

# Chromatofocusing Using Micropellicular Column Packings with Computer-Aided Design of the Elution Buffer Composition

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**Micropellicular, anion-exchange column packings are used in chromatofocusing to demonstrate the resolution and speed achieved when proteins are separated under these conditions. Linear or concave pH gradients are produced with simple mixtures containing four or fewer individual buffering species instead of the more commonly used polyampholyte buffers. Computer-aided design methods are demonstrated for selecting the composition of the elution buffer to produce a pH gradient of a desired shape. The method is applied to high-resolution, analytical- and preparative-scale separations involving horse myoglobin, human hemoglobin variants, and bovine carbonic anhydrase. A useful selection of buffering species is described capable of producing pH gradients of a variety of shapes in the range between pH 9.5 and 5.5.**

Chromatofocusing is a form of ion-exchange chromatography initially developed by Sluyterman and co-workers<sup>1–4</sup> where a retained pH gradient (i.e., a gradient that travels through the column more slowly than an unadsorbed elute) is produced inside a chromatography column as the buffering species in the elution buffer titrate weak-electrolyte functional groups on the column packing. To perform the method, the column is first equilibrated with a presaturation buffer that, for the case of an anion-exchange column packing incorporating weak-base functional groups, has a pH higher than the elution buffer. The feed sample is then introduced and the column subsequently eluted by making a stepwise change at the column inlet to the elution buffer. As a result of this procedure, a gradual pH gradient is produced and the proteins in the feed sample elute from the column in the order of their apparent isoelectric points, which are in general close to the actual isoelectric points.

The eluent typically used in chromatofocusing is a polyampholyte buffer containing a large number of individual polymeric buffering species. However, polyampholyte buffers tend to be expensive to use and, due to the way they are manufactured, to exhibit lot-to-lot variations in composition which may lead in turn to irreproducibilities in the shape of the pH gradient. To address these difficulties, a number of workers have investigated the use

of simple mixtures of buffering species (i.e., mixtures containing four or fewer individual species) in the elution buffer to form a gradual pH gradient in chromatofocusing.<sup>5–9</sup> The majority of these studies are based on the original observation of Sluyterman and Wijdnes<sup>2</sup> that a gradual, retained pH gradient can be produced by using an eluent containing relatively few buffering species if none of these buffering species are adsorbed (i.e., for the case of an anion-exchange column packing, only weak-base buffering species are present), in which case a stepwise change from the presaturation buffer to the eluent can be employed. Subsequently, it was pointed out by Frey and co-workers<sup>5,6</sup> that this method takes advantage of the single, non-self-sharpening (i.e., gradual) front that forms under these conditions. Alternatively, Sluyterman and co-workers also demonstrated that a gradual, retained pH gradient can be produced by employing external mixing of two or more elution buffers, in which case, these elution buffers can contain both adsorbed and unadsorbed buffering species. However, Sluyterman and co-workers did not fully develop these methods, evidently because of the difficulty in optimizing their performance, but instead pursued the use of polyampholyte elution buffers. Another approach to avoid the use of polyampholyte buffers is that of Hearn and Lyttle<sup>10</sup> and Hutchens et al.,<sup>11,12</sup> who employed as many as 30 individual adsorbed and unadsorbed buffering species in the elution buffer to approximate the behavior of a polyampholyte buffer.

In the present study, the chromatofocusing method described by Frey and co-workers<sup>5,6</sup> involving the use of simple mixtures of unadsorbed buffering species to form a single, non-self-sharpening front and a corresponding gradual, retained pH gradient is extended to the case of micropellicular (i.e., nonporous) anion-exchange column packings. The basic goal of this work is to exploit the lack of intraparticle pore diffusion in micropellicular particles and thereby perform chromatofocusing at resolutions and speeds not attainable using conventional porous high-performance liquid chromatography (HPLC) column packings. The use of

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pellicular particles having diameters of 40  $\mu\text{m}$  to accomplish high-speed liquid chromatography was first demonstrated by Horváth and co-workers.<sup>13,14</sup> Somewhat later, Unger and co-workers<sup>15,16</sup> demonstrated the similar use of micropellicular particles having diameters less than 5  $\mu\text{m}$ . Today, micropellicular column packings are widely employed to separate proteins and other biopolymers using reversed-phase chromatography or various traditional methods of ion-exchange chromatography.<sup>17,18</sup> However, only one previous study has investigated the use of micropellicular column packings in chromatofocusing.<sup>19</sup> Furthermore, this particular previous study utilized shrunken, cross-linked agarose particles that are not commercially available, together with a bed compression technique not in general use and employed separation times much longer than typical for chromatography performed with a micropellicular packing, so that the performance achievable using currently available micropellicular column packings is not apparent. In addition, in the present study, the local-equilibrium theory described by Bates et al.<sup>6</sup> will be used as the basis for a computer-aided design method for selecting the identities and concentrations of the buffering species in the elution buffer in order to produce a pH gradient of a desired shape.

