CAPILLARY ISOELECTRIC FOCUSING OF

PROTEINS WITH CARRIER AMPHOLYTE pH

GRADIENT AND IMMOBILIZED pH GRADIENT

By

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LIST OF SYMBOLS AND ABBREVIATIONS

α selectivity factor

$\Delta\mu_{ep}$ difference in electrophoretic mobilities of two adjacent zones

η viscosity

μ electrophoretic mobility

μ_{app} apparent mobility

μ_{eo} electroosmotic mobilitu

μ_{ep} electrophoretic mobility

ν migration velocity

σ_l standard deviation of the peak in unit length

σ_L^2 peak Variance

σ_t standard deviation of the peak in unit time

a radius of a spherical particle

D diffusion coefficient

E electric field strength

F_e electric force

F_f frictional force

H plate height

L total length of capillary

l	effective length
N	efficiency
q	charge of ions
R_s	resolution
t	migration time of a peak
t_0	migration time of a neutral marker
t_M	migration time of analyte
V	applied voltage
w_b	peak width at the base
w_h	peak width at the half-height
w_i	peak width at the inflection point
2D	two dimension
BF$_3$	boron trifluoride etherate
CE	capillary electrophoresis
GC	gas chromatography
MS	mass spectrometry
pI	isoelectric point
ACN	acetonitrile
AEG	polyacryloylaminoethoxy-ethyl-β-D-glycopyranoside
CGE	capillary gel electrophoresis
CZE	capillary zone electrophoresis
DMF	*N, N*-dimethylformamide
EOF	electroosmotic flow

ESI	eletrospray ionization
GMA	glycidyl methacrylate
HPC	hydroxypropyl cellulose
IpG	immobilized pH gradient
LIF	laser-induced fluorescence
PAA	polyacrylamide
PEG	polyethylene glycol 600
RPC	reverse-phased liquid chromatography
RSD	relative standard deviation
AAEE	acryloylaminoethoxyethanol
AIBN	2,2'-azobis(isobutyronitrile)
CIEF	capillary isoelectric focusing
CITP	capillary isotachophoresis
EDMA	ethylene glycol dimethacrylate
HPLC	high performance liquid chromatography
HPMC	hydroxypropylmethylcellulose
MAPS	γ-methacryloxypropyl-trimethoxysilane
MECC	micellar electrokinetic capillary chromatography
PDMA	polydimethylacrylamide
CHAPS	3-(3-cholamidopropyl)-dimethylammonio-1-propane sulfonate
GPTMS	γ - glycidoxypropyl trimethoxysilane
HDSPA	hexadecyldimethyl(3-sulfopropyl)ammonium hydroxide
TEMED	*N,N,N',N'*-tetramethylethylenediamine

CHAPTER I

BACKGROUND AND RATIONALE OF THE STUDY

Introduction

Isoelectric focusing (IEF) is an electrophoretic technique based on differences in isoelectric points (pI) of amphoteric solutes (e.g., proteins and peptides). It was first intercepted in 1967 by Olof Vesterberg and Torkel Wadstrom [1-6]. With the development of capillary electrophoresis (CE) as the instrumental version of the traditional slab gel electrophoresis, IEF was successfully transferred to the capillary format by Hjerten in 1985 [7]. Capillary isoelectric focusing (CIEF) combines the high resolving power of conventional gel isoelectric focusing with the advantages of CE instrumentation. As an important mode of CE, CIEF has been widely applied to the analysis of proteins and peptides [8-16]. CIEF is usually carried out with carrier ampholytes and more recently with immobilized pH gradient (IpG). Our research efforts in this dissertation pertain to assess the usefulness of both approaches (i.e., carrier ampholytes and IpG CIEF) and to offer some remedies to the existing problems in each type of CIEF. Chapter 2 deals with CIEF with carrier ampholytes, where commercially available ampholytes were used to provide the pH gradient. In this part of the dissertation, different capillary coating methods were investigated in order to achieve good separations. Chapter 3 deals with CIEF with immobilized pH gradient on novel

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monolithic capillary columns which were introduced and evaluated with standard proteins of differing pI values.

This chapter discusses some basic principles of CIEF. Also, it describes the essential factors influencing resolution, selectivity, and efficiency in CE, including the origin of electroosmotic flow (EOF) and methods to reduce EOF since the presence of this bulk flow is not advantageous in CIEF.

Some Basic Principles of CIEF

Capillary Electrophoresis (CE)

Before describing some of the basic principles of CIEF, a short overview of CE principles is necessary. This is because CIEF is a mode of CE, and some analytical parameters defined in CE can be applied to CIEF too.

Instrumentation: One of the important features of CE is the simplicity of its instrumentation. A schematic illustration of an in-house assembled CE instrument similar to the one used in our studies is shown in Figure 1. It consists of a few components, including (i) a high voltage power supply capable of delivering up to ± 30 kV, (ii) two electrolyte micro-reservoirs, (iii) a fused-silica capillary filled with the running electrolyte, (iv) an on-column detector (UV-Vis detector was used in our studies), (v) a data storage and processing device (e.g., a computing integrator), and (vi) a plexiglass safety box to protect the operator from high voltages.

Different CE modes: Capillary electrophoresis can be carried out in various modes of operation to achieve a given separation. The basic modes of CE are (i) capillary zone electrophoresis (CZE), (ii) capillary gel electrophoresis (CGE), (iii) micellar electrokinetic capillary chromatography (MECC), (iv) capillary isoelectric focusing (CIEF), and (v) capillary isotachophoresis (CITP).
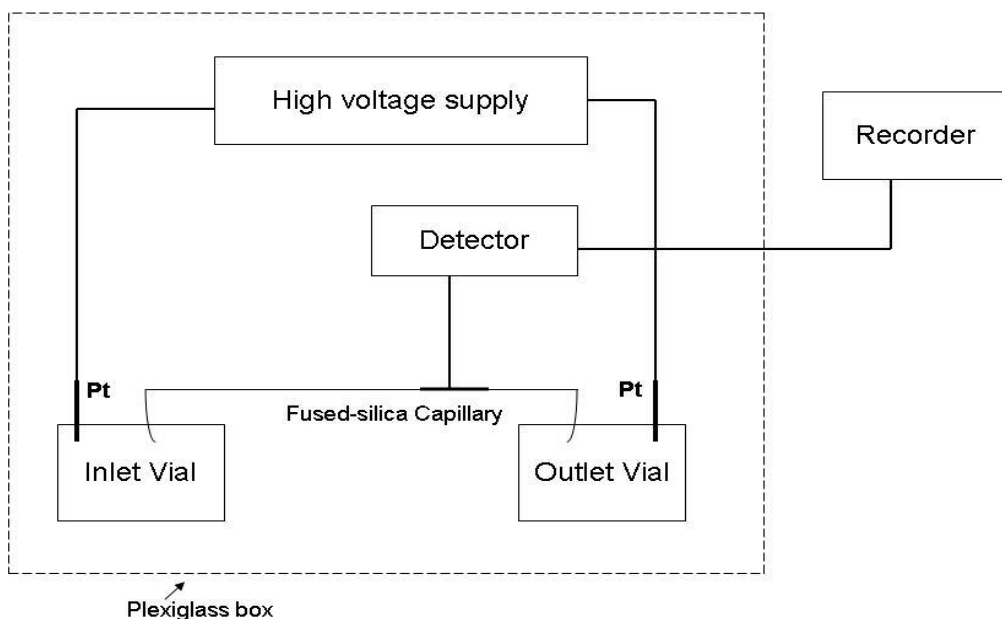
Figure 1. *A Schematic illustration of an instrument used in CE.*

Capillary zone electrophoresis (CZE) is the most commonly used mode of CE. The separation in CZE is based on the differences in the electrophoretic mobilities of the solutes. Separation of both anionic and cationic species is possible in the presence of EOF while neutral solutes migrate with EOF.

