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Gradient chromatofocusing high-performance liquid chromatography II. Theoretical aspects

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Abstract

This article is Part II of a series describing a newly-developed gradient chromatofocusing high-performance liquid chromatography (HPLC) technique. Theoretical aspects of the technique are discussed. In gradient chromatofocusing, the column pH gradient with respect to column distance can be varied without necessarily affecting the outlet pH gradient with respect to time. Factors influencing the value of the slope of the column pH gradient are identified through derived equations and a computer simulation model. A newly-identified parameter is introduced, column travel time, which can be uniquely varied in gradient chromatofocusing. Experiments show increased conversion of fibrinogen to denatured forms with increased column travel time. Another unique aspect of gradient chromatofocusing is that the mobile phase buffer concentration can be manipulated without necessarily affecting the outlet pH gradient slope, giving the technique expanded versatility for optimizing the separation. In the present work, the $pI_{apparent}$ for fibrinogen is found to increase with increased mobile phase buffer concentration.

Keywords: Chromatofocusing; Gradient elution; pH gradients; Mobile phase composition; Fibrinogen

1. Introduction

A newly-developed gradient chromatofocusing HPLC technique is described in Part I [1]. This technique greatly extends the operational capabilities of conventional chromatofocusing; producing linear pH gradients with common buffer components, and offering flexible control of pH gradient slope through manipulation of an external gradient. Part I [1] gives practical examples of mobile phase conditions used to generate particular linear pH gradients exiting from a weak anion-exchange HPLC column (outlet pH gradient). This outlet pH gradient results from the superimposition of an external pH gradient (inlet pH gradient) onto a column which has a pH gradient distributed throughout its length (column pH gradient). The column pH gradient results from an internally-generated mechanism, based on the buffering action that the weak anion-exchange column exerts on any pH change brought upon it by the mobile phase and vice versa [2]. It is important to note that the column pH gradient is a gradient with respect to column distance, while the outlet pH gradient is a gradient with respect to time. These more intricate details of gradient chromatofocusing are the subject of the present article, which covers several theoretical aspects of gradient chromatofocusing.

A new chromatographic parameter is identified from the theory developed in this work, the travel time of a protein in the column. The column travel time in conventional or gradient chromatofocusing equals, to a first approximation, the time it takes for the $pH=pI_{protein}$ band to travel through the column.

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It is presumed that the protein travels within this band when it moves down the column, since the protein has a net zero charge at this pH. It is duly noted, however, that anomalous behavior has been widely reported for proteins on ion exchangers and in chromatofocusing, including retention on ion exchangers of proteins at their p*I* values [3,4] and elution of proteins at pHs different than their p*I* values in chromatofocusing [5,6]. The effect of the column travel time on the gradient chromatofocusing of fibrinogen is studied in the present work.

The effect of the mobile phase buffer concentration on the apparent p*I* is also reported.

2. Experimental

Experimental conditions were the same as specified in Part I [1].

3. Results and discussion

3.1. Model for gradient chromatofocusing

3.1.1. Description of model

Gradient chromatofocusing can be depicted by modifying a model employed for conventional chromatofocusing, which calculates the pH as a function of column distance and time [2]. The process of conventional chromatofocusing (described here for a weak anion-exchange column) involves the equilibration of the ion-exchange column with a high pH buffer, followed by a step change to a low pH buffer after the injection of the sample. The model for conventional chromatofocusing calculates how the pH changes within the column with respect to time and distance by segmenting the column into a specified number of sections. The pH is then calculated for each particular section after the shifting of mobile phase from the adjoining upstream section of the column, as specified by Eq. (1):

$$\mathrm{pH} = \frac{\mathrm{pH_m} + R_c \mathrm{pH_s}}{1 + R_c} \quad (1)$$

where $\mathrm{pH_m}$ is the pH of the mobile phase in the upstream section of the column and $\mathrm{pH_s}$ is the pH of the stationary phase in the downstream section of the column, both being the equilibrium pH in each section prior to the shift of the mobile phase. R_c is the ratio of buffer capacities per unit column length of the stationary phase over the mobile phase. This model assumes that equilibrium is attained after shifting each mobile phase section into adjoining stationary phase sections, with the equilibrium pH in each column section resulting from the mutual titration of the mobile and stationary phases. The mobile and stationary phases within a particular column section assume the same pH at equilibrium, as calculated by Eq. (1).