## THEORY

**Calculation of the pH Gradient.** Consider chromatofocusing, which employs a single, non-self-sharpening pH front where local-equilibrium conditions apply and for the case of a stepwise change from the presaturation buffer to the elution buffer at the column entrance. Under these conditions, the velocity of a pH level in the column when the only adsorbed species is an inert ion (i.e., an ion that does not participate in acid–base equilibrium, such as the chloride ion) is given by the following relation:<sup>5</sup>

$$v_{\text{pH}} = v_{\text{fluid}} \left[ 1 + \frac{(1 - \alpha)\epsilon}{\alpha} + \frac{(1 - \alpha)(1 - \epsilon)}{\alpha} \frac{dq_s^*}{dC_s} \right] \quad (1)$$

In eq 1,  $v_{\text{pH}}$  is the velocity of a particular pH value inside the column,  $v_{\text{fluid}}$  is the liquid-phase interstitial velocity,  $\alpha$  and  $\epsilon$  are the column and particle porosities, and  $dq_s^*/dC_s$  is the slope of the adsorption isotherm for the inert ion S at the given pH in a liquid phase containing the buffering species at their concentrations in the elution buffer. In the remainder of this study, it will be assumed for simplicity that  $\epsilon = 0$  so that  $q_s^*$  becomes identical to  $q_{\text{st}}^*$  used previously.<sup>5,6</sup>

To simplify the application of eq 1, the term  $dq_s^*/dC_s$  can be written as<sup>6</sup>

$$\frac{dq_s^*}{dC_s} = \frac{dq_s^*/d\text{pH}_{\text{fluid}}}{dC_s/d\text{pH}_{\text{fluid}}} = \frac{\beta'_{\text{ads}}}{\beta_{\text{fluid}}} \quad (2)$$

where  $dq_s^*/d\text{pH}_{\text{fluid}}$  is the buffering capacity of the adsorbed

phase based on changes in the liquid-phase pH, i.e.,  $\beta'_{\text{ads}}$ , and  $dC_s/d\text{pH}_{\text{fluid}}$ , denoted as  $\beta_{\text{fluid}}$ , is the buffering capacity in the liquid phase. Equations 1 and 2 can be combined to yield

$$t(\text{pH}) = (L/v_{\text{fluid}}) \left[ 1 + \frac{(1 - \alpha)\epsilon}{\alpha} + \frac{(1 - \alpha)(1 - \epsilon)}{\alpha} \frac{\beta'_{\text{ads}}(\text{pH})}{\beta_{\text{fluid}}(\text{pH})} \right] \quad (3)$$

where  $t(\text{pH})$  is a function that gives the time for a particular pH value to exit the column and  $L$  is the column length. For convenience in applying eq 3, it is assumed that  $\beta_{\text{fluid}}$  for a specific buffer composition and  $\beta'_{\text{ads}}$  for a specific column packing are functions solely of the liquid-phase pH. The buffering capacities of mono- and dibasic buffering species are given by<sup>20</sup>

$$\beta_{\text{fluid,monobasic}} = 2.303 \frac{C_{\text{buffer}} K_a C_{\text{H}^+}}{(K_a + C_{\text{H}^+})^2} \quad (4)$$

$$\beta_{\text{fluid,dibasic}} = 2.303 C_{\text{buffer}} K_{a1} C_{\text{H}^+} \frac{C_{\text{H}^+}^2 + 4K_{a2} C_{\text{H}^+} + K_{a1} K_{a2}}{(C_{\text{H}^+}^2 + K_{a1} C_{\text{H}^+} + K_{a1} K_{a2})^2} \quad (5)$$

The buffering capacity of the liquid phase can then be calculated as

$$\beta_{\text{fluid}} = \beta_{\text{fluid,species 1}} + \beta_{\text{fluid,species 2}} + \beta_{\text{fluid,species 3}} + \dots \quad (6)$$

To apply the above relations, a calibration experiment with a known buffer composition can be performed and the function  $t(\text{pH})$  measured experimentally. With  $\beta_{\text{fluid}}(\text{pH})$  calculated using eqs 4–6, the function  $\beta'_{\text{ads}}(\text{pH})$  can then be determined from eq 3 for a specific column packing. With  $\beta'_{\text{ads}}(\text{pH})$  thus determined, the time at which a pH value exits the column for any elution buffer composition can be obtained using eqs 3–6.

In general, local-equilibrium theories such as the one described above are well suited to predict the shapes of non-self-sharpening fronts since mass-transfer and other kinetic effects are relatively unimportant under these conditions. In the present study, this was confirmed by measuring the pH gradient produced for a given set of conditions but at various flow rates of up to 5 mL/min and showing that the gradients were basically identical when the eluent pH was plotted as a function of the volume eluted (data not shown).

**Computer-Aided Design of the Elution Buffer Composition.** Computer-aided design of a specific pH gradient shape can be accomplished by defining an appropriate objective function and constraints that characterize the desired gradient and then solving the resulting constrained optimization problem using the concentrations of buffering species in the elution buffer as independent variables. For example, consider the following objective function:

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$$F = (S_1 - bS_2)^2 + (S_2 - bS_3)^2 + (S_3 - bS_4)^2 + (S_4 - bS_5)^2 \quad (7)$$

where  $S_1$  to  $S_5$  denote the slopes of the pH gradient in small pH ranges spaced evenly across the gradient and  $b$  denotes a curvature factor. If the function  $F$  is minimized for a specified value of  $b$  using the concentrations of the buffering species as independent variables subject to the constraints that the value of one of the slopes  $S_i$  lies in a certain range and that the midpoint of the gradient lies within a certain time interval, then an elution buffer composition can be determined that yields a pH gradient of a desired shape in a certain pH range and with a specified average transit time. In particular, the above strategy yields a linear pH gradient if  $b = 1$ . Software that performs these calculations is available upon request directly from the authors or at the website <http://www.research.umbc.edu/~dfrey1/bufferdesign>.

