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Development of chromatofocusing techniques employing mixed-mode column packings for protein separations

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ABSTRACT

Recent studies reported in the literature using mixed-mode chromatography (MMC) column packings have shown that multiple modes of interactions between the column packing and proteins can be usefully exploited to yield excellent resolution as well as salt-tolerant adsorption of the target protein. In this study, a mixed-mode separation method using commercially available column packings was explored which combines the techniques of hydrophobic-interaction chromatography and chromatofocusing. Two different column packings, one based on mercapto-ethyl-pyridine (MEP) and the other based on hexylamine (HEA) were investigated with regard to their ability to separate proteins when using internally generated, retained pH gradients. The effects of added salt and urea on the behavior of the retained pH gradient and the protein separation achieved when using MMC column packings for chromatofocusing were also investigated. Numerical simulations using methods developed in previous work were shown to agree with experimental results when using reasonable physical parameters. These numerical simulations were also shown to be a useful qualitative method to select the compositions of the starting and elution buffers in order to achieve desired shapes for the pH and ionic strength gradients. The use of the method to fractionate blood serum was explored as a prototype example application.

1. Introduction

In recent years there has been an increasing interest in developing mixed-mode chromatography (MMC) methods for downstream processing in the biopharmaceutical industry. In particular, MMC is becoming a promising method to improve the selectivity achieved for protein separations [1,2]. The most commonly used MMC method for protein separations is the combination of hydrophobic-interaction chromatography (HIC) and ion-exchange chromatography (IEC) [3–7]. A particularly successful class of MMC, termed hydrophobic charge induction chromatography (HCIC), has been proposed by Burton and Harding [8–12]. In contrast to more common applications of MMC where multiple interaction modes simultaneously influence the adsorption of molecules, HCIC is based on the pH-dependent behavior of ligands that are more hydrophobic and uncharged at neutral or higher pH and ionize at lower pH so that during gradient elution the nature of the interactions between the protein and column packing varies significantly with time. More specifically, in HCIC proteins bind to the column packing ligands by hydrophobic interaction at the beginning of the process, and with a reduction of the fluid phase pH the bound proteins will be eluted by electrical charge repulsion. In addition to using a combination of HIC and IEC, other forms of MMC have also been developed, such as those that combine hydrophilic-interaction chromatography (HILIC) and IEC [13–16].

Mixed-mode chromatography has several significant advantages as compared to traditional single-mode chromatography when applied to the purification of proteins. Since proteins are amphiphilic molecules with both hydrophobic and hydrophilic surfaces, MMC can improve selectivity and also potentially achieve so-called "salt-independent" adsorption where proteins are able to adsorb onto the column packing at moderately high salt concentrations due to hydrophobic interactions, particularly if the multimodal components of the protein and column packing are complementary. Consequently, MMC can facilitate process step transitions such as performing ion-exchange chromatography directly on filtered cell extract without an intermediate desalting step [17]. Another advantage of MMC is that it may facilitate a reduction in the number of chromatographic steps by performing orthogonal chromatography techniques in a single column [6,7]. Reducing the number of chromatographic steps in this way is likely to increase yield and reduce the processing time so that the overall throughput is improved.

Despite the significant amount of past research regarding MMC for protein separation and purification described previously in the literature, no studies have been reported on the use in these

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systems of retained pH gradients that are entirely generated internally, which is a technique generally termed "chromatofocusing." One recent study, however, separated proteins by using a mixed-mode column packing and an externally produced pH gradient that evidently was partly retained [18]. This study also demonstrated the ability of pH gradient elution to achieve better resolution as compared to the salt gradient elution for the case of separating α-lactalbumin, trypsin inhibitor, and β-lactoglobulin A using a mixed-mode cation exchanger.

Chromatofocusing is a variant of IEC where, in contrast to standard forms of IEC which employ an unretained salt gradient, a retained pH gradient is formed entirely inside the column by utilizing the buffering capacity of the column packing and the adsorption characteristics of the buffering species. The original version of this technique was developed by Sluyterman and coworkers who employed polyampholyte elution buffers similar to those used in isoelectric focusing [19,20]. The method has been investigated and further developed by a number of researchers over the last decade who have used simple mixtures of buffering species instead of polyampholyte buffers to form the pH gradient [21–31]. In this study, chromatofocusing will be extended to the use of mixed-mode column packings by employing these methods along with numerical simulations to aid in the selection of the buffer composition. In addition, it will be demonstrated in this study that additives such as urea or a neutral salt can be employed to usefully adjust the protein retention behavior.

Another goal of this work is to explore the use of chromatofocusing with a mixed-mode column packing for the fractionation of blood plasma. The blood plasma fractionation industry produces a number of commercial therapeutic proteins such as immunoglobulins and albumin, and it is by far the largest segment in global therapeutic protein manufacturing in terms of mass produced [32]. The Cohn process, which incorporates cold ethanol fractionation, is the oldest and most widely used method for blood plasma fractionation [33], but the method often exhibits poor yield and the albumin produced generally has relatively low purity [34]. For these reasons, chromatography in combination with ultrafiltration has been widely investigated since the 1980s as a means to improve the purity and yield in blood plasma fractionation. Among the possible alternatives, dye-ligand affinity chromatography [35] and immobilized metal chelate affinity chromatography [36] have shown considerable promise, although the higher cost of these methods has inhibited their widespread use so that there is a need to develop lower cost chromatographic methods for plasma fractionation.

2. Experimental

2.1. Materials

Myoglobin from equine skeletal muscle, cytochrome C from horse heart, lysozyme from chicken egg white, α chymotrypsinogen A from bovine pancreas, and bovine serum albumin were products M0630, C2506, L7651, C4879, A7638, respectively, obtained from Sigma–Aldrich (St. Louis, MO, USA). Rabbit blood serum (i.e., blood plasma with the clotting factors removed) was obtained from Covance Inc. (Princeton, NJ, USA) and stored at −20 °C until use.

Tris(hydroxymethyl)-aminomethane (Tris), 2-(N-morpholino)-ethanesulfonic acid (MES), 2-(cyclohexylamino)-ethanesulfonic acid (CHES), N-tris(hydroxymethyl)-methyl-3-amino-propanesulfonic acid (TAPS), N-tris(hydroxylmethyl)-methyl-2-aminoethanesulfonic acid (TES), 3-(N-morpholino)-propanesulfonic acid (MOPS), citric acid, urea, ethanol, PBS, NaOH, NaCl and HCl were also obtained from Sigma–Aldrich. Formic acid was obtained from J.T. Baker (Philipsburg, NJ, USA).