Capillary gel electrophoresis (CGE) has been mainly used for the separation of large molecular weight biopolymers, e.g., proteins and DNA. The main separation mechanism is based on differences in solute size as the charged solutes migrate through the pores of gel-filled capillary column.

Micellar electrokinetic capillary chromatography (MECC) is a modification of CZE to separate neutral solutes. The separation is based on the differential partitioning of the analytes into the micellar "pseudo-stationary" phase. Hydrophobic solutes undergo strong interaction with the micellar phase and are more retarded than the less hydrophobic ones.

In CIEF, solutes are separated on the basis of their isoelectric point (pI) values. A pH gradient is formed within the capillary using ampholytes with pI values that cover the desired pH range of the CIEF experiment. Ampholytic analytes (e.g., proteins) will migrate in the electric field until they reach the point in the pH gradient where the pH equals their pI. At this point, the solutes cease to move and they are focused. A mobilization step is then required to pass the focused analytes in front of a detector. This is usually achieved by pressure mobilization or salt mobilization.

Capillary isotachophoresis (CITP) uses two buffer systems in order to create a state in which the separated zones all move at the same velocity. The zones remain

sandwiched between the leading electrolyte (contains ions with higher mobility than the solute ions) and the terminating electrolyte (contains ions with lower mobility than the solutes).

Migration in CE

Electrophoretic Mobility: In all the above CE modes, the electrophoretic migration is an important parameter. When a constant electric field is applied across a capillary, ions in the capillary, whether positively or negatively charged, would experience electrical force F_e which is proportional to the electric field strength E and the charge q of the ion as follows

$$F_e = qE \qquad (1)$$

The electric field strength, on the other hand, is given by

$$E = \frac{V}{L} \qquad (2)$$

where V is the applied voltage and L is the total length of the capillary. This driving force causes migration of the ion toward the electrode of opposite sign. In its migration, the ion also experiences frictional forces which counteract the ion's movement. The frictional force F_f for a spherical particle with radius a is expressed by Stokes' law as

$$F_f = 6\pi\eta a v \qquad (3)$$

where η is the viscosity of the solution and v is the migration velocity of the ion. The migration velocity of an ion is further expressed as

$$v = \mu E \qquad (4)$$

where μ is the electrophoretic mobility, a characteristic property of the ion collectively affected by the medium and temperature.

When steady state is reached, the driving force F_e equals frictional force F_f. By combining the above equations, μ can be expressed as

$$\mu = \frac{q}{6\pi\eta a} = \frac{v}{E} \qquad (5)$$

the unit of μ is $cm^2V^{-1}s^{-1}$. It can be seen that electrophoretic mobility is controlled by the charge and size of the ion, and by the viscosity of the medium.

Electroosmotic Flow (EOF): Due to the ionization of the silanol group (SiOH) to the anionic form (SiO⁻), the surface of fused-silica capillaries possess an excess of negative charges at pH > 3.5. As a result, anionic species in the running electrolyte are repelled from the surface, while electrolyte counterions are attracted to the capillary wall. Ions next to the wall are immobile forming the compact region of the electric double layer. Due to thermal motion, some of the counterions leave the compact region and diffuse further from the wall to form the mobile region of the electric double layer. Figure 2 illustrates the electric double layer at the surface of the fused-silica capillary. When an electric field is imposed tangentially to the surface of the capillary, it causes hydrated counterions in the diffuse region to migrate toward the oppositely charge electrode and to drag solvent with them. This flow is known as the EOF and is the driving force for many modes of CE. However, the presence of EOF is not suitable for CIEF, a condition which requires the elimination/reduction of EOF.

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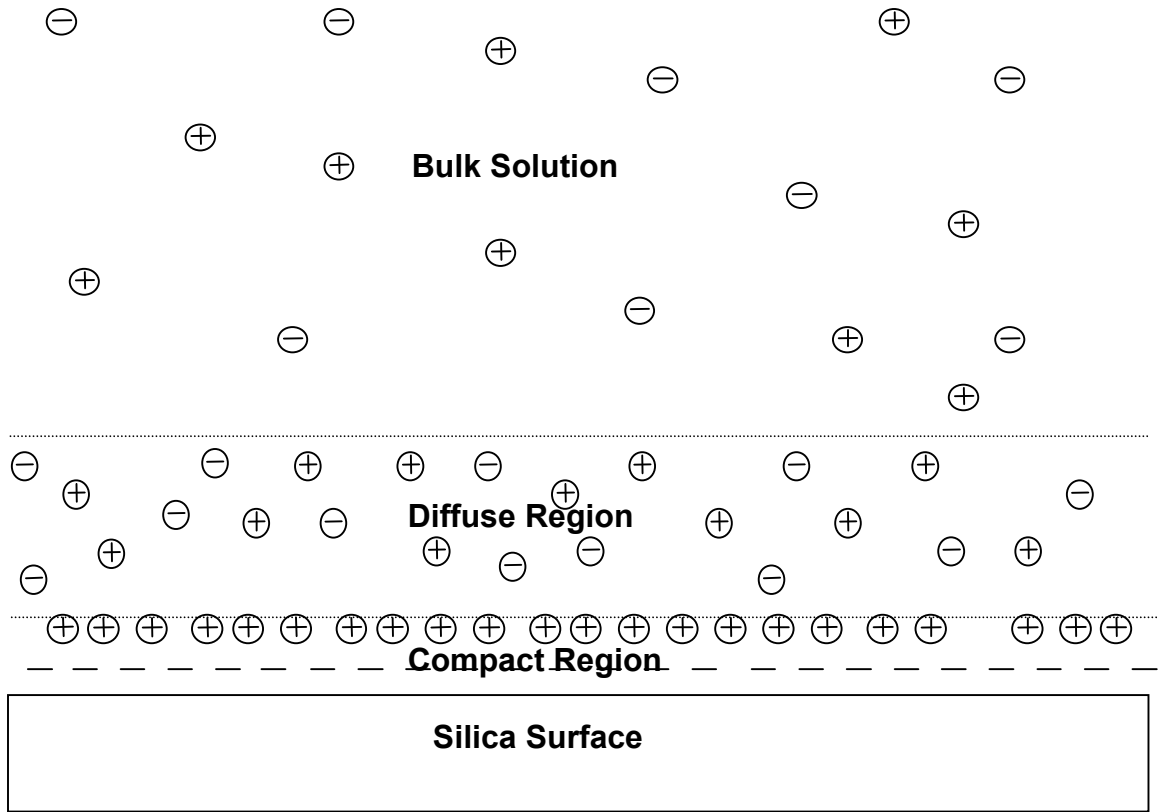
Figure 2. *Illustration of the electric double layer at the surface of the fused-silica capillary.*