## EXPERIMENTAL SECTION

The buffering species used in this work are diethanolamine (DEA), imidazole, Trizma base (Tris, tris(hydroxymethyl)aminomethane), and piperazine, all obtained from Sigma (St. Louis, MO). To produce the elution and presaturation buffers, mixtures of these species were titrated with HCl to the desired pH and the mixture then vacuum filtered through a 0.2- $\mu$ m membrane filter. The columns used were a  $3.5 \times 0.46$  cm i.d. TSKgel DEAE-NPR column and a  $7.5 \times 0.46$  cm i.d. TSKgel DNA-NPR column, both manufactured by Tosoh Biosep (Montgomeryville, PA), and a  $25 \times 0.4$  cm i.d. ProPac WAX-10 analytical column and a  $5 \times 0.4$  cm i.d. ProPac WAX-10G guard column, which were gifts from Dionex (Sunnyvale, CA). The Tosoh Biosep columns contain 2.5- $\mu$ m pellicular polymethacrylate particles derivatized with diethylaminoethyl (DEAE) functional groups, and the Dionex columns contain 10- $\mu$ m pellicular particles composed of ethylvinylbenzene cross-linked with 55% divinylbenzene and also derivatized with DEAE functional groups. According to the manufacturer, the particles in the Dionex columns were tentacle-type in which the DEAE functional groups are attached to the particle using a lengthy spacer arm.<sup>21</sup> The proteins used were horse myoglobin, bovine carbonic anhydrase, and human hemoglobins A<sub>0</sub> and S<sub>1</sub>, all obtained from Sigma (Product Nos. M 0630, C 3934, H 0267, and H 0392, respectively) and human hemoglobin variants A, C, S, and F obtained from PerkinElmer Life Sciences (Boston, MA).

The chromatography equipment used was either a model 510 HPLC pump and a model 490E programmable absorbance detector (Waters, Milford, MA) or a model P4000 SpectraSystem pump and a model UV2000 SpectraSystem UV-Vis absorbance detector (Thermo Separation Products, San Jose, CA). The chromatography system was equipped with a low-dead-volume, in-line pH-sensing cell (Sensorex, Stanton, CA) and a model 701A Ionalyzer (Orion, Beverly, MA) to record the pH of the column effluent. Sample introduction and the formation of a stepwise change from the presaturation buffer to the elution buffer at the column inlet was accomplished using two model 9010 valves (Rheodyne, Rohnert Park, CA). The various components of the chromatographic systems after the sample injector were connected

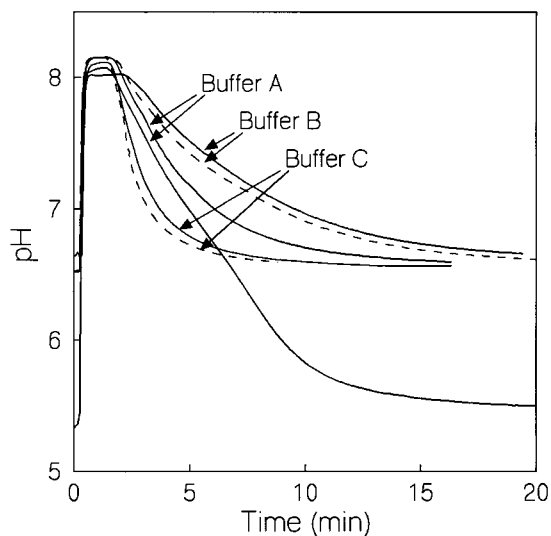


Figure 1. Comparison of calculated (dashed line) and experimentally measured (solid line) pH gradients using a DEAE-NPR column at a flow rate of 1 mL/min. Buffer A: 2 mM DEA, 2 mM Tris, 2 mM imidazole, and 2 mM piperazine. Buffer B: 2 mM DEA, 2 mM Tris, 1 mM imidazole, and 2 mM piperazine. Buffer C: 2 mM DEA, 2 mM Tris, 5 mM imidazole, and 2 mM piperazine. The columns were presaturated at either pH 8.0 or 8.2 as shown and were eluted at pH 6.5 or 5.5. The experimental result for buffer A in the range between pH 8.2 and 6.5 was used to determine  $\beta'_{\text{ads}}$  (pH), which was then used to predict theoretically the pH gradients for buffers B and C.

using 0.007-in.-i.d. tubing in order to minimize extracolumn band broadening.

## RESULTS AND DISCUSSION

**Comparison between Calculated and Experimentally Measured pH Gradients.** Figure 1 illustrates a comparison between theoretically predicted and experimentally measured pH gradients for the DEAE-NPR column. To perform the experiments shown, the column was first presaturated with a given buffer titrated with HCl to either pH 8.0 or 8.2 and then eluted in a stepwise manner using the same buffer titrated with HCl to pH 6.5 or 5.5 as the eluent. More specifically, buffer A was used in the range between pH 8.2 and 6.5 to determine  $\beta'_{\text{ads}}$  (pH) for the column packing and consisted of 2 mM each of DEA, Tris, imidazole, and piperazine. Buffers B and C in the figure were the same as buffer A except that in buffer B the imidazole concentration was 1 mM instead of 2 mM while for buffer C the imidazole concentration was 5 mM instead of 2 mM.

As shown in Figure 1, the pH gradients calculated using eqs 3–6 when buffers B and C are employed are in good agreement with the experimental results, with the small discrepancies observed likely due to the approximation that  $\beta'_{\text{ads}}$  is a function solely of the liquid-phase pH. The figure also indicates that the curvature of the pH gradient can be readily adjusted by making appropriate changes in the composition of the elution buffer. In particular, the use of buffer A leads to a pH gradient of modest curvature while the use of buffers B and C leads to gradients that are less curved and more curved, respectively, than the gradient resulting from the use of buffer A. The data in Figure 1 also show that nearly linear gradients where the elution pH is as low as pH 5.5 can be produced using buffer A and that pH perturbations

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caused by particle swelling observed in previous work with agarose-based column packings are absent, evidently due to the greater structural rigidity of the packings used in this study. Figure 1 finally indicates that although the DEAE-NPR column, like all the columns used in this study, is not intended by its manufacturer to be used for chromatofocusing, it can nevertheless be used to produce a retained pH gradient suitable for this purpose.