All buffer compositions are described in the figure captions corresponding to each experiment, and the buffer solutions were prepared using distilled water and were degassed by vacuum filtering using disposable filter units with nylon membranes having 0.2 μm pores (Part No. 0974024A, Thermo Fisher Scientific, Waltham, MA, USA). To produce a sample for injection, proteins were dissolved into a starting buffer and filtered with a nylon syringe filter having 0.2 μm pores (Part No. 431215, Corning Life Sciences, Lowell, MA, USA).

2.2. Columns

MEP HyperCel and HEA HyperCel particles (Pall Life Sciences, Port Washington, NY, USA) which were 90 μm in diameter were slurry packed into a 10-cm long glass Omnifit column (Diba Industries, Danbury, CT, USA) with 1.0 cm internal diameter and with one end fitting being adjustable in length. The column was packed using PBS buffer at a flow rate of 4 ml/min, and the packing process was terminated when the height of the bed became constant. The final height of the packed bed produced by this process varied from 3.8 to 6.6 cm. After packing, the column was washed with 20% (v/v) ethanol in deionized water for overnight at a flow rate of 0.1 ml/min.

2.3. Equipment

Experiments were performed using a LC Packings Ultimate HPLC instrument (now Thermo Scientific Dionex, Sunnyvale, CA, USA) and an Orion (now Thermo Scientific Orion, Beverly, MA, USA) model 520A pH meter. A Model FC49K 50 μl internal volume flow cell and a Model 450CD pH electrode (Sensorex, Garden Grove, CA, USA) were used to directly measure the pH of the column effluent. The same pH meter and electrode were used for measuring both the elution buffer pH and column effluent pH in order to enhance the accuracy of the pH measurements. All the chromatography experiments were controlled by Chromeleon software version 6.6 (Thermo Scientific Dionex).

2.4. Chromatofocusing experiments

To perform an experiment, the column was initially equilibrated with the starting buffer. The feed sample was then introduced into the column, and the column was subsequently eluted with a stepwise change to the elution buffer.

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

All materials used for SDS-PAGE were obtained from Sigma–Aldrich. To prepare each 10 ml of 12% SDS-PAGE separation gel, 3.4 ml distilled water, 2.5 ml of 1.5 M Tris–HCl (pH 8.8), 0.05 ml of 20% (w/v) SDS, 4 ml of 30% acrylamide/bis-acrylamide (37.5:1), 0.05 ml of 10% ammonium persulfate and 0.01 ml N,N,N′,N′-tetramethylenediamine (TEMED) were mixed. To prepare 10 ml of stacking gel, 6 ml of Milli-Q water, 2.5 ml of 0.5 M Tris–HCl (pH 6.8), 1.33 ml of 30% acrylamide/bis-acrylamide (37.5:1), 0.05 ml of 10% ammonium persulfate and 0.01 ml TEMED were mixed. To prepare a 1.0 mm thick mini-gel, 4.8 ml of separation gel and 2 ml of stacking gel was used. The 10× stock solution of running buffer consisted of 15.0 g Tris, 72.0 g glycine, and 5.0 g SDS in 500 ml deionized water. The 2× stock solution of reducing sample buffer consisted of 1.0 ml of 0.5 M Tris–HCl (pH 6.8), 1.6 ml 10% (w/v) SDS, 2.0 ml glycerol, 0.08 ml 1.0% bromophenol blue, 0.4 ml β-mercaptoethanol, and 2.92 ml deionized water. The electrophoresis was performed at a voltage of 150 V for 1 h using

a Mini-PROTEAN Tetra Cell (Bio-Rad Labs, Hercules, CA, USA). The gel was stained by using the silver staining method.

3. Results and discussion

3.1. Differences between mixed-mode chromatofocusing and standard chromatofocusing

Fig. 1 illustrates conceptually some of the major differences between mixed-mode chromatofocusing (MMCF) and the traditional version of chromatofocusing. In both cases, an internally produced, retained pH gradient (i.e., a gradient which travels through the column more slowly than an unadsorbed molecule) at low ionic strength is employed to focus and elute proteins at a fluid phase pH, termed the "apparent" isoelectric point (pI_{app}), which generally differs from the true isoelectric point. The column packing used in traditional versions of chromatofocusing, such as one incorporating the diethylaminoethyl (DEAE) functionality, is charged through the whole pH range of interest. In general, for a functional group such as DEAE which carries a positive charge, a protein is adsorbed onto the column packing by electrostatic attraction when the pH is higher than its isoelectric point (pI), and it is correspondingly desorbed from the column packing by electrostatic repulsion when the pH is lower than its pI. A focusing effect therefore occurs in a subregion in the column between electrostatic attractive and repulsive regions as shown in the figure.

In contrast to the case of traditional chromatofocusing, the charge on the functional group used in MMCF tends to vary to a greater extent due to its having a pK_a value close to the pH range spanned by the gradient. As illustrated in Fig. 1, for a weak-base functionality this group therefore becomes nearly uncharged at a relatively high pH, and the protein is consequently adsorbed onto the column through hydrophobic interactions and subsequently eluted by an electrostatic repulsion effect as the pH in the elution buffer is decreased. As discussed by Shen and Frey [37], the value of pI_{app} in traditional chromatofocusing is generally near the true pI of a protein so that proteins tend to elute in the order of their isoelectric point subject to the influences of charge regulation and other secondary effects. However, as again illustrated in Fig. 1, the pI_{app} of a protein in MMCF may be very different from its true pI due to the effect of mixed-mode interactions. Consequently, the presence of both hydrophobic and electrostatic interactions in MMCF allows the separation of proteins having similar pIs in a manner that cannot be achieved using traditional version of chromatofocusing.