The same model, with a slight modification, can be employed for gradient chromatofocusing. The only difference in the models is the pH of the inlet mobile phase steadily changes with time in the gradient chromatofocusing model, in contrast to the conventional chromatofocusing model, where the pH of the inlet mobile phase does not change with time after the initial step change to a lower pH buffer.

3.1.2. Results from computer simulation studies

Fig. 1a gives the results for a computer simulation of the above described model for gradient chromatofocusing. Fig. 1a shows the complete pH profile within the column as a function of distance and time, illustrating the two types of gradients present within gradient chromatofocusing. The pH gradient with time (arrow 1) shows a surface sloping downward as the time increases. Of interest are the two dimensional plots at the column's outlet (5 cm) and inlet (0 cm), which are the outlet and inlet pH gradients, respectively. The pH gradient with distance (arrow 2) shows a surface sloping upward as the column distance increases. A two dimensional plot results when any particular time is selected (parallel to arrow 2), which gives the column pH gradient for that particular time. Fig. 1a underscores the counteracting nature of the two gradients. The slope of the pH gradient with respect to time at any particular column location is negative, while the slope of the column pH gradient at any particular time is positive.

Fig. 1b gives a better perspective for visualizing the magnitude of the column pH gradient, with the thickness (i.e., vertical height) of the plot at a particular time equalling the pH difference between the column's outlet and inlet. The steepness of the

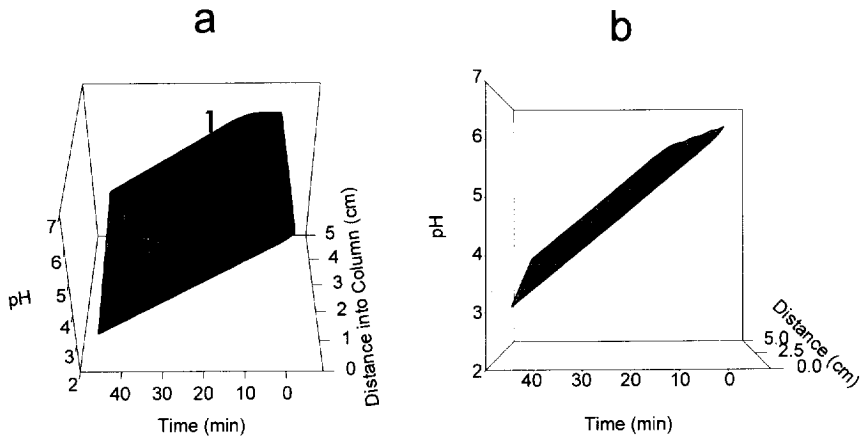

Fig. 1. (a) Three dimensional plot of pH with respect to time and column distance (from inlet) for the gradient chromatofocusing technique, viewed from a 40.7° vertical perspective. Plot illustrates two distinct pH gradients; the pH gradient with time, which has a negative slope (arrow 1), and the pH gradient with distance, which has a positive slope (arrow 2). (b) Same plot as (a), viewed from a 0.0° vertical perspective. This view allows for ready visualization of the magnitude of the column pH gradient slope. The slope of the column pH gradient equals the vertical height of the plot in (b) (which is the pH difference between the outlet and inlet at a particular t) divided by the column length. This calculation assumes that there is a linear distribution of pH in the column. Plots were generated by a computer simulation of the model for gradient chromatofocusing, as described in the text. Parameters used for the simulation are the experimental conditions used for Fig. 3b of Part I [1], being: equilibration pH, 6.3; pH window, 0.2 pH units; inlet pH gradient slope, 0.069 pH/min; column length, 5.0 cm; column dead volume, 0.27 ml; flow-rate, 0.6 ml/min; and number of segments that column is divided into, 10. A value of 25 was used for R_c, which was determined to produce the approximate difference of the outlet and inlet pH gradients noted in Fig. 3b of Part I [1].

column pH gradient slope is proportional to this pH difference. Refer to the caption of Fig. 1b for how the slope of the column pH gradient is calculated. It is possible not to have any column pH gradient component, as would be expected for runs using a strong ion-exchange column. In this case, the pH gradient surface in Fig. 1a would still have a negative slope with respect to time, but would be flat with respect to distance. From the perspective of Fig. 1b, the plot would have no thickness (vertical height).