Analytical Parameters

Migration Time and Apparent Mobility: Migration time refers to the elution time of a peak recorded by the instrument. The observed mobility of a charged analyte is the apparent mobility μ_{app} which is the summation of electrophoretic mobility μ_{ep} and electroosmotic mobility μ_{eo}, that is,

$$\mu_{app} = \mu_{ep} + \mu_{eo} \qquad (6)$$

Since the apparent mobility ν_{app} is given by

$$\nu_{app} = \frac{l}{t_M} \qquad (7)$$

where l is the length from inlet end to the detection point and it is also called the effective length, and t_M is the migration time of the analyte peak, the apparent electrophoretic mobility μ_{app} can be expressed as

$$\mu_{app} = \frac{v_{app}}{E} = \frac{lL}{t_M V} \qquad (8)$$

Upon rearranging eqn 8, the migration time in relation to μ_{app} can be expressed as

$$t_M = \frac{lL}{\mu_{app} V} \qquad (9)$$

The electroosmotic mobility μ_{eo} is determined by measuring the migration time t_0 of a neutral marker, such as acetone or dimethyl sulfoxide, and is expressed by:

$$\mu_{eo} = \frac{v}{E} = \frac{lL}{t_0 V} \qquad (10)$$

From eqns (6), (8) and (10), the electrophoretic mobility, μ_{ep}, of a certain analyte can therefore be expressed as

$$\mu_{ep} = \mu_{app} - \mu_{eo} = \frac{lL}{V}\left(\frac{1}{t_M} - \frac{1}{t_0}\right) \qquad (11)$$

Separation Efficiency: In CE, column efficiency is a measure of solute band broadening during its migration through the capillary. It is expressed by the plate number which is given by the following equation,

$$N = \left(\frac{l}{\sigma_L}\right)^2 \qquad (12)$$

where σ_L is the standard deviation of the peak in unit of length. Under ideal conditions, longitudinal molecular diffusion can be considered as the only contribution to standard deviation σ_L. According to Einstein's law of diffusion, equation (13) applies

$$\sigma^2_L = 2Dt_M = \frac{2DlL}{\mu_{app}V} \qquad (13)$$

where D is the diffusion coefficient of the analyte. By substituting the above equation into equation (12), column separation efficiency can be expressed as

$$N = \frac{\mu_{app}Vl^2}{2DlL} = \frac{\mu_{app}Vl}{2DL} \qquad (14)$$

From eqn (14), we can see that N increases with increasing voltage V, increasing μ_{app} and decreasing D. Therefore, in principle, large molecules with low values of D can yield higher N than small molecules. However, increasing the applied voltage is limited by Joule heating, which causes band broadening and reduces N.

As in chromatography, N can be calculate from the electropherogram by

$$N = 4\left(\frac{t_M}{w_i}\right)^2 = 5.54\left(\frac{t_M}{w_h}\right)^2 = 16\left(\frac{t_M}{w_b}\right)^2 \qquad (15)$$

where w_i, w_h, and w_b are the peak widths for a Gaussian peak at the inflection point, half height and base, respectively.

Selectivity Factor and Resolution: The selectivity factor α for two adjacent zones is given by

$$\alpha = \frac{\Delta\mu_{ep}}{\overline{\mu_{app}}} = \frac{\Delta\mu_{ep}}{\overline{\mu_{ep}} + \mu_{eo}} \qquad (16)$$

$\Delta\mu_{ep}$ is the difference in electrophoretic mobilities of 2 adjacent zones, $\overline{\mu_{app}}$ and $\overline{\mu_{ep}}$ are the average apparent mobilities and electrophoretic mobilities of 2 adjacent zones, respectively.

The resolution of 2 adjacent peaks is given by

$$R_s = \frac{\sqrt{N}}{4} \frac{\Delta\mu_{ep}}{\mu_{app}} = \frac{1}{4\sqrt{2}} \Delta\mu_{ep} \sqrt{\frac{Vl}{DL(\overline{\mu_{ep}} + \mu_{eo})}} \quad (17)$$

The above equation simplifies to

$$R_s = 0.177\Delta\mu_{ep} \sqrt{\frac{V}{D(\overline{\mu_{ep}} + \mu_{eo})}} \quad (18)$$

when L= l. This equation shows that increasing the applied voltage increases Rs but the square root tends to level off the effect and R_s is easily adjusted by manipulating $\Delta\mu_{ep}$. This is usually achieved by adjusting the pH and adding additives to the running electrolyte.

As in chromatography R_s is calculated from the electropherogram by:

$$R_s = \frac{t_2 - t_1}{2(\sigma_1 + \sigma_2)} \quad (19)$$

where σ_1 and σ_2 are the respective standard deviations of the two neighboring peaks.

Band Broadening Factors in CE: Band broadening in CE can arise from several contributing factors, which include initial zone width caused by sample injection, molecular diffusion, electrodispersson, Joule heating and solute adsorption to the capillary walls.   The observed band broadening expressed as the plate height H is given by

$$H = h_{inj} + h_{diff} + h_{cond} + h_{Joule} + h_{ads} \quad (20)$$

To minimize band broadening, initial sample zone must be kept as small as possible. Band broadening due to axial diffusion should be reduced by shortening analysis time, which can be done by using high voltage and short capillary.   Using capillaries with smaller inner diameter can reduce temperature and mobility gradient induced by Joule

heat across the tube radius. The use of buffer additives and chemical modification of the capillary wall can reduce the band broadening caused by wall adsorption.

CIEF Operational Aspects

Sample Preparation and Injection: Sample preparation includes selection of the appropriate ampholyte composition, adjustment of sample salt levels, and dilution or concentration of the sample to the proper protein levels required for detection [17]. The ionic strength of the sample should be as low as possible, preferably lower than 50 mM. Because of the concentration effect of CIEF, the higher the salt concentration the higher the risk of protein sample precipitation. The problem can be solved by desalting by dilution, dialysis, gel filtration or ultra-filtration. The ampholyte composition should be selected based upon the desired separation range. In situations where an enhanced resolution of proteins with similar pI values is desired, the use of narrow pH range ampholyte mixtures may be considered. The final protein concentration in the sample and ampholyte mixture will depend on sensitivity requirements and the solubility of protein components under focusing conditions. Usually, a final concentration of 0.5 mg/ml per protein can provide adequate sensitivity and satisfactory focusing and mobilization results. After preparation, the sample and ampholyte mixture is introduced into the capillary. This can be accomplished by pressure injection. For good quantitative precision, a sufficient amount should be loaded into the capillary to ensure that the tube contains a homogeneous mixture of sample.   Therefore, the capillary should be injected with at least 3-5 times the column volume of samples.