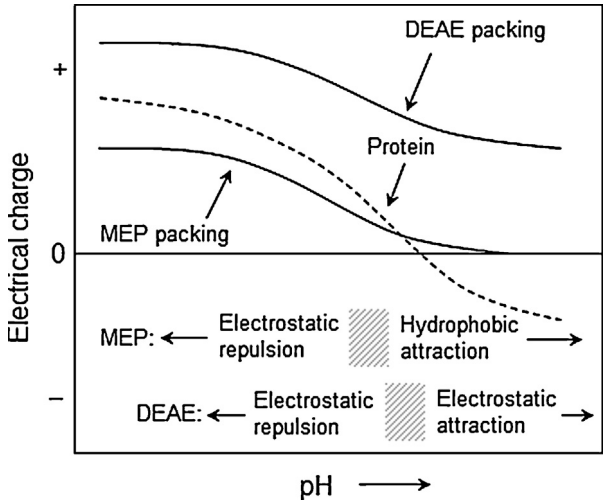

Fig. 1. Illustration of the differences between the mixed-mode chromatofocusing and traditional chromatofocusing. Regions where electrostatic attraction, electrostatic repulsion, and hydrophobic attraction dominate are depicted on the bottom portion of the figure. Shaded rectangles illustrate the focusing position of the protein.

3.2. Comparison between calculated and experimentally measured pH gradients

In order to develop mixed-mode chromatofocusing techniques, numerical simulation methods developed in previous work [21,38] were used in this study as a qualitative guide for producing a desired shape for the pH and ionic strength gradients by adjusting the compositions of the elution and starting buffers. Control of the shape of the pH gradient in MMCF is important since the pH gradient ultimately determines the manner in which proteins are separated. However, it is often a challenge to relate the gradient shape to the conditions used since the pH gradient in MMCF is produced by a complex interaction of several types of phenomena, such as the acid–base dissociation of the buffering species and column packing functional groups present and the adsorption behavior of buffering species. Numerical simulations are consequently often helpful to develop an appropriate chromatographic process for a given purpose, especially since in many cases the nature of the composition transitions are sufficiently complex that a simple approach, such as the use of local-equilibrium theory, can be difficult to apply [30].

The numerical simulation method employed in this work is based on the description of equilibrium shown in Fig. A1 of the Supplementary Content for this article (see Appendix A) where an ionizable buffering species is assumed to distribute between the fluid and adsorbed phases so that acid–base equilibrium and charge electroneutrality in both phases as well as interphase adsorption equilibrium can be accounted for. This equilibrium description is then incorporated into a numerical simulation method implemented using the method of characteristics that predicts the pH effluent profile that exits from the column. Details of the numerical methods used and definitions of the physical parameters in Table 1 are given in the Supplementary Content for this article and by Strong and Frey [21] and Frey et al. [38].

Fig. 2A and Fig. 2B shows a comparison between experimentally measured and theoretically predicted pH gradients for a column containing the MEP HyperCel mixed-mode column packing. In order to fit the data for these two cases, the functional groups attached to the column packing in the simulations were represented as a family of three subtypes with pK_a values of 3.5, 4.3 and 5.5, as compared to the intrinsic pK_a of 5.25 for pyridine and the pK_a of 4.8 for MEP HyperCel reported by Pall Life Science. This difference was attributed to the polymeric nature of the column packing where pK_a values of functional groups are influenced by neighboring functional groups that are charged, so that the use of more than one subtype of functional group fits experimental data better than the presumption of a single functional group. Other parameters used in the simulations are shown in Table 1.

As shown in Fig. 2A and Fig. 2B, the pH profiles observed experimentally agree reasonably well with the theoretically calculated profiles since the general shapes of pH fronts – i.e., whether they are self-sharpening (abrupt), non-self-sharpening (gradual) or composite in nature – are usually correctly predicted, and the average positions and widths of these fronts are also correctly predicted with an error that is generally less than 20% in terms of the relevant elution volumes. In particular, Fig. 2A shows the case where a single non-self-sharpening front is formed while Fig. 2B illustrates a more complex set of conditions where several types of fronts are formed that are separated by two intermediate plateaus.

The theoretically predicted effluent concentration profiles for the buffering species for Case 1 in Fig. 2B are shown in Fig. 3. According to the theoretical calculations in this figure, the portion of the pH gradient that extends from pH 7.5 to 3 corresponds to a single composite front consisting of an upstream non-self-sharpening section

Table 1
Physical properties used to fit experimental data for MEP HyperCel.

Properties for Fig. 2A:

	R_1	R_2	R_3	
pK_R	3.5	4.8	5.3	
q_R (M)	0.02	0.1	0.02	
	Na^+	Cl^-	Tris	Citrate
pK_a	NA	NA	8.06	4.75, 5.41
$K_B{}^+{}_{,ads}$	1	NA	NA	NA
$K_A{}^0{}_{,ads}$	NA	1.0	1.0	1.2
$K_A{}^-{}_{,ads}$	NA	1.0	1.0	0.8
$K_A{}^{-2}{}_{,ads}$	NA	NA	NA	1.0

Properties for Fig. 2B:

	R_1	R_2	R_3	
pK_R	3.5	4.8	5.3	
q_R (M)	0.02	0.1	0.02	
	Na^+	Formic acid	TAPS	MES
pK_a	NA	3.7	8.4	6.15
$K_B{}^+{}_{,ads}$	1	NA	NA	NA
$K_A{}^0{}_{,ads}$	NA	2.4	1.2	1.4
$K_A{}^-{}_{,ads}$	NA	2.4	1.2	1.4

NA: Not applicable.
R_1, R_2, R_3: Functional group subtypes on column packing.

adjoined to a downstream self-sharpening section. Furthermore, it can be seen in Fig. 2B that the detailed shape of the experimental profiles differs to some extent from the calculated profiles, most likely due to the simplified form of the equilibrium expressions used where the equilibrium parameters employed (see Eq. A4 of Appendix A) are assumed to be constants rather than functions of composition. Nevertheless, it is evident that the numerical simulations represent, at least semi-quantitatively, the experimental behavior when using reasonable physical parameters. This also suggests that the numerical methods employed here when further developed with more precise equilibrium relations are likely to be useful for quantitative computer-aided design, as opposed to their more qualitative use described here. This is further illustrated by comparing the experimental results and theoretical calculations for Cases 1 and 2 in Fig. 2B which show the effect of reducing the concentration of one of the buffering species used (MES) by a factor of two. As illustrated, the theoretical calculations predict to a reasonable extent the effect of this change on the observed pH profile. Note finally that Fig. 3 also shows that the ionic strength, as indicated by the sodium ion concentration, is nearly constant throughout the pH gradient. The system shown therefore is suitable for illustrating the effect of pH changes on the electrostatic and hydrophobic interactions at a nearly fixed ionic strength.