The fact that the gradients shown in Figure 1 can be produced using a column with DEAE functional groups with an intrinsic  $pK_a$  of 9.5 is likely due to electrostatic interactions between functional groups. As described by Tanford,<sup>22</sup> when a weak-electrolyte functional group on a polyelectrolyte becomes charged, it becomes progressively harder (i.e., requires a lower pH) to protonate adjacent functional groups due to electrostatic repulsion effects. In this way, a monofunctional polyelectrolyte can provide a buffering capacity over an extended pH range as shown.

In general, values for  $\beta'_{ads}$  for the pellicular column packings used in this study are smaller by a factor of  $\sim 2$  than values of this parameter for porous packings.<sup>6</sup> Since a truly nonporous particle would be expected to have a much smaller buffering capacity than a porous particle, this indicates that although the pellicular particles used are impermeable to proteins, they do have a pore structure of sufficient size to admit small ions and permit internal functionalization with DEAE groups. This is also consistent with the reported size exclusion limit of these particles of 500 Da.<sup>23</sup>

**Analytical-Scale Separation of Human Hemoglobins.** As discussed in the introduction, micropellicular column packings offer significant advantages for accomplishing high-speed separations of proteins. To demonstrate the use of these packings in chromatofocusing, the gradient of intermediate curvature in Figure 1 was employed with the DEAE-NPR column to separate a mixture of human hemoglobins  $A_0$  and  $S_1$ , where  $HbA_0$  is the normal form of human hemoglobin and  $HbS_1$  is a mutated form associated with sickle cell disease and involving the single-point mutation Glu  $\rightarrow$  Val at the sixth position of both hemoglobin  $\beta$ -chains.

As shown in Figure 2A, the pH gradient formed using buffer A and the DEAE-NPR column accomplishes the complete separation of the two forms of hemoglobin in  $\sim 4$  min. The resolution achieved can be determined quantitatively by the relation<sup>24</sup>

$$R_S = \frac{\Delta t}{2\sigma_{A_0} + 2\sigma_{S_1}} \quad (8)$$

where  $\sigma_i$  is the standard deviation of the peak shape and  $\Delta t$  is the time interval between the peak maximums. If it is assumed that the peak shapes are Gaussian and that the shape distortion caused by overlapping peaks can be ignored, then  $\sigma_i$  can be evaluated as the peak half width at 60% of the peak height. The resolution achieved in Figure 2A on this basis was determined to be 3.2, which is significantly greater than the resolution of 1.2 achieved in a previous study by virtually the same chromatofocusing method but using porous particles of standard size for HPLC, i.e., porous particles that were 10  $\mu m$  in diameter.<sup>25</sup>

Figure 2B illustrates experimental results for the separation of human hemoglobin variants when the DNA-NPR column is used

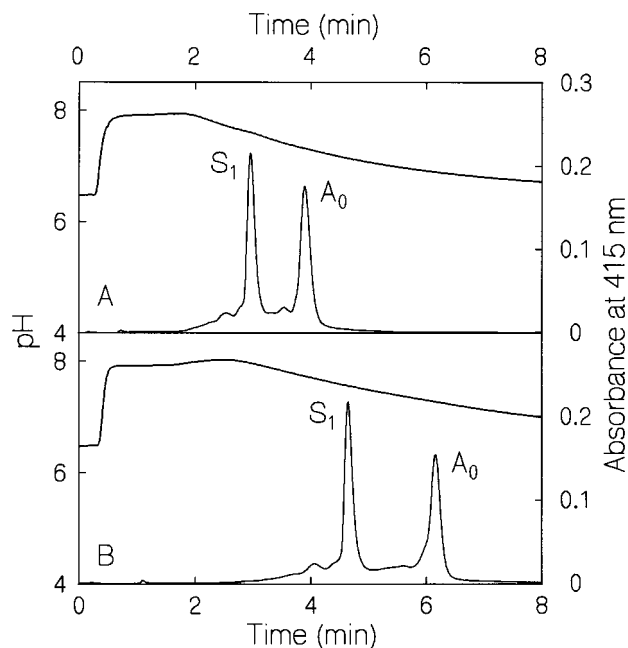


Figure 2. Chromatofocusing of 0.04 mg of a sample of human hemoglobins  $A_0$  and  $S_1$ . The presaturation buffer was a mixture of DEA, Tris, imidazole, and piperazine each at 2 mM at pH 8.2. The column was eluted with a pH 6.5 buffer having the same composition at a flow rate of 1 mL/min: (A) 3.5-cm-long DEAE-NPR column; (B) 7.5-cm-long DNA-NPR column.

with all other conditions the same as in Figure 2A. As described in the Experimental Section, the DNA-NPR column contains the same packing as the DEAE-NPR column, but is approximately twice the length. As shown by comparing panels A and B of Figure 2, the separation of the two hemoglobin variants improves considerably as the column length increases. More specifically, the resolution achieved between variants in Figure 2B was determined to be 5.1, which when compared to the resolution of 3.2 observed for the DEAE-NPR column indicates that the resolution, and therefore also the inverse of the bandwidth when expressed in pH units, is approximately proportional to  $L^{1/2}$ . This observation is in agreement with the theory of Sluyterman,<sup>1</sup> which states that the bandwidth in terms of pH units is proportional to square root of gradient steepness, which is in turn proportional to the inverse of  $L^{1/2}$ . Figure 2B also indicates that the baseline bandwidths (i.e., 4 times the half-width expressed in pH units at 60% of the band height, denoted as  $\Delta pH$ ) are 0.05 pH unit, which implies that proteins differing in apparent isoelectric points by this amount should be completely separated using the conditions shown. These widths are significantly less than the baseline bandwidths of 0.1–0.2 pH unit typically observed for chromatofocusing using porous HPLC column packings of standard size.<sup>25–27</sup>