Focusing: After the analyte mixture dissolved in a dilute solution of the ampholytes is introduced into the column, the focusing step begins with the immersion of the capillary in the anolyte (a strong acid, such as phosphoric acid) and catholyte (a strong base, such as sodium hydroxide) solutions, followed by application of high voltage. Under the influence of an electric field, hydrogen ions begin to migrate from the anode reservoir toward the cathode reservoir. Hydroxide ions from the cathode begin to move in the opposite direction. If a component of the ampholyte or the analyte has a net negative charge, it migrates toward the positive anode. As it migrates it passes into continuously lower pH regions, where progressive protonation of the species occurs and lowers its negative charge. At last, it reaches the pH where its net charge is zero. This pH corresponds to the isoelectric point of the ampholyte. Migration of the species then stops. This process goes on for each ampholyte species and ultimately provides a continuous pH gradient throughout the capillary. The analyte ions also migrate until they reach their isoelectric points. These processes then result in the separation of each analyte into a narrow band that is located at the pH of its isoelectric points. The isoelectric focusing separations are based upon differences in equilibrium properties of the analytes (K_a, K_b) rather than on differences in rates of migration. Once each analyte has migrated to a region where its net charge is neutral, the positions of bands become constant and no longer change with time. Figure 3 shows the process of focusing.
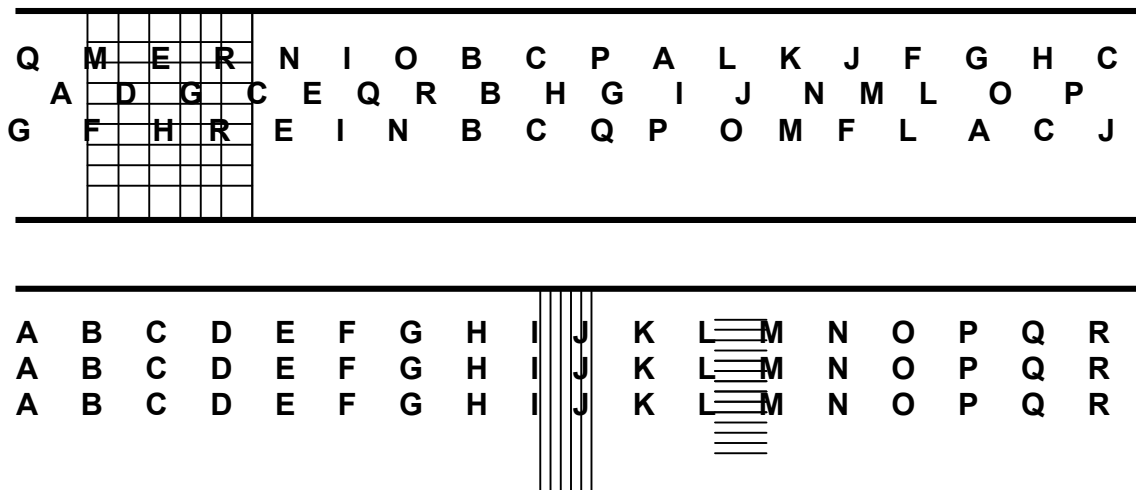
Figure 3. *Illustration of the focusing process. This illustration shows the distribution of ampholytes (A,B,C,D,E,F,G,H,I,J,K,L,M,N,O,P,Q,R and their pIs are from lowest to highest) and a two-component (one is expressed by "---- " and the other is expressed by "||||") sample at two different times: at the beginning of focusing (top) and after the focusing is finished (bottom).*

Optimization of focusing consists of determining the focusing time and field strength, which yield fully focused zones in a reasonable time. Incomplete focusing is evidenced by duplicate peaks for a single protein, which may be mistakenly assigned as a separate species. Increasing the focusing time after steady state has been achieved will increase the risk of precipitation. In practice, field strengths of 300-600 V/cm are adequate [18].

Mobilization: After focusing, the zones can be migrated or mobilized from the capillary by several approaches so that the sample zones pass through the detection point, and are detected. Mobilization can be accomplished in combination with focusing, a process that is described below (one-step CIEF), but it is more frequently performed as a separate step following focusing (two-step CIEF). Two techniques for mobilization in

two-step CIEF have been described, namely chemical mobilization and pressure mobilization.

Chemical or salt mobilization was introduced first [7]. It is carried out by changing the chemical composition of anolyte or catholyte, causing a shift in the pH gradient that results in electrophoretic migration of focused zones past the detection point. During the focusing step, equal number of H^+ and OH^- ions would enter the opposite ends of the capillary, so that the pH gradient would remain stable. The most common chemical mobilization method consists of adding a neutral salt such as sodium chloride to the anolyte or catholyte [7]. Sodium ions serve as the non-proton cation in anodic mobilization and chloride functions as the non-hydroxyl anion in cathodic mobilization. At the beginning of mobilization, current initially remains at the low value observed at the end of focusing, but gradually begins to rise as the chloride ions enter the capillary. Later in mobilization, when chloride is present throughout the column, a rapid rise in current shows the completion of mobilization. Ideally, mobilization should cause focused zones to maintain their relative position during migration. In practice, it will cause a pH change at the capillary end when ions move into the column. The rate of change depends on the amount of co-ion moving into the column, the mobility of the co-ion, and the buffering capacity of the carrier ampholytes [19]. The actual pH gradient changes across the capillary, becoming shallower in the direction opposite to mobilization. Neutral and basic proteins are efficiently mobilized towards the cathode with sodium chloride, and mobilization times correlate well with pIs [20]. However, acidic proteins are mobilized with lower efficiency and usually come out with zone broadening. Figure 4 illustrates the process of chemical mobilization.

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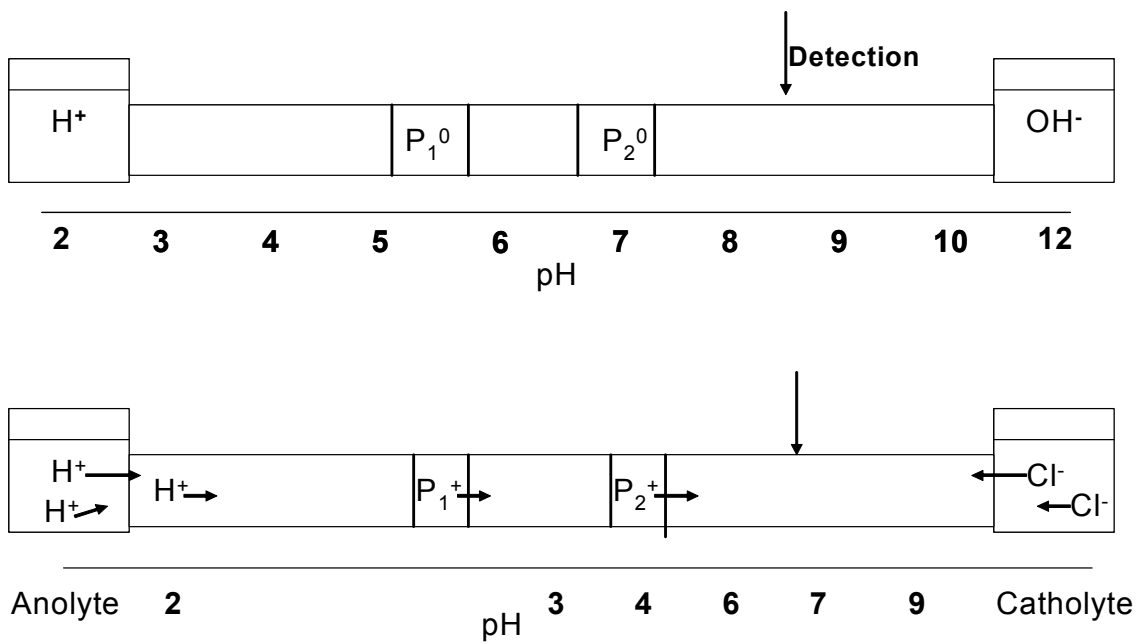
Figure 4. *Two-step CIEF with the chemical mobilization process. (The top one shows the situation when the focusing is finished and the bottom one shows the mobilization begins.)*