Fig. 4 shows a comparison between the experimentally measured and the theoretically predicted pH gradients for the case of the HEA HyperCel column packing. As in the case for Fig. 2B, a family of three subtypes of functional groups was employed in the simulations to represent the column packing, but in this case the pK_a values were taken as 7.0, 8.0 and 8.5, as compared to the instrinsic pK_a of hexylamine of 10.5 and the pK_a of 8.0 for HEA HyperCel reported by Pall Life Science. This difference was again attributed to the polymeric nature of the column packing. Other parameters used in this simulation are shown in Table 2. As shown in the figure, the theoretically calculated pH profile again agrees reasonably well with the pH gradient observed experimentally, except that all the fronts were predicted to be self-sharpening, whereas the experimental results indicate the first front is predominantly non-self-sharpening in character. These results therefore confirm that the pH profile formed using the mixed-mode column can be predicted with the simplified description of phase equilibrium used here at least qualitatively by using numerical simulations, although in some instances the character of the front in terms of its self-sharpening or non-self-sharpening behavior may not be entirely accurate.

As a final consideration, it can be observed in Figs. 2–4 that a variety of different types of pH fronts (i.e., self-sharpening, non-self-sharpening, and composite) can be produced depending on the conditions employed. The choice as to which type of gradient shape is preferable for a given application depends on the objectives of protein separation problem being addressed. For example, in previous work it has been shown that self-sharpening pH fronts can be employed to focus proteins into highly concentrated bands, and even accomplish displacement chromatography, while non-self-sharpening fronts (i.e., gradual pH gradients) are useful for separating a contaminant from a target protein when the pI_{app} values are very similar [21,23,27–29,39]. In the remainder of this study the behavior of proteins in various types of retained pH gradients will be studied with the shapes of the gradients employed largely unoptimized due to the general nature of the studies being performed. However, in Section 3.6 a retained pH gradient consisting of multiple stepwise pH fronts will be specifically developed for the purpose of fractionating blood serum into narrow p*I* range fractions.

3.3. Separation of proteins using mixed-mode chromatofocusing

Experimental results for the separation of myoglobin, cytochrome C, lysozyme and α chymotrypsinogen A in a column packed with MEP HyperCel and using the same starting and elution buffer system used in Fig. 2A, but with different specific buffer compositions, are shown in Fig. 5. The p*I* values reported in the literature [40] and the pI_{app} values determined here for the four proteins are indicated in Table 3. As shown, the pI_{app} values of the proteins are much lower than their respective p*I* values, except for myoglobin whose pI_{app} value was somewhat higher than its p*I* value. More specifically, myoglobin was eluted in the first column void volume so that it was not retained on the column, which is consistent both with its low hydrophobicity

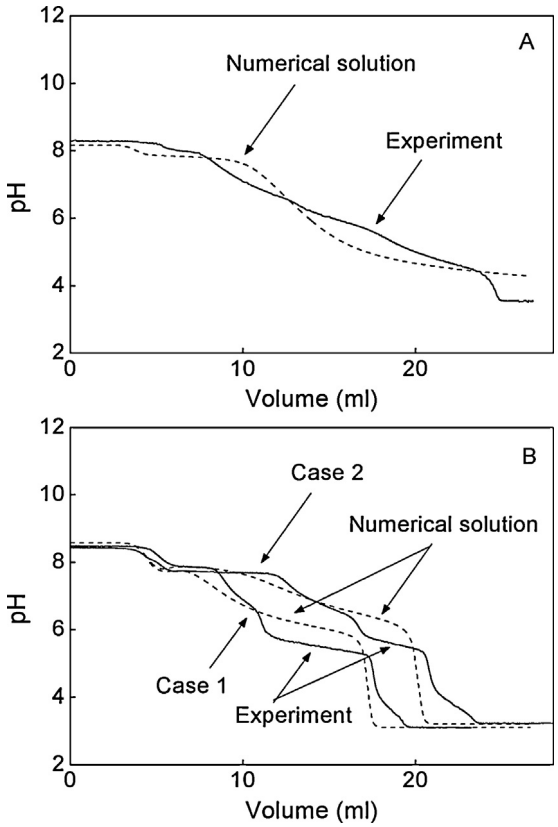

Fig. 2. Comparison of theoretically calculated (dash line) and experimentally measured (solid line) pH profiles formed on a column packed with MEP HyperCel. (A) The starting buffer was 25 mM Tris titrated with HCl to pH 8.2 and the elution buffer was 25 mM sodium citrate at pH 3.5. The column was 6.4 cm × 1.0 cm I.D. and the flow rate was 0.4 ml/min. (B) The starting buffer is the same for each case shown and contains 10 mM NaOH titrated with TAPS to pH 8.5. The elution buffer for the Case 1 contains 10 mM NaOH and 10 mM MES titrated with formic acid to pH 3.1. The result for the Case 1 was used to determine the column-packing physical properties shown in Table 1. The elution buffer for Case 2 was designed on the basis of computer simulations to extend the intermediate gradual section to longer times and contains 10 mM NaOH and 5 mM MES titrated with formic acid to pH 3.2. The column was 6.6 cm × 1.0 cm I.D. and the flow rate was 0.4 ml/min.

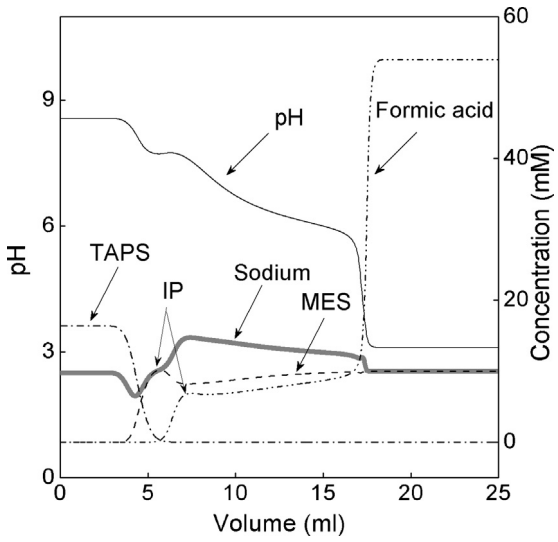

Fig. 3. Calculated concentration profiles of buffering species for Case 1 in Fig. 2B. The symbol IP denotes the intermediate pH plateaus.