It is also of interest to compare the resolutions obtained in Figure 2B with those obtained by capillary isoelectric focusing, although a number of differences in the methods must be considered when this comparison is being made. In particular,

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Figure 2B exhibits the general trend observed in this and previous work that the difference in the apparent isoelectric points between hemoglobin variants observed in chromatofocusing is approximately twice the difference in actual isoelectric points observed in isoelectric focusing.<sup>25</sup> More specifically, the apparent isoelectric points for the two hemoglobin variants in the figure are 7.55 and 7.27 as compared to the actual isoelectric points of 7.25 and 7.10, respectively. This suggests that the resolution shown in Figure 2B is comparable to resolutions typically obtained for hemoglobin variants by standard forms of capillary isoelectric focusing where protein bandwidths of 0.02 pH unit are observed but where analysis times are significantly longer than those in the figure.<sup>28</sup> Alternatively, bandwidths in Figure 2 are similar to those reported by Wu et al.<sup>29</sup> and Tragas and Pawliszyn,<sup>30</sup> who performed capillary isoelectric focusing with an analysis time similar to that in the figure by using whole-column-imaging detection.

Recently, improved methods for capillary isoelectric focusing where baseline bandwidths for hemoglobin variants of 0.01 pH unit have been reported.<sup>31</sup> However, these methods require specialized capillary coatings and very dilute samples and appear not to be in general use. Furthermore, the separation time achieved using these methods is considerably longer than that in Figure 2B so that direct comparison between methods is difficult.

Panels A and B of Figure 3 illustrate the separation of hemoglobin variants obtained using the WAX-10 and WAX-10G columns, respectively, together with the same buffer system used in Figure 2. As shown, the WAX-10 column achieves a separation nearly equivalent to that achieved with the DNA-NPR column described above with baseline bandwidths for the two hemoglobin variants again being  $\sim 0.05$  pH unit and with a resolution between variants of 3.6. As expected, a lower resolution was obtained using the shorter WAX-10G column albeit at a faster separation time of less than 1 min. As shown in Figure 3B, although the WAX-10G column is intended by its manufacturer to be used as a guard column, it can nevertheless be used alone to achieve very high-speed separations at moderate resolutions.

The results in Figures 2 and 3 can also be compared to those achieved using standard ion-exchange chromatography in which an externally produced ionic strength gradient is employed at constant pH. In particular, Weitzhandler et al.<sup>21</sup> investigated the separation of these variants using a cation-exchange version of the WAX-10 column employed in this study. These workers obtained a resolution similar to that shown in Figure 3A but with a longer separation time of 18 min.

Burke et al.<sup>32</sup> also used micropellicular ion-exchange chromatography to separate hemoglobin variants, although the particular column used by these workers is no longer commercially available. At moderate flow rates, the resolution achieved by Burke et al. was somewhat less than that shown in Figure 2B. However, at higher flow rates, these workers achieved a resolution somewhat

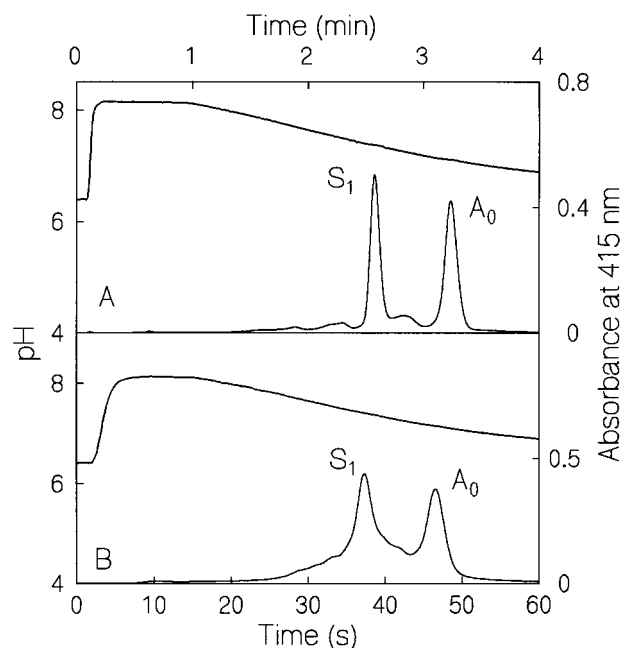


Figure 3. Chromatofocusing of a sample of human hemoglobins  $A_0$  and  $S_1$ . (A) 25-cm-long WAX-10 column. Total protein sample injected was 0.2 mg and the flow rate was 5 mL/min. (B) 5-cm-long WAX-10G guard column. Total protein sample injected was 0.05 mg, and the flow rate was 4 mL/min. Other conditions are the same as in Figure 2.

greater than that in Figure 3B in a comparable separation time of less than 1 min. This difference appears to be partly due to the fact that the column used by Burke et al. was manufactured with the intent that it be used alone for analytical chromatography, even though it had similar dimensions, and contained particles of similar diameter, in comparison to the WAX-10G guard column used to obtain the results in Figure 3B. In addition, extracolumn dead volume was minimized by Burke et al. to an extent greater than in this study. Instead, in this study, standard HPLC equipment was employed, and the extracolumn components were chosen for convenience in producing stepwise gradients at the column entrance. The result is that, as demonstrated below, for the analytical columns used in this study, plate heights and therefore also the resolution were relatively independent of flow rate, whereas when the short WAX-10G guard column was used alone with no attached analytical column, the plate height increased and the resolution decreased somewhat with flow rate. Nevertheless, the results in Figure 2B suggest that the performance of the method developed in this study is at a minimum comparable to that achieved by Burke et al. when the two methods are performed with similar chromatography equipment.