3.2. Column pH gradient

The effect of the column pH gradient in chromatofocusing has not been investigated previously. This is because the column pH gradient cannot be varied in conventional chromatofocusing without changing the outlet pH gradient, which means it is not possible to isolate effects due to the column pH gradient alone. Gradient chromatofocusing offers the unique capability of being able to vary the column pH gradient without necessarily changing the outlet pH gradient, thus giving better capability for studying the column pH gradient parameter. An expression is derived below incorporating factors that affect the magnitude of the slope of the column pH gradient.

The buffer capacity of the mobile phase (β_M) is defined as [7]:

$$\beta_M = \frac{dB}{d\,pH} \tag{2}$$

where dpH is the change in pH of a section of mobile phase induced by an increment of base (dB) added to that section of mobile phase. The parameter dB is proportional to the axial distance in the column (dx) through which the mobile phase proceeds (i.e., $dB \propto dx$). The greater the column distance that the mobile phase traverses, the greater the number of hydrogen ions taken up from the mobile phase by the column, which means more base is added into the mobile phase. Therefore,

$$dB = k(x,t)dx \tag{3}$$

where $k(x,t)$ is a proportionality factor and has units

of moles per unit distance. The factor $k(x,t)$ accounts for parameters that affect the ability of the stationary phase to titrate the mobile phase, including; the buffer capacity of stationary phase, the difference in pH between adjoining sections of the mobile and stationary phases prior to the mobile phase shift, and kinetic factors (flow-rate, diffusion, etc.). The-variables x and t are the column distance (from inlet) and time, respectively.

Eq. (4) is derived from Eqs. (2) and (3):

$$\rho_c(x,t) = \frac{k(x,t)}{\beta_M} \tag{4}$$

where $\rho_c(x,t) = \mathrm{d\ pH}/\mathrm{d}x$ is the slope of the column pH gradient. One conclusion of Eq. (4) is borne out in Fig. 3 of Part I [1], which shows a larger slope for the column pH gradient (indicated by a larger separation of the linear portions of the outlet and inlet plots) when a mobile phase of lower buffer concentration is used.

The computer simulation studies are instructive in clarifying factors in the $k(x,t)$ term of Eq. (4), which ultimately affect the value of ρ_c. Three factors have been identified. The following lead to an increase in ρ_c: an increased value for R_c (see Eq. (1)), an increased value for the pH window (the difference in pH between the equilibrated column [prior to the initiation of the external gradient] and the starting pH of the inlet pH gradient), and an increased ratio of the inlet pH gradient slope over the flow-rate.

The effect of the column pH gradient on the gradient chromatofocusing of fibrinogen is presented below. The column pH gradient has been identified as being pertinent to the ability of chromatofocusing to focus proteins into concentrated bands on the column [2]. However, the column pH gradient also affects another fundamental aspect of the chromatofocusing process, the column travel time. This parameter is newly identified in the present work and is discussed below.

3.3. Column travel time

An equation can be derived relating the slopes of the two pH gradients (outlet and column) to the travel time for a particular pH section to move from the inlet to the outlet of an anion-exchange column. This is presumably the time it takes for a protein to proceed through the column, once it is desorbed from the beginning of the column at the $\mathrm{pH} \approx \mathrm{p}I_{\mathrm{protein}}$ portion of the gradient. Equation parameters are explained below. The parameter pH denotes the pH value of a section of mobile phase at x, while pH + d pH denotes the pH of the section of mobile phase at $x + \mathrm{d}x$, all at a particular t. With continuous flow of mobile phase through the column, the interactions between mobile and stationary phases will change the pH value of the column section at $x + \mathrm{d}x$, from pH + d pH to pH during an interval of time ($\mathrm{d}t$). Thus, $\mathrm{d}t$ denotes the time required for a shift of the section at pH = pH from x to $x + \mathrm{d}x$.

The value of the slope of the column pH gradient (ρ_c) between x and $x + \mathrm{d}x$ is:

$$\rho_c(x,t) = \frac{\mathrm{d\ pH}}{\mathrm{d}x} \tag{5}$$

while the change of pH with change of time (ρ_o) is:

$$\rho_o(x,t) = -\frac{\mathrm{d\ pH}}{\mathrm{d}t} \tag{6}$$

The negative sign in Eq. (6) and in the equations following indicates that the pH at any particular x decreases as t increases (for an anion-exchange column).