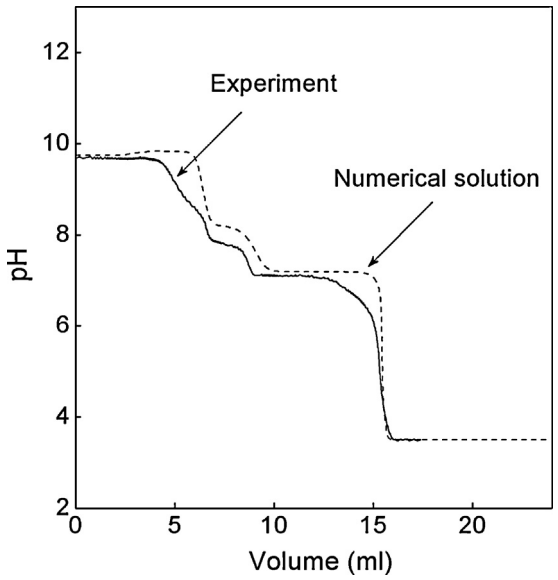

Fig. 4. Comparison between theoretically calculated (dash line) and experimentally measured (solid line) pH gradients formed on a 3.8 cm × 1.0 cm I.D column packed with HEA HyperCel. The starting buffer contains 10 mM NaOH titrated with CHES to pH 10. The elution buffer contains 10 mM NaOH, 20 mM TAPS and 15 mM TES titrated with formic acid to pH 3.5. The flow rate was 0.4 ml/min.

Table 2
Physical properties used to fit experimental data for HEA HyperCel.

	R_1	R_2	R_3
pK_R	7.0	8.0	8.5
q_R (M)	0.02	0.07	0.03

	Na^+	Formic acid	CHES	TAPS	TES
pK_a	NA	3.7	9.3	8.4	7.5
$K_{B^+,\,ads}$	1	NA	NA	NA	NA
$K_{A^0,\,ads}$	NA	2.0	1.0	1.0	1.4
$K_{A^-,\,ads}$	NA	2.0	1.0	1.0	1.4

NA: Not applicable.
R_1, R_2, R_3: Functional group subtypes on column packing.

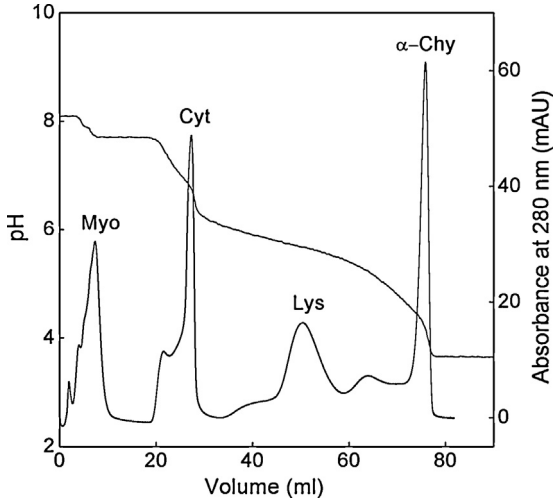

Fig. 5. Experimental separation of myoglobin, cytochrome C, lysozyme and α chymotrypsinogen A accomplished using a 50 μl feed sample containing 12 mg/ml of approximately equal mixture of the proteins and a 6.4 cm × 1.0 cm I.D column packed with MEP HyperCel. The starting buffer was 5 mM Tris titrated to pH 8.2 with HCl. The elution buffer was 5 mM citric acid titrated to pH 3.5 with NaOH. The flow rate was 0.15 ml/min.

Table 3
Comparison of p*I* and pI_{app} values for the four proteins used in this study.

	Protein	p*I*	pI_{app}
1	Myoglobin	7.0	7.7
2	Cytochrome C	10.6	6.7
3	Lysozyme	11	5.7
4	α Chymotrypsinogen A	9.5	4.2

[41,42], and relatively high pI_{app} value of 8.5 [28]. The elution order of the other three proteins can be explained by considering both their hydrophobicity and electrostatic interactions. Since the hydrophobicity of cytochrome C is the lowest of these three proteins [41,42], it was eluted before lysozyme even though its p*I* is lower than that for lysozyme. Also, α chymotrypsinogen A has the highest hydrophobicity and the lowest p*I* among the proteins, so that it was the last protein to be eluted from the column.

The results shown in Fig. 5 indicate the presence of the mixed-mode mechanism. Each protein, except for myoglobin, exits the column at a pH much lower than its p*I*, so that they are all positively charged and consequently electrostatically repelled from the column packing as they travel through the column. It is therefore evident that the hydrophobic interactions which counteract the electrostatic repulsion are responsible for the observed retention on the column. The results also show that mixed-mode chromatofocusing leads to a unique retention order for proteins that differs from standard chromatofocusing. Furthermore, it will be shown in the next two sections that the balance between the hydrophobic attractive and electrostatic repulsive forces can be adjusted using additives to the fluid phase in order to "tune" the separation achieved.

3.4. Effect of added salt on the protein separation in mixed-mode chromatofocusing

In general, adding a neutral salt to the starting and elution buffers can be expected to both increase hydrophobic attractive forces [41] and to decrease electrostatic repulsive forces [16], the net effect of which is to shift the elution position of a protein to a downstream location on the gradient. On the other hand, an increase in the salt concentration will reduce the Donnan potential in the adsorbed phase, which will tend to shift the elution position of a protein to an upstream location [37]. Consequently, the overall effect of the addition of a neutral salt may shift a protein to either an upstream or downstream location on the gradient depending on the specific properties of the protein. In this study, sodium chloride was chosen as the neutral salt to add since it is one of the most commonly used salts in chromatography and it is also in the middle of the Hofmeister lyotropic series so that its effects are expected to be typical of added neutral salts in general.

Fig. 6A shows the effect of the adding 0.1 M NaCl to both the elution and starting buffers on the pH gradient in a column packed with MEP HyperCel and using the same starting and elution buffers as in Fig. 2A. As shown in the figure, the shapes of the pH gradients are similar after addition of salt, although the results also indicate that salt addition causes the pH gradient to exit the column at somewhat shorter times. Fig. 6B shows the effect of the added salt on the elution of proteins when using a similar buffer system as in Fig. 5, and with the same four proteins as used in that figure.