**Chromatofocusing of Bovine Carbonic Anhydrase.** Figure 4 shows the chromatofocusing of a commercial preparation of bovine carbonic anhydrase using the WAX-10 column. To perform the experiment, buffer A was used to presaturate the column at pH 8.4 and the same buffer at pH 5.2 was used for elution. However, only the pH gradient from pH 8.4 to 6.5 is shown in the figure since no elutes were evident at lower pH values. As illustrated, one major peak and at least eight minor peaks are resolved in the chromatogram. The figure also indicates that the bandwidth of the major peak is 0.14 pH unit, which is somewhat

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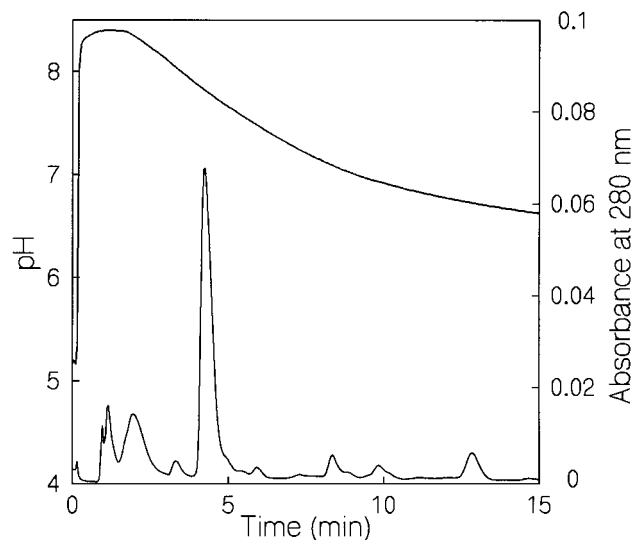


Figure 4. Chromatofocusing of a commercial preparation of bovine carbonic anhydrase using the WAX-10 column. The buffers used were the same as in Figure 2 except that the pH of the presaturation and elution buffers was 8.4 and 5.2, respectively. The amount of protein sample injected was 0.2 mg, and the flow rate was 2 mL/min.

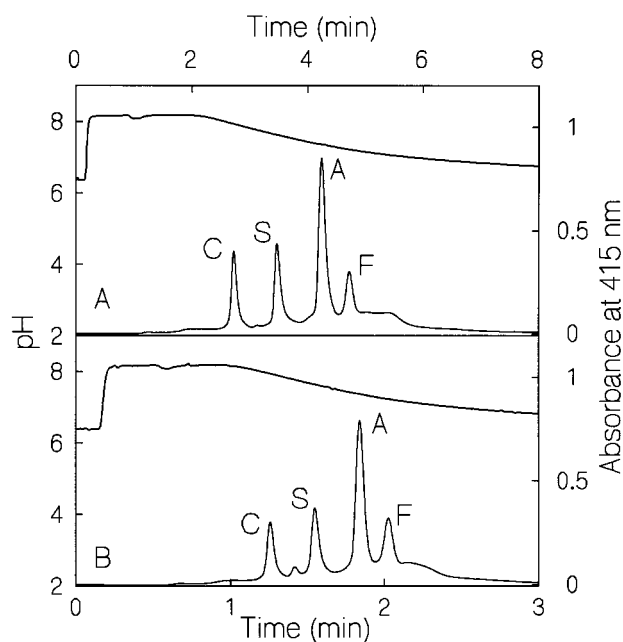


Figure 5. Separation of human hemoglobin A, C, F, and S using the WAX-10 column. The amount of hemoglobin sample injected was 0.5 mg. The buffering species and the pH of the elution and presaturation buffers were the same as in Figure 2. The concentration of each buffering species was 4 mM. Flow rate: (A) 2 and (B) 5 mL/min.

larger than that observed for hemoglobin variants. This suggests that bandwidths vary for different proteins due to extraneous factors such as secondary equilibrium effects.

#### Preparative-Scale Separation of Human Hemoglobins.

Figures 5 and 6 show the separation of a mixture of human hemoglobin A, C, F, and S using the WAX-10 column. In Figure 5A, 0.5 mg of hemoglobin sample was loaded onto the column and the concentration of the buffering species was 4 mM. The high resolution achieved using these conditions is indicated by the baseline resolution of the A and F forms of hemoglobin, which

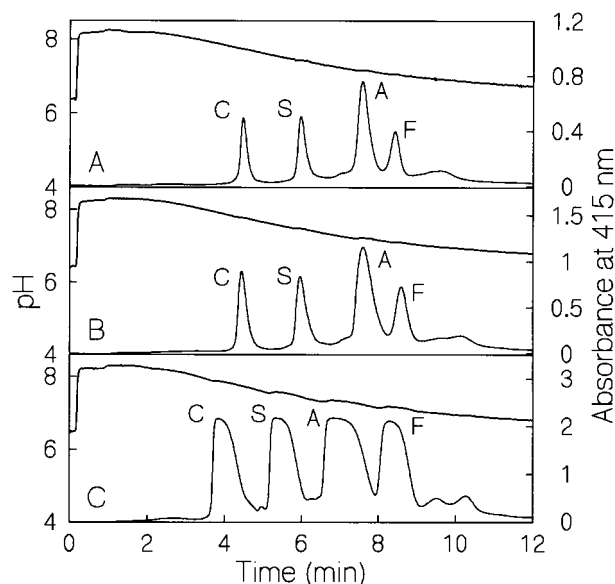


Figure 6. Separation of human hemoglobin A, C, F, and S using the WAX-10 column. The flow rate was 2 mL/min. Other conditions were the same as in Figure 2. Hemoglobin sample injected: (A) 1.0, (B) 2.0, and (C) 8.0 mg.

differ in isoelectric points by only 0.05 pH unit. The figures also indicate that the A variant elutes earlier than the F variant although the isoelectric point of the latter is higher than that of the former. The result is that the resolution between the S and F variants is enhanced in chromatofocusing relative to isoelectric focusing due to the interchange of the elution order of the A and F variants.

