsterdam, 1990, Chapter 1.
[12] O. Mikeš, High-Performance Liquid Chromatography of Biopolymers and Biooligomers, Part A: Principles, Materials and Techniques, Elsevier, Amsterdam, 1988, Chapter 2.
[13] A. Murel, S. Vilde, M. Pank, I. Shevchuk, O. Kirret, J. Chromatogr. 347 (1985) 325.
[14] A. Murel, S. Vilde, M. Pank, I. Shevchuk, O. Kirret, J. Chromatogr. 362 (1986) 101.
[15] J.C. Strong, D.D. Frey, J. Chromatogr. A 769 (1997) 129.
[16] R.C. Bates, D.D. Frey, J. Chromatogr. A 814 (1998) 43.
[17] P.G. Righetti, T. Caravaggio, J. Chromatogr. 127 (1976) 1.
[18] D. Malamud, J.W. Drysdale, Anal. Biochem. 86 (1978) 620.
[19] M.T.W. Hearn, A.N. Hodder, P.G. Stanton, M.I. Aguilar, Chromatographia 24 (1987) 769.
[20] H.F. Walton, in: E. Heftmann (Ed.), Chromatography, Fundamentals and Applications of Chromatography and Related Differential Migration Methods, Part A: Fundamentals and Techniques, 5th Edition, Elsevier, Amsterdam, 1992, Chapter 5.
[21] G. Wagner, F.E. Regnier, Anal. Biochem. 126 (1982) 37.
[22] W. Kopaciewicz, M.A. Rounds, J. Fausnaugh, F.E. Regnier, J. Chromatogr. 266 (1983) 3.
[23] F.E. Regnier, R.M. Chicz, in: K.M. Gooding, F.E. Regnier (Eds.), HPLC of Biological Macromolecules, Marcel Dekker, New York, 1990, Chapter 4.
[24] F.E. Regnier, Science 238 (1987) 319.
[25] T.A. Horbett, J.L. Brash, in: J.L. Brash, T.A. Horbett (Eds.), Proteins at Interfaces: Physicochemical and Biochemical Studies, ACS Symposium Series, Vol. No. 343, American Chemical Society, Washington, DC, 1987, Chapter 1.
[26] S.C. Goheen, J.L. Hilsenbeck, J. Chromatogr. A 816 (1998) 89.
[27] Y. Liu, D.J. Anderson, J.R. Shainoff, J. Chromatogr. A 753 (1996) 63.

[28] W.R. Melander, Z. El Rassi, Cs. Horváth, J. Chromatogr. A 469 (1989) 3.
[29] M.L. Heinitz, L. Kennedy, W. Kopaciewicz, F.E. Regnier, J. Chromatogr. 443 (1988) 173.
[30] D.H. Leaback, H.K. Robinson, Biochem. Biophys. Res. Commun. 67 (1975) 248.
[31] M.P. Henry, in: W.S. Hancock (Ed.), High Performance Liquid Chromatography in Biotechnology, Wiley, New York, 1990, Chapter 10.
[32] S. Hjertén, J.-P. Li, J. Chromatogr. 475 (1989) 167.