Conti *et al.* [21] reported that the use of high concentrations of NaCl solution in salt mobilization resulted in the deterioration of the capillary coating which may be due to the formation of strong alkaline boundaries. Neutral salts other than NaCl can be used for mobilization. A recent study [18] demonstrated that the use of sodium tetraborate improved the resolution of a series of hemoglobin variants, and that reduced salt concentration enhanced resolution at the expense of increased mobilization time.

Pressure mobilization utilizes positive pressure or negative pressure (i.e., vacuum) as the force that moves the focused proteins zones towards the detection point. During pressure mobilization, it is necessary to apply an electric field across the capillary in order to maintain focused protein zones. The main disadvantage of this type of mobilization is the parabolic shape of the hydrodynamic flow, which can cause band broadening and a decrease in resolution. To solve this problem, half of the electric force as used in the focusing step can be used. The main advantage is that pressure mobilization increases mobilization efficiency at the far end of the capillary, and it offers good linearity of migration time *vs.* pI plots (linear correlation coefficient of 0.9991 has been reported [22]) and reproducibility comparable to chemical mobilization [23]. Figure 5 illustrates the process of pressure mobilization.

This technique was first described by Hjerten and Zhu [7]. Since their pioneering studies, other forms of pressure have been used, e.g., compressed gas [22], pressure created by height difference of liquid levels contained in the reservoirs ("gravity mobilization"), and vacuum [24].
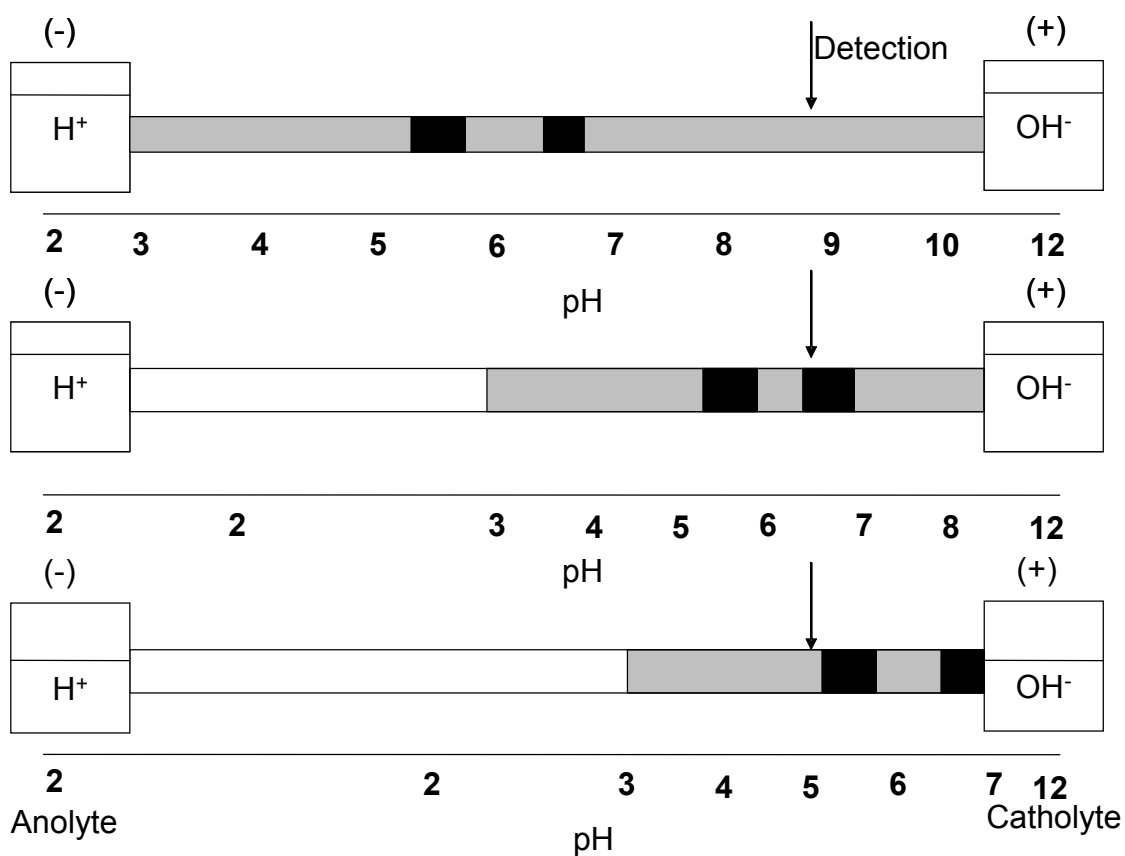
Figure 5. *Two-step CIEF with the pressure mobilization process. (The top one shows the situation when the focusing is finished, the two components in a sample mixture are focused and cease to move; the middle one shows the movement of the first focused sample zone passing the detection window under the influence of pressure; the bottom one shows the movement of the second focused sample zone passing the detection window under the influence of pressure. All of the three show the movement of the pH gradient along the capillary axis)*

One-step CIEF: CIEF with significant levels of EOF is a one-step process, with focusing and mobilization occurring at the same time. This technique has been used with both uncoated capillaries and with capillaries coated to reduce (but not eliminate) EOF [25]. After immersion of the capillary inlet in anolyte, high voltage is applied at field strengths of 300-600 V/cm, the pH gradient is formed, and proteins are focused as the sample is swept towards the detection point.

Thormann *et al.* [26] developed the procedure using hydroxypropylmethylcellulose (HPMC) coated columns. The addition of HPMC served to dynamically coat the fused silica wall, thereby reducing protein adsorption and EOF [19]. Mazzeo and Krull improved the technique by filling the entire length of the column with ampholyte and sample mixture [27, 28]. Improved mobilization of acidic proteins was achieved using commercial C8-coated capillaries in which the EOF varied less with pH [28].