As shown in Figs. 5 and 6, the value of pI_{app} for the proteins is significantly influenced by the salt concentration. In particular, Fig. 6B indicates that increasing the salt concentration interchanged the elution order for myoglobin and cytochrome C. This illustrates that changes in salt concentration may affect the retention behavior of different proteins in different directions, so that the salt concentration can be used to modify significantly the separation of proteins

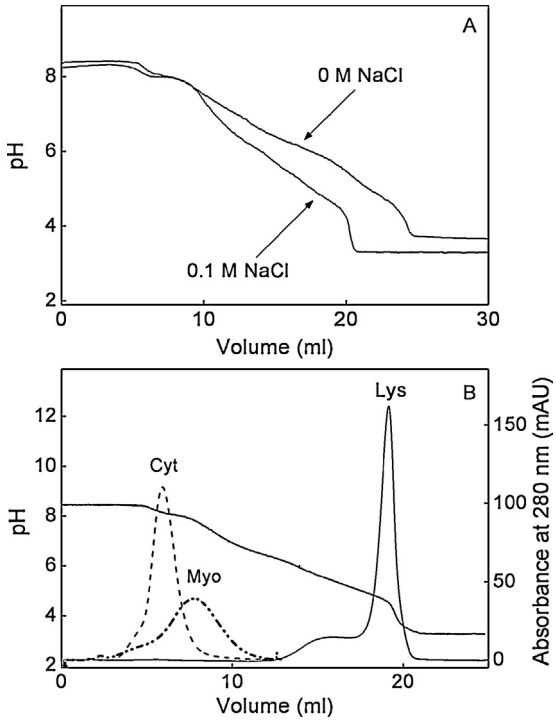

Fig. 6. (A) Effect of added salt on the pH gradient when using a 6.4 cm × 1.0 cm I.D column packed with MEP HyperCel. The starting buffer was 25 mM Tris titrated to pH 8.2 with HCl. The elution buffer was 25 mM citric acid titrated to pH 3.5 with NaOH. Conditions are the same in both experiments except that 0.1 M NaCl was added to both the elution and starting buffers in one experiment. The flow rate was 0.4 ml/min. (B) Elution of myoglobin, cytochrome C, and lysozyme accomplished by using a 50 μl feed sample containing 1.0 mg/ml of each protein on the same column used in Panel A. The flow rate was 0.4 ml/min. The starting buffer was 25 mM Tris and 0.1 M NaCl titrated to pH 8.2 with HCl. The elution buffer was 25 mM citric acid and 0.1 M NaCl titrated to pH 3.5 with NaOH.

in MMC. In contrast, in traditional chromatofocusing the addition of salt has been shown to nearly always reduce retention times since in this case only electrostatic interactions are involved [43,44]. Consequently, the separation "tuning" shown in Fig. 6B using the salt concentration illustrates the unique possibilities that exist for optimizing the separation when using a MMC column packing for chromatofocusing. The results described here involving the change in the pI_{app} with ionic strength are also consistent with the recent work by Holstein et al. [45] which showed that for a related type of mixed-mode column packing the retention time for lysozyme was significantly greater in an increasing salt gradient as compared to a standard ion-exchange column packing, while for cytochrome C this retention time difference was much smaller.

3.5. Effect of urea on the protein separation in mixed-mode chromatofocusing

In addition to neutral salts, urea can also be used to modify the protein elution behavior in MMCF. The addition of urea to the starting and elution buffers used in MMCF potentially alters several factors which affect the separation achieved, including the following: (1) an increase in the electrostatic interactions due to a decrease in the dielectric constant in the fluid phase [46], (2) a decrease in the hydrophobic interactions due to the shielding by urea of hydrophobic surfaces and to the reduction by urea of the hydrogen bonding between water molecules [46,47], and (3) an increase in the pK_a values of both the buffering species and the amino acid side chain groups of the proteins [48]. Although the overall effect of these factors is difficult to predict *a priori*, the first and second factors taken together suggest that the addition of urea

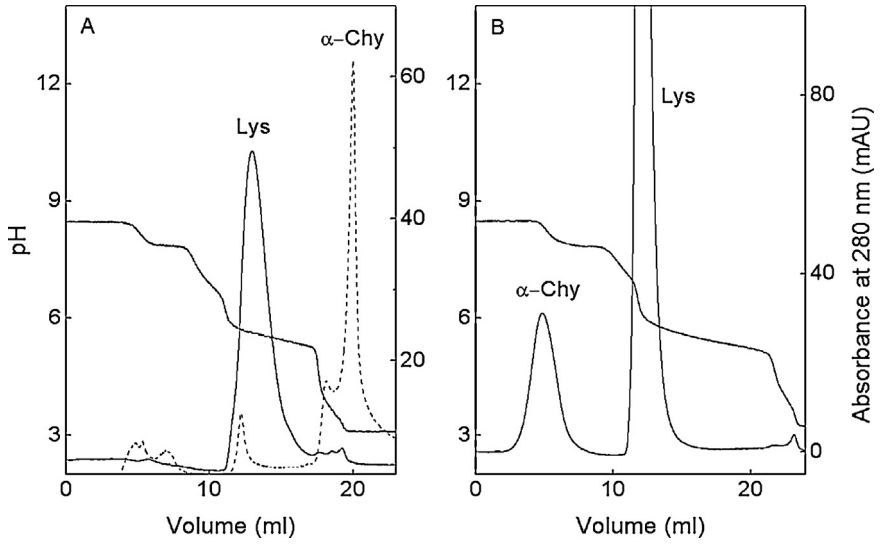

Fig. 7. Effect of urea on the elution of lysozyme and α chymotrypsinogen A accomplished using a 50 μl feed sample containing 1.0 mg/ml of each protein and a 6.6 cm × 1.0 cm I.D column packed with MEP HyperCel. The flow rate was 0.4 ml/min. (A) The starting buffer was 10 mM NaOH titrated with TAPS to pH 8.5 and the elution buffer was 10 mM NaOH and 10 mM MES titrated with formic acid to pH 3.1. (B) The starting buffer was 2 M Urea and 10 mM NaOH titrated with TAPS to pH 8.5 and the elution buffer was 2 M Urea, 10 mM NaOH and 10 mM MES titrated with formic acid to pH 3.4.

will generally cause a protein to elute at a position that is more upstream on the gradient than it would otherwise. Furthermore, the second factor given above indicates that the addition of urea tends to increase the solubility of a protein at a given fluid phase pH, which is likely to be beneficial in most cases.