Figure 5B illustrates results using the same conditions as used in Figure 5A except that the flow rate was increased to 5 mL/min. As shown, the four hemoglobin variants are completely separated in a much shorter time of almost 2 min with little reduction in resolution. This indicates that, for the longer columns used in this study, extracolumn band broadening is negligible and the resolution achieved is largely unaffected by the flow rate, which is one of the useful characteristics of micropellicular column packings.

In panels A and B of Figure 6, similar conditions were used as in Figure 5A but with 2 and 4 times the sample loading, respectively. As shown, despite the increase in protein loaded, the resolution is basically unchanged, or even somewhat increased, as compared to Figure 5A, with the smallest bandwidth observed in this study for hemoglobin A (0.03 pH unit) being observed in Figure 6A. Finally, Figure 6C shows results using the same conditions as in Figure 6A but with a total of 8 mg of protein sample, which is 16 times the amount of protein employed in Figure 5A and nearly 80 times the loading of protein recommended by the column manufacturer.<sup>33</sup> The results indicate that although baseline resolution of the A and F variants is still accomplished, the band shapes are affected by the very high protein loading used and become non-Gaussian in character. Furthermore, the highest resolution of the two minor bands that elute after hemoglobin F can be seen in Figure 6 to occur at the highest protein loading.

(33) Installation Instructions and Troubleshooting Guide for the ProPac WAX-10G Guard Column and the ProPac WAX-10 Analytical Column, Document No. 031697-01, Dionex, Sunnyvale, CA, 2000, p 7.

The results in Figure 6C also indicate that the pH gradient is largely unaffected by the presence of large quantities of protein. This appears to be due to the fact that, as mentioned previously, the column packing used has pores large enough for internal functionalization with DEAE groups so that it is able to contribute substantially to the overall buffering capacity despite being impermeable to proteins. In addition, the band shapes in Figure 6C are nearly rectangular and do not exhibit the tailing from nonlinear equilibrium effects that generally typify mass overloading. This is likely due to the fact that in chromatofocusing proteins are only moderately bound to the column packing during their entire transit time through the column so that conditions are never far from linear equilibrium. Furthermore, the tentacle nature of the column packing used would seem to be highly conducive to maintaining linear equilibrium even at high protein loadings. Related features of tentacular column packings have recently been summarized by DePhillips and Lenhoff.<sup>34</sup> The result of these characteristics is that the chromatofocusing method developed in this study is highly suitable for the high-resolution preparative-scale purification of proteins.

**Separation of Proteins with Computer-Aided Design of the Elution Buffer.** The previous examples involved the resolution of protein variants that either elute in a narrow pH range so that the separation is mainly determined by the local slope of the pH gradient, and minor amounts of curvature in the overall gradient do not affect the results, or where later eluting variants have very similar isoelectric points so that a curved gradient with a lower slope at later times can be used to achieve a high resolution of these variants. In this section, a relatively steep, linear pH gradient is produced using computer-aided design that is appropriate for the high-speed separation of a protein mixture with a broader range of apparent isoelectric points.

Figure 7 illustrates the use of the computer-aided design method described in the Theory section to produce a linear pH gradient of a desired slope in the range from pH 9.5 to 6.5. To apply the method, buffer A in Figure 1 was titrated to pH 9.5 and used to presaturate the DEAE-NPR column. The column was then eluted with the same buffer titrated to pH 6.5, and the effluent pH profile was measured and used to determine  $\beta'_{\text{ads}}(\text{pH})$ . The constrained optimization problem described in the Theory section was then solved to determine the concentrations of the four buffering species making up buffer A that produce a linear pH gradient with a midpoint slope between 0.36 and 0.42 pH unit/min and a transit time between 2.3 and 2.5 min for the specified mobile-phase flow rate of 1 mL/min and assuming  $\epsilon = 0$ . The resulting optimized buffer composition was predicted to be 2.4 mM Tris, 1.5 mM imidazole, and 11.6 mM piperazine with diethanolamine absent from the mixture.

As shown in Figure 7, the pH gradient observed experimentally when using the computer-designed buffer system has the desired characteristics and agrees well with the theoretically calculated pH profile. The figure further illustrates the separation of horse myoglobin and the A<sub>0</sub> and S<sub>1</sub> variants of hemoglobin using the gradient. As shown, the three proteins are well separated with a resolution between hemoglobin variants of 3.3, which is nearly the same as in Figure 2A.

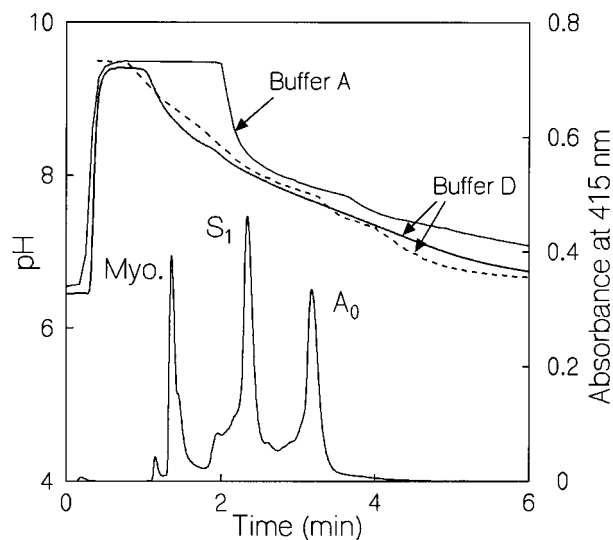


Figure 7. Comparison of calculated (dashed line) and experimentally measured (solid line) pH gradients using a DEAE-NPR column and a flow rate of 1 mL/min. Buffer A: 2 mM DEA, 2 mM Tris, 2 mM imidazole, and 2 mM piperazine. Buffer D: 2.4 mM Tris, 1.5 mM imidazole, and 11.6 mM piperazine. The experimental result for buffer A was used to determine  $\beta'_{\text{ads}}(\text{pH})$ , which was then used to predict theoretically the pH gradient for buffer D. Also shown is the separation of 0.06 mg of a sample of horse myoglobin and human hemoglobins A<sub>0</sub> and S<sub>1</sub> using buffer D.