A comparative study of one-step CIEF with EOF mobilization, two-step CIEF with pressure mobilization, and two-step CIEF with chemical mobilization was performed by Schwer [29]. All three techniques provided satisfactory reproducibility, particularly when using internal protein standards. One-step CIEF provided the shortest analysis times, but a correlation of pI with migration time was linear only over a narrow range due to variations in EOF caused by pH shifts during the analysis. Pressure mobilization provided good linearity of pI vs. migration time but required high field strengths during mobilization to counter loss of resolution due to laminar-flow band

broadening. Chemical mobilization exhibited the highest resolution of the three techniques at the expense of longer analysis time.

CIEF Theoretical Foundations

Since CIEF has some particular operational aspects which are different from those of other modes of CE, it is obvious that CIEF has also some particular theoretical foundations, which make CIEF a unique approach for electrokinetic separations. Thus, this section is devoted to describing the theoretical foundations and the essential equations for CIEF.

Principle of Chemical Mobilization: Hjerten et $al.$ derived expressions which describe the theoretical basis of electrokinetic mobilization [7]. The electroneutrality condition at steady state during focusing is given by

$$C_{H^+} + \Sigma C_{NH_3^+} = C_{OH^-} + \Sigma C_{COO^-} \qquad (21)$$

where $C_H{}^+$, $\Sigma C_{NH3}{}^+$, $C_{OH}{}^-$ and $\Sigma C_{COO}{}^-$ are the concentrations in equivalents per liter of protons, hydroxyl ions, and positive and negative groups in the carrier ampholytes, respectively. Equation (21) is the charge balance equation derived from the rule of neutrality. Mobilization can be achieved by disturbing the charge equilibrium via adding a positive term to the left side of the equation, which then becomes:

$$C_{X^{n+}} + C_{H^+} + \Sigma C_{NH_3^+} = C_{OH^-} + \Sigma C_{COO^-} \qquad (22)$$

Where X^{n+} (n is the valency) represents a cation. This equation illustrates one method for achieving anodic mobilization, that is, by replacing the anolyte used for focusing with a cation which can enter the tube electrophoretically.

The analogous expression for cathodic mobilization becomes:

$$C_{H^+} + \Sigma C_{NH_3^+} = C_{OH^-} + \Sigma C_{COO^-} + C_{Y^{m-}} \quad (23)$$

where Y^{m-} is an anion.

When the voltage is kept constant during isoelectric focusing, the current decreases in the focusing step due to the increasing resistance of the generated pH gradient. During the electrophoretic mobilization, the change in current is negligible at the beginning, gradually increasing to the end of the experiment representing the entry of the mobilizing cation or anion into the whole capillary column [19].

CIEF Separation Equation: CIEF is described as the electrophoresis in a pH gradient formed between a cathode and an anode with the cathode at a higher pH than the anode. Due to the presence of amino acid residues, proteins have amphoteric properties and will be positively charged at pH values below their pIs and negatively charged at pH above their pIs. This condition favors the migration of individual proteins toward the region where the pH is equal to their pIs. Under the influence of the electrical force the pH gradient will be established by the carrier ampholytes, and the protein species migrate and focus at their pIs. Eventually, a steady state is established. From the factors that decide the widths of the protein zones and distance between the zones, Shen and Smith [19, 30] derived an equation for the resolution [expressed as ΔpI, equation (25)] of two similar proteins, based on the following assumptions: 1) straight and continuous pH

gradients, dpH/dx; 2) constant field strength, E; 3) the two different proteins have the same diffusion coefficient, D; 4) the electrophoretic mobility change with pH, dμ/dpH, is constant and the same for both proteins; and 5) two closely spaced proteins are considered separated when the position of their peak maxima differs by 3 standard deviation or more.

The minimum difference in isoelectric points for two proteins to be resolved is expressed as [30]

$$\Delta pI = -3 \left(\frac{D\left(\frac{dpH}{dx}\right)}{E\left(\frac{d\mu}{dpH}\right)} \right)^{\frac{1}{2}} \qquad (24)$$

In other words, the smaller the ΔpI, the better the separation and the higher the resolution. In equation (24), $D = \frac{RT}{\eta f}$, where η is viscosity of the medium, R is the gas constant, T is the absolute temperature and f is the frictional coefficient given by $f = 6\pi\eta r$ where r is the radius of the molecule.

From the above equations, it can be seen that by reducing the diffusion, D, the resolution will increase. With a given separation, the only way to accomplish this is to increase the viscosity of the medium. Increased viscosity will also affect the mobility (μ) of the proteins, which makes the separation longer and decrease the resolution by decreasing the dμ/dpH. The shallower the gradient pH, dpH/dx, the further apart will two proteins be and better separated. However, too shallow a gradient results in too long focusing. High field strength (E) will not only increase the resolution but will also speed

up the experiment. However, there is a limit as to how much E can be increased due to the overheating problems associated with the high voltage.

Overview of Progress in CIEF in Recent Years

Since the first experiments on CIEF performed by Hjerten and co-workers in the mid-1980s [17], several papers have appeared about the CIEF technique and its applications. Comprehensive reviews have been published by Hille *et al.* [31], Shen and Smith [32], Moini [33], Shimura [34], and Mukai et al. [35] which discuss the different aspects of CIEF and its coupling to other techniques, especially to mass spectrometry (MS). Other recent reviews by Kilar [36-38] and Rodriguez-Diaz *et al.* [18, 19, 25], have discussed the technical aspects of CIEF. Sheng and Pawliszyn introduced a whole-column imaging detection system which is made up of a whole-column optical absorption imaging detector with a camera to capture the image, including an imaging lens and a charge-coupled device sensor [39-44]. Shimura *et al.* introduced fluorescence-labeled peptide pI makers [45-47] and laser-induced fluorescence (LIF) detection received wide applications [48-53]. The combination of CIEF with MS detection is one of the major challenges for studying proteomics. It is the driving force for researchers to optimize CIEF. Here, we will briefly discuss the development of two aspects of CIEF which are closely related with our research. One is the capillary coating approaches to reduce EOF, and the other is to solve the problems caused by the presence of ampholytes in the sample mixture when CIEF is coupled with on-line detection techniques.

To obtain a good resolution in CIEF, it is essential that EOF is reduced to a very low level to prevent the sample zones from moving out of the capillary before they are focused. Both covalent and dynamic coatings have been used for CIEF, but covalent coatings offer the advantages of enhanced stability and prevent the focused zones from contamination by the coating additive, which can also interfere with zone detectability.

A viscous polymeric coating is ideal for the reduction of the magnitude of EOF, while the use of a neutral, hydrophilic coating material reduces protein-capillary wall interactions. Although the use of neutral, hydrophobic polymers, anionic and cationic polymers (e.g., 2-acrylamido-2-methylpropane sulphonate, polyethyleneimine) have been reported [54, 55] to reduce EOF, they displayed a variety of limitations (e.g., hydrolysis, poor pH stability) when the coated capillary columns were applied to CIEF. The most commonly used coating procedure has been described by Hjerten *et al.* [7, 17]. In this procedure, a bifunctional silane such as γ-methacryloxypropyl-trimethoxysilane (MAPS) is reacted with the silanol groups on the inner walls of the capillary. After covalent attachment of this reagent, the acryl group is reacted with acrylamide in the presence of TEMED and ammonium persulfate to form a monolayer coating of linear polyacrylamide covalently attached to the inner capillary surface [7]. Figure 6 displays the different reaction steps of this coating. Capillaries coated with this type of procedure exhibit a reduction in both EOF and protein adsorption to the capillary surface. Based on this method, a number of acrylmide derivatives [e.g. polydimethylacrylamide (PDMA), acryloylaminoethoxyethanol (AAEE), polyacryloylaminoethoxy-ethyl-β-D-glycopyranoside (AEG)], have also been evalutated [56, 57]. Chiari *et al.* [58-61] presented a systematic investigation of the hydrolytic stability of AAEE coating.