Figs. 7 and 8 show the effect of urea addition on the separation of proteins in columns packed with MEP HyperCel and HEA HyperCel, respectively. Panels A and B in both figures illustrate results without and with urea addition, respectively. As shown in these figures, adding 2 M urea to the buffers used does not significantly affect the pH gradient shape. However, it can also be seen that the value of pI_{app} was influenced by urea by different amounts for different proteins. More specifically, Fig. 7 shows that adding urea increased the pI_{app} values of both lysozyme and α chymotrypsinogen A. It can also be seen that the pI_{app} value of α chymotrypsinogen A increased significantly while only a small increase in the pI_{app} value of the lysozyme was observed. Note that the elution pH used was slightly different when comparing Fig. 7A and Fig. 7B (i.e., 3.1 versus 3.4). It was assumed here that this difference did not affect the conclusions obtained.

The results shown in Fig. 8 indicate that the separation of lysozyme and α chymotrypsinogen A cannot be achieved with the buffer system used without the addition of urea when the HEA HyperCel column packing is used since the pI_{app} values of the two proteins are nearly equal in this case. In contrast, the addition of urea to the buffers used permits a significant degree of separation between these two proteins as shown in panel B in Fig. 8. Furthermore, it was also noted that the mass recovery of proteins based on the UV absorbance at 280 nm measured in the column effluent varied from 60% to 90% without adding urea, while the addition of

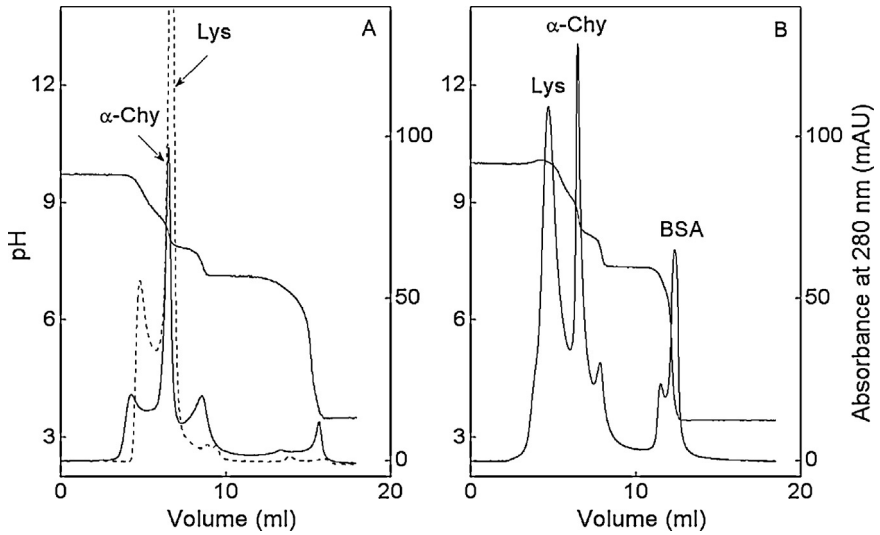

Fig. 8. Effect of urea on the elution of lysozyme, α chymotrypsinogen A and BSA (B only) accomplished using a 50 μl feed sample containing 1.0 mg/ml of each protein and a 3.8 cm × 1.0 cm I.D column packed with HEA HyperCel. The flow rate was 0.4 ml/min. (A) The starting buffer was 10 mM NaOH titrated with CHES to pH 10 and the elution buffer was 10 mM NaOH, 20 mM TAPS and 15 mM TES titrated with formic acid to pH 3.5. (B) The starting buffer was 2 M Urea and 10 mM NaOH titrated with CHES to pH 10 and the elution buffer was 2 M Urea, 10 mM NaOH 20 mM TAPS and 15 mM TES titrated with formic acid to pH 3.5.

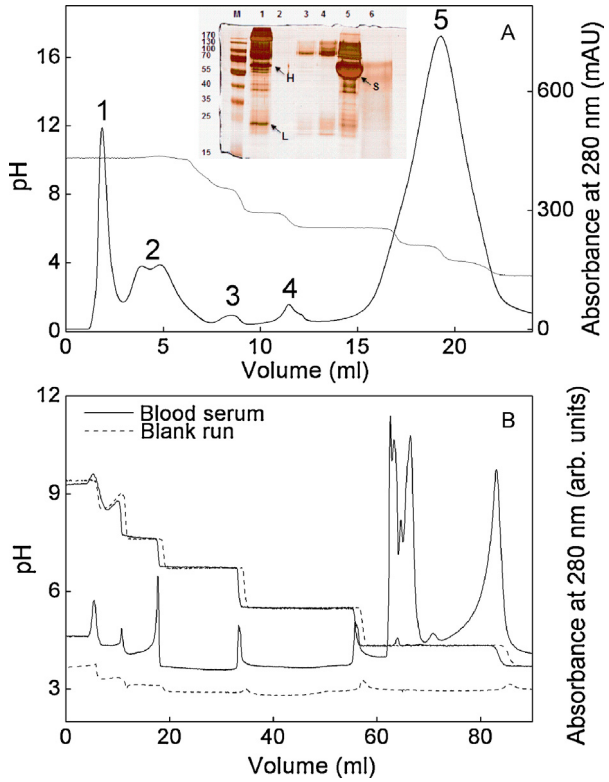

Fig. 9. (A) Separation of 470 μl of rabbit blood serum on a 3.8 cm × 1.0 cm I.D column packed with HEA HyperCel. The flow rate was 0.4 ml/min. The starting buffer was 2 M urea and 10 mM NaOH titrated with CHES to pH 10. The elution buffer was 2 M urea, 18 mM TAPS, 12 mM MOPS, 10 mM MES, 4 mM acetic acid and 4 mM formic acid titrated with HCl to pH 3.2. Fractions from the column were further separated by 12% SDS-PAGE with silver staining as shown by the inset gel image. Lanes 1–5 in the gel image correspond to the fractions as indicated in the chromatogram. Lane 6 shows the collected fraction from 1 M NaOH washing buffer. (H) Heavy chain of IgG, (L) light chain of IgG, (S) serum albumin. (B) Separation of 100 μl of rabbit blood serum on a 6.2 cm × 1.0 cm I.D column packed with HEA HyperCel. The flow rate was 0.1 ml/min. The starting buffer was 20 mM NaOH titrated with CHES to pH 10. The elution buffer was 10 mM NaOH, 10 mM TAPS, 7.5 mM TES, 5 mM MES, 2.5 mM acetic acid and 2.5 mM formic acid titrated with HCl to pH 3.2.

urea generally increased the mass recovery of proteins. This observation was particularly apparent for the more hydrophobic column packing HEA HyperCel used in Fig. 8.