## CONCLUSIONS

It is demonstrated that commercially available, weak-base micropellicular column packings can be used to conduct high-speed, high-resolution chromatofocusing of proteins at both the analytical and preparative scales with simple mixtures of four or less buffering species employed in the elution buffer. The performance of the method was evaluated specifically for both analytical- and preparative-scale separations involving horse myoglobin, bovine carbonic anhydrase, and human hemoglobin variants, although previous work on similar chromatofocusing methods utilizing column packings consisting of porous particles indicates that the method should be applicable to a broad range of protein separation problems.<sup>5,35,36</sup> It is also demonstrated that calculated pH gradients based on local-equilibrium theory agree well with experimental results and that computer-aided design methods can be used to determine buffer compositions that produce a desired shape for the pH gradient. In particular, the use of the four buffering species diethanolamine, imidazole, Tris, and piperazine permits the formation of pH gradients of a variety of useful shapes in the range from pH 9.5 to 5.5. This pH range in turn reflects the  $\text{pK}_a$  values of the buffering species used, which ranged from 9.72 to 5.76.

As described in the Results and Discussion section, for the particular case of separating hemoglobin variants, the chromatofocusing method developed in the present study achieves a performance comparable to, and possibly better than, that achieved using standard methods for either capillary isoelectric focusing or ion-exchange chromatography with an ionic strength

(34) DePhillips, P.; Lenhoff, A. M. *J. Chromatogr. A* **2001**, *933*, 57–72.

(35) Chromatofocusing with Polybuffer and PBE. Amersham Pharmacia Biotech, Uppsala, Sweden, 1984; pp 25–30.

(36) Alexander, N. M.; Neeley, W. E. *J. Chromatogr. Biomed. Appl.* **1982**, *230*, 137–141.



gradient when these two methods are performed with similar separation times. This implies that the chromatofocusing method described here may be preferable to these alternatives, depending on the specific separation problem under consideration. For example, the chromatofocusing method described here is likely to be preferable to capillary isoelectric focusing when it is desired to perform a preparative-scale separation or to separate proteins based on their isoelectric points but where it is necessary to avoid the use of polyampholyte buffers. It may also be preferable to ion-exchange chromatography with an ionic strength gradient when it is desired to elute proteins at low ionic strength or when large feed samples need to be employed since for chromatofocusing the bandwidth is largely unaffected by the feed sample size due to the focusing effects that are present. For these reasons, the chromatofocusing method described in this study is likely to be a useful separation technique that is complementary to capillary isoelectric focusing and standard methods of ion-exchange chromatography for accomplishing the high-speed, high-resolution separation of proteins.

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#### LIST OF SYMBOLS

$b$	gradient curvature factor (—)
$c_{\text{buffer}}$	liquid-phase concentration of a buffering species (mol/L)
$C_{\text{H}^+}$	liquid-phase concentration of hydrogen ion (mol/L)
$C_{\text{S}}$	liquid-phase concentration of inert ion S (mol/L)
$F$	objective function, the sum of the square of the difference between neighboring slopes on the pH gradient (pH units <sup>2</sup> s <sup>-2</sup> )
$K_{\text{a}}$	acid–base dissociation constant for a monobasic species (mol/L)
$K_{\text{a1}}, K_{\text{a2}}$	acid–base dissociation constant for a dibasic species (mol/L)

$L$	column length (cm)
$\text{pH}_{\text{fluid}}$	pH in the liquid phase (pH units)
$\Delta\text{pH}$	baseline bandwidth (pH units)
$q_{\text{S}}^*$	equilibrium adsorbed-phase concentration of inert ion S based on the volume of solid material inside the particle (mol (L of solid material) <sup>-1</sup> )
$q_{\text{S,t}}^*$	equilibrium adsorbed-phase concentration of inert ion S based on the total volume of particle (mol (L of total particle) <sup>-1</sup> )
$R_{\text{S}}$	resolution as defined by eq 8 (—)
$S_i$	slope of the pH gradient in the $i$ th pH range (pH units/s)
$t(\text{pH})$	transit time for a pH value (s)
$t_{\text{R}}$	retention or separation time (s)
$v_{\text{fluid}}$	interstitial or linear velocity (cm/s)
$v_{\text{pH}}$	velocity of a pH value (cm/s)

#### Greek Symbols

$\alpha$	column void fraction (—)
$\beta_{\text{fluid}}$	buffering capacity of the liquid phase (mol (L $\times$ pH unit) <sup>-1</sup> )
$\beta_{\text{fluid,monobasic}}$	buffering capacity of a monobasic buffering species in the liquid phase (mol (L $\times$ pH unit) <sup>-1</sup> )
$\beta_{\text{fluid,dibasic}}$	buffering capacity of a dibasic buffering species in the liquid phase (mol (L $\times$ pH unit) <sup>-1</sup> )
$\beta_{\text{fluid,species } i}$	buffering capacity of buffering species $i$ in the liquid phase (mol (L $\times$ pH unit) <sup>-1</sup> )
$\beta'_{\text{ads}}$	buffering capacity of the adsorbed phase (mol (L of total particle $\times$ pH unit) <sup>-1</sup> )
$\epsilon$	particle porosity (—)
$\sigma_i$	standard deviation of a Gaussian band (s)

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