Usually, the coatings must be easy to prepare, and should offer (i) a high reproducibility from run-to-run and day-to-day, (ii) good stability, and (iii) high separation efficiency over a wide pH range. A number of research reports have been published to meet those criteria, but it is not easy to meet all of them at the same time.

Huang and Richards [62] used a commercially available capillary coated with a neutral polymer (polyacrylamide) and showed consecutive runs of several proteins with pI values 2.75-9.45 with good migration time reproducibility (< 2% RSD for 10 consecutive replicates). The neutral, hydrophilic coating inside the capillary wall consisted of covalently bonded layers, which followed the coating procedure of Hjerten and Zhu [7]. They found that this coating was successful in reducing protein adsorption and EOF but had a life time of only a few days and exhibited separation efficiencies far below than that predicted by CIEF separation theory and equation as described above.

Tang *et al.* [63] reported the use of hydroxypropyl cellulose-coated capillaries for routine CIEF of recombinant immunoglobulins. They showed an RSD in peak area < 2 % intraday and < 8 % interday. The RSD for mobilization times of the various IgG peaks was < 1 % intraday and < 3 % interday. However, the major problem of this coating was the fact that it did not efficiently suppress EOF at higher pH (pH > 8.5), which limited its application for acidic protein separations.

With a highly hydrophilic and hydrolysis-stable acrylamide derivative (e.g., AAEE) [64, 65], Talmadge *et al.* [66] have been able to perform several hundred analyses by CIEF with zwitterions mobilization. It was an innovative work in the sense

Step one:

$$\overset{\displaystyle |}{\underset{\displaystyle |}{Si}}-OH \quad + \quad (CH_3O)_3Si\,CH_2CH_2CH_2-\overset{\displaystyle O\quad CH_2}{\overset{\displaystyle \|\quad\ \|}{C\text{-}O\text{-}C\text{-}CH}}{}_3$$

$$\longrightarrow \quad -\overset{\displaystyle |}{\underset{\displaystyle |}{Si}}-O-\overset{\displaystyle |}{\underset{\displaystyle |}{Si}}-CH_2CH_2CH_2-\overset{\displaystyle O\quad CH_2}{\overset{\displaystyle \|\quad\ \|}{C\text{-}O\text{-}C\text{-}CH}}{}_3$$

Step two:

$$-\overset{\displaystyle |}{\underset{\displaystyle |}{Si}}-O-\overset{\displaystyle |}{\underset{\displaystyle |}{Si}}-CH_2CH_2CH_2-\overset{\displaystyle O\quad CH_2}{\overset{\displaystyle \|\quad\ \|}{C\text{-}O\text{-}C\text{-}CH}}{}_3 \quad + \quad CH_2=CH\text{-}\overset{\displaystyle }{\underset{\displaystyle \underset{O}{\|}}{C}}\text{-}NH_2$$

$$\xrightarrow[\text{TEMED}]{(NH_4)_2S_2O_8} \quad -\overset{\displaystyle |}{\underset{\displaystyle |}{Si}}-O-\overset{\displaystyle |}{\underset{\displaystyle |}{Si}}-CH_2CH_2CH_2-\overset{\displaystyle O}{\overset{\displaystyle \|}{C}}\text{-}O\text{-}C\text{-}CH_3$$

$$CH_3-CH\text{-}\overset{\displaystyle }{\underset{\displaystyle \underset{O}{\|}}{C}}\text{-}NH_2$$
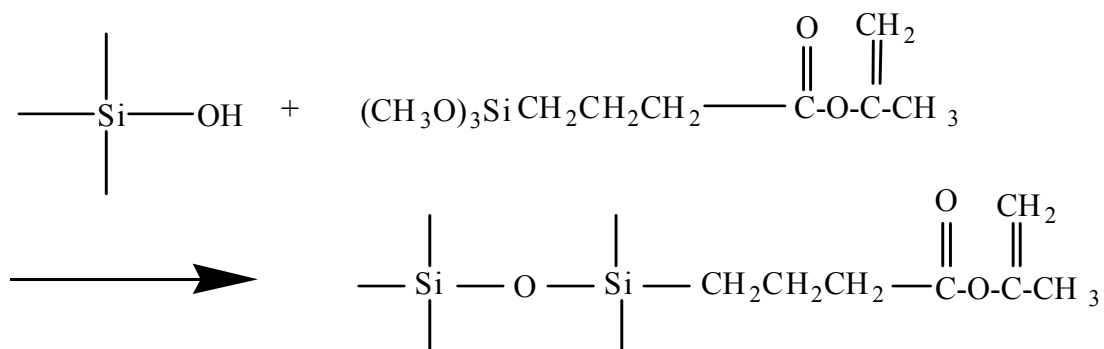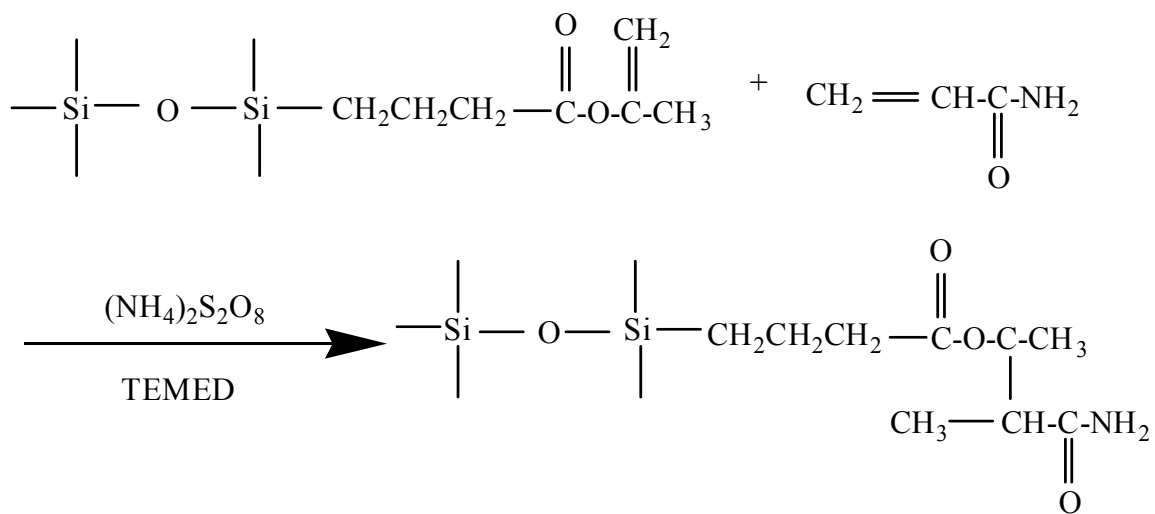
Figure 6. *The polyacrylamide coating chemistry. (In step one, MAPS reacted with the silanol groups on the inner walls of the capillary and in step two, the acryl group reacted with acrylamide in the presence of TEMED and ammonium persulfate forming a monolayer coating.) [7].*

that they introduced some novel polymers and performed a series of studies to optimize the synthetic conditions required to yield an efficient coating. However, synthesizing a new polymer in the laboratory was laborious and time-consuming. Furthermore, the synthesis procedure usually required an *in situ* polymerization reaction that was difficult to control, and had adverse effects on the reproducibility and quality of the preparation.