3.6. Applications to blood serum fractionation

In this section MMCF will be investigated on a preliminary basis as an alternative chromatographic method for blood serum fractionation. Chromatofocusing as a first-dimension separation method coupled with reversed-phase chromatography as a second-dimension separation method has been used previously for the two-dimensional proteomic analysis of blood serum [49], so the use of MMCF incorporating both electrostatic and hydrophobic interactions for blood serum fractionation as explored here seems reasonable. In addition, the fact that the method investigated here employs a single stepwise change in buffer composition at the column inlet to form a complex, internally generated pH gradient makes this method well suited for the large-scale operation typical of the blood plasma fractionation industry due to its simplicity of operation.

The HEA HyperCel column packing was used in the work described here since it has been used previously to fractionate immunoglobulin and albumin, albeit using an unretained gradient formed externally [50]. Fig. 9 shows the fractionation achieved for rabbit blood serum (i.e., blood plasma with the clotting factors removed) by employing the MMCF method using a retained stepwise pH gradient. The starting and elution buffers compositions for the gradient shown were selected by modifying a buffer composition previously described by Shen et al. [51] (see "Recipe 5" in this reference). These modifications were accomplished by adding formic acid as an additional buffering species and also adjusting the buffering species concentrations using numerical simulations as an approximate guide so that a sequence of relatively evenly spaced self-sharpening pH transitions was produced.

In comparison to Fig. 9A, Fig. 9B employs a longer column and a lower flow rate in order to maximize the resolution obtained by increasing the number of theoretical equilibrium plates in the column. Fig. 9A also illustrates sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) results for the collected effluent fractions which indicate that the albumin component of the blood serum feed sample is contained in peak 5 of Fig. 9A along with other acidic proteins [52]. This result is in agreement with past work using unretained pH gradients formed by the external mixing of buffers which demonstrates that bovine serum albumin elutes from a column containing HEA HyperCel near pH 3.0 [53]. In contrast, the SDS-PAGE results also indicate that the more basic proteins that are present, such as the immunoglobulins, are present in peak 1 of Fig. 9A. The SDS-PAGE results shown in Fig. 9A further indicates that the immunoglobulin and albumin components are present in single fractions (i.e., fractions 1 and 5, respectively) with minimal cross contamination of the other fractions with these two components. Despite their preliminary nature, the results shown in Fig. 9 demonstrate that the MMCF method developed in this study may have useful applications for blood plasma fractionation, such as for an initial fractionation step that is followed by additional purification steps to recover high-purity blood proteins.

It is useful also to compare the results given in this section, as well as in the previous section with past results obtained by other workers. In particular, the results given in Fig. 9 can be compared with results given by Levison and Brieau [50] for a similar system except that unretained pH and ionic strength gradients produced externally were used. These workers obtained elution pH values for immunoglubins and albumin that were somewhat less than the pI_{app} values observed in Fig. 9 and a resolution between proteins that was also less than that obtained in Fig. 9. However, it should be noted that the higher resolution observed in Fig. 9A may be partly due to the addition of urea and the lower ionic strength employed, and not just due to the different type of pH gradient used. Furthermore, the results in Fig. 8 can be compared with results given by Kaur et al. [53] and Schwartz [54] for a similar system except that unretained gradients produced externally were employed. These workers again observed elution pH values for proteins that were somewhat less than the pI_{app} values shown in Fig. 8. However, in contrast to the previous case, the protein resolution obtained by Kaur et al. was greater than that observed in Fig. 8. It seems evident therefore that the use of a retained pH gradient, as compared to the use of an unretained gradient, may either increase or decrease the resolution of proteins depending on the particular system under consideration, in which case these two techniques can be considered to be complementary in nature.

4. Conclusions

In this work it is demonstrated that the technique of chromatofocusing in which a retained, internally generated pH gradient is used to separate proteins can be extended to the case where mixed-mode column packings are employed. This method, termed here "mixed-mode chromatofocusing" (MMCF), is more powerful and flexible compared to traditional chromatofocusing methods since more than one retention mechanism is employed. It is shown here

that the elution order of proteins in MMCF is the result of a combination of hydrophobic and electrostatic interactions so that the true p*I* and apparent p*I* values can differ significantly. It is also shown that the complexity of MMCF can in some cases be usefully exploited to improve the separation. For example, in MMCF the separation of proteins can be modified by the addition of salt or urea to alter the balance between hydrophobic attractive forces and electrostatic repulsive forces between proteins and the column packing and without significantly changing the shape of the pH gradient.

It is also demonstrated here that the effluent pH profile calculated numerically on the basis of relatively simple equilibrium relations agrees reasonably well with experimental results since the front types are usually correctly predicted, and the average positions and widths of these fronts are also correctly predicted with an error that is generally less than 20% in terms of the relevant elution volumes. It is apparent therefore that the numerical simulations illustrated here can be used, at least qualitatively, to determine the eluent buffer compositions that produce a desired shape for the pH and ionic strength gradients, as well as to account for various related considerations such as the effect of added neutral salts on the pH gradient shape. It is also demonstrated here that MMCF may have useful applications for blood plasma fractionation.

The chromatographic methods described in this study all involve the use of a pH gradient which is dynamically formed entirely inside the column and without the aid of any external mixing. These methods therefore maximize the role of dynamic focusing effects on the separation achieved in comparison to alternative pH gradient elution methods where the gradient is formed partly or wholly by external mixing. Without need of an external gradient, this method is well suited for large-scale operation since gradients produced solely by a single stepwise changes in the column influent, as done here, are generally preferred over gradients produced by external mixing as the scale of operation increases due to their reproducibility and ease of automation [55].

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.10.090.

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