The shifting time ($\mathrm{d}t$) of a particular pH through a unit distance ($\mathrm{d}x$) can be calculated from a combination of Eqs. (5) and (6), as given by Eq. (7):

$$\frac{\mathrm{d}t}{\mathrm{d}x} = -\frac{\rho_c(x,t)}{\rho_o(x,t)} \tag{7}$$

The travel time of a section of mobile phase with a particular pH through a particular portion of the column can be calculated by Eq. (8):

$$\int \mathrm{d}t = -\int \frac{\rho_c(x,t)}{\rho_o(x,t)} \mathrm{d}x \tag{8}$$

Eq. (8) is a general expression which is applicable regardless of the function of either gradient.

For the case of linear and parallel inlet and outlet pH gradients (see Fig. 3 in Part I [1], after the plateau region), ρ_c and ρ_o are assumed to be both constant. In this case, Eq. (8) can be simplified to:

$$\int \mathrm{d}t = -\frac{\rho_c}{\rho_o} \int \mathrm{d}x \tag{9}$$

Thus, the travel time (T_t) of a section of mobile phase with a particular pH through the entire column length (l) can be calculated by:

$$T_t = -\frac{\rho_c}{\rho_o} l \quad (10)$$

The column travel time for a particular pH value is graphically shown in Fig. 1b, being the horizontal length of the plot for a particular pH value, from the column inlet boundary (lower limit of the plot) to the column outlet boundary (upper limit of the plot).

Eq. (10) also calculates the travel time for a protein in the column, which is presumed to travel in one particular pH band as it traverses the column. It should be noted that the column travel time is different from the retention time, as it does not include the time that the analyte dwells on the ion exchanger prior to its initial desorption. In Eq. (10), ρ_o is calculated from the slope of the outlet pH gradient plot (dashed line after the plateau region in Figs. 2 and 3), and ρ_c is calculated as described in the caption for Fig. 1b. It is apparent from Eq. (10) that conditions resulting in the same outlet pH gradient can yield different column travel times if the values for ρ_c differ.

3.4. Effect of ρ_c on the gradient chromatofocusing of fibrinogen

The effect of ρ_c on the gradient chromatofocusing of fibrinogen is seen by comparing Figs. 2 and 3, which show different chromatograms for different values of ρ_c (slopes of the outlet pH gradients are similar). Fig. 2 shows the chromatogram resulting from the lower ρ_c run, consisting of an early eluting peak (22 min) and three later eluting peaks. The former peak has been identified as the native form of fibrinogen, while the latter three peaks are attributed to denatured or degraded fibrinogen forms [8]. The chromatographic pattern changes with the higher ρ_c run in Fig. 3, where there is a noticeable shift of peak area from the early eluting native peak to the later eluting denatured/degraded peak(s). The effect of the column pH gradient on the column travel time is the most likely explanation for the results obtained. These results suggest that fibrinogen denatures with an extended travel time in the column (11

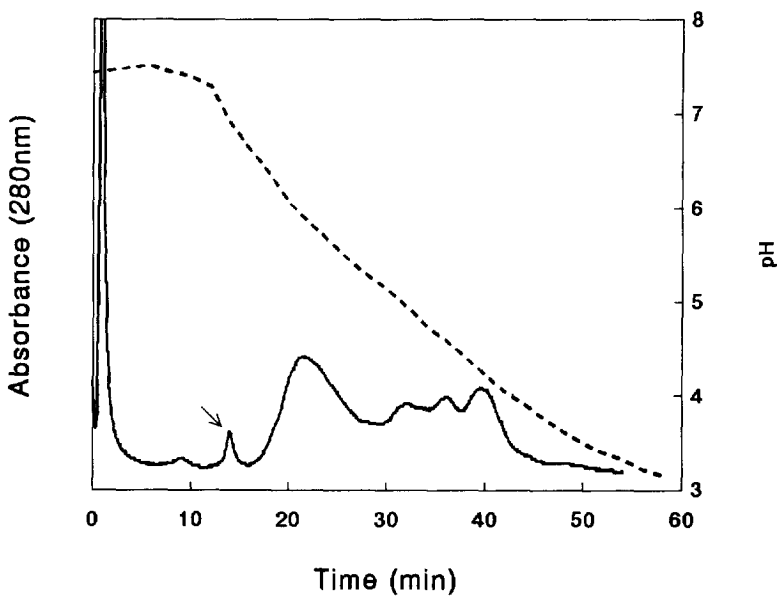

Fig. 2. Chromatogram of 200 μg fibrinogen using gradient chromatofocusing. The buffers and method of generation of the pH gradient were the same as described in Fig. 7 in Part I [1]. The outlet pH gradient is shown by the dashed line. The travel time is calculated from Eq. (10) to be 5.9 min. The chromatographic blank showed a small buffer peak at the arrow. AUFS is 0.05 AU.

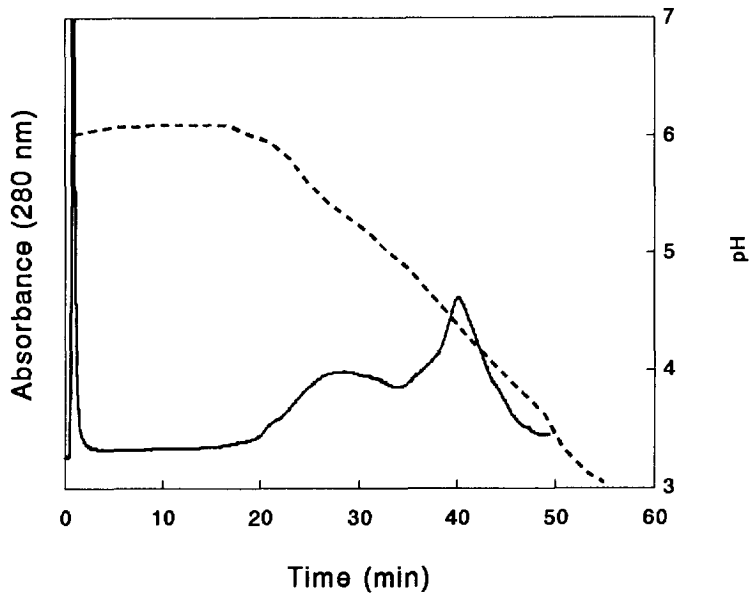

Fig. 3. Chromatogram of 200 μg fibrinogen with an extended travel time (11 min as calculated by Eq. (10)) compared to Fig. 2. The buffers and method of generation of the pH gradient were the same as described in Fig. 3b in Part I [1]. The outlet pH gradient is shown by the dashed line. AUFS is 0.075 AU.

min in Fig. 3 compared to 5.9 min in Fig. 2). Further study is warranted concerning these effects.

3.5. Effect of mobile phase buffer concentration

Another issue of conventional chromatofocusing, which is also an issue in the present work, is the apparent p*I*. The value for the apparent p*I* of a protein (being the pH at which the protein elutes from the column) can either be lower than the true p*I* due to a Donnan potential effect [2,5], or higher than the true p*I* due to a displacement effect by competing ions [6], as determined by conventional chromatofocusing on an anion-exchange column.

The influence of mobile phase buffer concentration on the apparent p*I* of proteins was investigated in the present work. This was done by comparing the results obtained for three different buffer systems. Each buffer system included buffer A (consisting of piperazine–HCl, pH 6.4) and buffer B (consisting of acetic, lactic and chloroacetic acids). Buffer component concentrations for buffer A and buffer B, respectively, were 10 m*M* and 5 m*M* (each) for buffer system 1, 25 m*M* and 12.5 m*M* (each) for buffer system 2, and 40 m*M* and 25 m*M* (each) for buffer system 3. Thus, the mobile phase buffer concentration increases in going from buffer system 1 to 2 to 3.

The outlet pH gradient was produced to give the same slope (0.09±0.01 pH/min) for each buffer system by choosing an appropriate rate of external mixing. The apparent p*I* values for the major peak of fibrinogen were 5.31, 5.54, and 5.87 for buffer system 1, 2, and 3, respectively. The apparent p*I* with buffer system 3 was close to the reported p*I* value (5.85) for fibrinogen [9].

It may be argued that an increase in the buffer concentration of the mobile phase would be accompanied by an increase in ionic strength of the mobile phase, which could explain the trend of higher apparent p*I* values through an anion displacement mechanism. Another experiment was done, to determine if this was the cause for the higher apparent p*I* values at higher mobile phase buffer concentrations. The results for the gradient chromatofocusing of fibrinogen using buffer system 2 with added NaCl (concentrations of 20 m*M* and 45 m*M* for buffers A and B respectively) are given in Fig. 4.

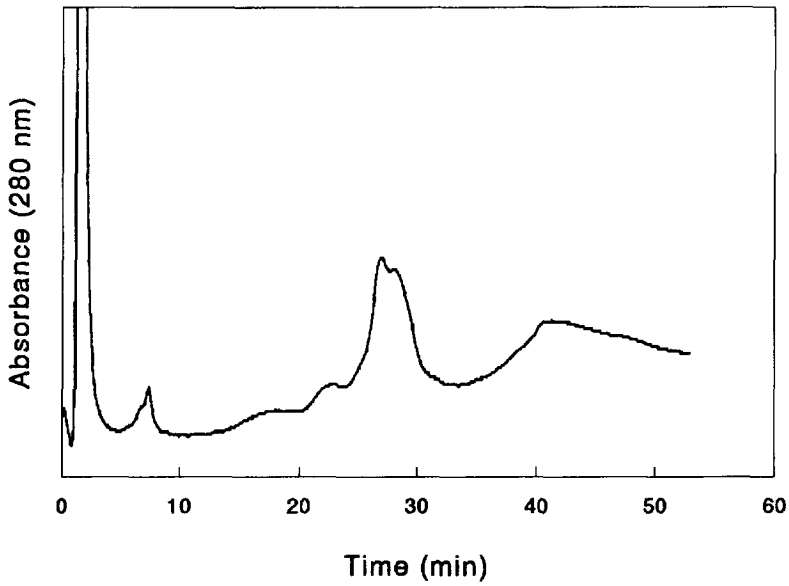

Fig. 4. The effect of NaCl on the gradient chromatofocusing of fibrinogen (200 μg). The column was pre-equilibrated with buffer A: 20 m*M* NaCl and 25 m*M* piperazine–HCl, pH 6.4. Buffer B consisted of acetic, lactic and chloroacetic acids, 12.5 m*M* each, and 45 m*M* NaCl. The external gradient was 10% B to 15% over 5 min, then 15% to 75% over 30 min, and finally 75% to 80% B over 20 min. The flow-rate was 0.6 ml/min. Refer to Fig. 4a (lower plot) in Part I [1] for an estimation of the first 35 min of the outlet pH gradient. AUFS is 0.05 AU.

The chromatographic profile was different in comparing Fig. 4 with Fig. 2, with two additional small peaks being resolved before the first major peak in Fig. 4. However, all peaks eluted at pHs below 5.5, and thus the increase of the apparent p*I* of fibrinogen that occurs with increased buffer concentration is not likely explained by a displacing effect.

Other considerations could affect the apparent p*I* of a protein during the chromatographic process, which inevitably complicate the interpretation of the experimental results. Leavis and Rothstein [10] studied the solubilities of fibrinogen as a function of pH, ionic strength, and the type of anionic species. Their results revealed that the p*I* of fibrinogen shifts towards a lower value with increasing ionic strength, the extent of this shift being a function of the specific anion. These results were obtained by experiments at equilibrium conditions and thus reflect possible thermodynamic effects on p*I*. Kinetic effects are also anticipated, owing to the nature of the chromatographic process. The rate of protonation of a protein may be a critical factor in its desorption from the ion-exchanger using gradient pH elution, thus affecting the apparent p*I* value.

4. Conclusions

In Part I [1], the advantages of gradient chromatofocusing over conventional chromatofocusing are presented. Conditions and composition of the mobile phase are specified that produce linear outlet pH gradients. It is also demonstrated that the slope of the outlet pH gradient could be readily manipulated through external gradient control. In Part II, theoretical aspects involving the column pH gradient and the effect of mobile phase buffer concentration on elution are discussed. Factors affecting the column pH gradient are identified through theory and computer modeling.

Unique capabilities of gradient chromatofocusing in studying fundamental aspects of the chromatographic process are identified in Part II. The parameter of column travel time is introduced, with an expression being derived showing its dependence on the values for the slope of the column and the outlet pH gradients. An increase in column travel time is thought to be the most likely explanation for a noted shift in the chromatographic profile from native to denatured protein peaks with an increase in ρ_c, as

demonstrated in the gradient chromatofocusing of fibrinogen.

Also addressed in Part II is the effect of the mobile phase buffer concentration on protein retention. Gradient chromatofocusing has unique capabilities in investigating and utilizing this parameter to achieve optimization of chromatography. Unlike conventional chromatofocusing, gradient chromatofocusing has the ability to vary mobile phase buffer concentration while keeping the slope of the outlet pH gradient constant. Results for fibrinogen show that its apparent p*I* increases with increased concentration of buffer in the mobile phase, at least for the range of buffer concentrations used in this work.

Acknowledgments

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