Based on the above overview, it is still attractive to look for coating chemistry that can meet all those criteria mentioned above. Our work in Chapter II was focused on improving the capillary coating technique to achieve good separations and high reproducibility and stability.

Although some problems exist as far as capillary coatings are concerned, CIEF still received wide applications because of its concentration properties. Moreover, CIEF coupled with other separation technologies is currently gaining popularity. This is called multi- dimensional separations, and they are powerful analytical techniques suitable for proteomics.

The successful coupling of CIEF in the first dimension to micellar electrokinetic capillary chromatography (MECC) in the second dimension was made by a 10-port valve interface with two conditioning loops. In the loops, salt and other unwanted first dimension effluent components were eliminated by dialysis and carrier ampholytes were added [39]. Peak broadening during dialysis did not have significant impact on the CIEF separation due to its concentrating effect. The dialysis interface was then used for an easier coupling of CIEF to other techniques, e.g., to capillary non-gel sieving electrophoresis [67] and to CZE [68].

Yang *et al.* introduced an online two-dimensional system consisting of CIEF and CGE including a dialysis interface by a hollow fiber to remove the mobilizing salt before gel-electrophoresis [69]. During this procedure, a single CIEF band was separated into several peaks due to different molecular weights. The resulting electropherogram is quite different from that of either CIEF or CGE. Therefore, more information about the studied protein (e.g., hemoglobin variants) could be obtained.

The integration of CIEF with capillary reversed-phase liquid chromatography (RPC) for two dimensional (2D) proteomics separation was developed by Chen *et al.* [70] and further studied by Zhou and Johnston [71] and Wang *et al.* [72]. After focusing was completed, the analytes were sequentially and hydrodynamically transferred into the second separation dimension through a micro-injector with a 200 nL internal injection loop. The high separation efficiency and comprehensive resolution of this combined CIEF-RPC system was demonstrated by analyzing *Drosophila* proteomics during steroid-induced programmed cell death.

Among all 2D methods which involved CIEF, coupling to MS has shown remarkable results using electrospray ionization (ESI) as the interface. This coupling allows the direct identification of analytes by molecular mass [73], selectivity enhancement [31], and insight into the molecular structure by MS/MS techniques. The recent advances in this aspect were reviewed by Banks [74] and Wehr [75, 76].

Stepwise mobilization was investigated by Zhang *et al.* [77] to improve separation analysis and simplify coupling with multistage MS analysis since it allows more effective temporal control of protein elution from CIEF columns. A modified configuration for

CIEF-ESI-MS using a coaxial sheath flow interface was described in order to facilitate the automation of on-line analysis. The system was tested with standard protein mixtures.

Yang *et al.* [78] demonstrated a CIEF-ESI-MS using a separation capillary connected with a short tapered micro-electrospray emitter capillary by a micro-dialysis membrane tube. The micro-dialysis junction provided the electric connection across the dialysis membrane for the electric field needed for the CIEF separation and the electrospray process. According to the authors, coaxial liquid-sheath flow configuration is mostly used for the coupling CIEF separation and ESI. The micro-dialysis junction they introduced is better than the coaxial liquid sheath interface as evidenced by the simplicity in operation procedures, the enhancement in detection sensitivity, and the linear correlation between standard protein migration time and pI in CIEF-ESI-MS [69, 78].

On-line combination of CIEF with ESI-MS was applied to the analysis of *Escherichia.coli* proteins by Tang et. al. [79]. Polyacrylamide-coated columns were used for CIEF separation and mobilization was achieved by a combination of electrophoresis and pressure. At the end of CIEF, focused proteins zones were analyzed by ESI-MS. The procedure exhibited high resolving power and separation speed but reduced detection sensitivity. The presence of carrier ampholytes in ESI not only caused a marked reduction in the protein ion intensity but also resulted in a decrease of the net charge of protein ions in the mass spectra compared to the average charge state measured in the absence of carrier ampholytes [80, 81]. Later, with the help of a free-flow electrophoresis device that can remove carrier ampholytes, this system was well applied

to the separation and analysis of hemoglobin variants [81] and transferrin glycoforms [82].

As can be seen from the above discussion, the further development of CIEF-MS could certainly open the door to the real proteomic analysis [83]. However, the presence of ampholytes in the sample mixture is a hindrance. Yang *et al.* introduced a procedure to make the repeatedly usable immobilized pH gradient (IpG) in a monolithic capillary column [84], which brought hope to completely solve this problem. Our research in Chapter III is directed on furthering the progress of IpG.

Rationale and Scope of the Study

Based on the above overview of the progress made in CIEF during the last five years, the CIEF technique seems to be quite powerful and promising in protein separations. However, the exploitation of the full potentials of CIEF is yet to come and will largely depend on further improvement in the operational aspects of the technique and a better understanding of the underlying phenomena. In this regard, our research has focused on the optimization of CIEF separation conditions, including the examination of different capillary coating strategies and the introduction of an immobilized pH gradient. While Chapter II summarizes the results on capillary coatings for performing CIEF with carrier ampholytes, Chapter III describes the development and evaluation of monolithic capillary columns with immobilized pH gradient (IpG). Although CIEF with carrier ampholytes and coated capillaries was easy to operate and yielded efficient separations with salt mobilization, the operation of CIEF with IpG monolith brought about improved

reproducibility and a good chance for CIEF to serve as the first dimension in multidimensional separation when coupling CIEF with other separation techniques. Carrier ampholytes are considerably expensive and always reduce detection sensitivity due to their high absorbance at low wavelengths. Some work have been previously done to perform CIEF without ampholytes, e.g., the electrolysis of water [85] and thermally engendered pH gradient [86]. They have been attractive for a long time but there still exist different problems. On the other hand, the IpG CIEF in monolithic columns, which was very recently initiated by Yang *et al.* [85] still require more optimization. Our investigation in Chapter III is an attempt to furthering the progress of IpG CIEF with monolithic capillary columns.

Conclusions

This chapter has outlined the scope and rationale of the research. The basic principles and fundamental equations for both CE and CIEF are described and presented. Some of these equations and principles have been utilized throughout this dissertation. In addition, an overview of the recent progress made in CIEF is provided. These background materials are essential in order to facilitate for the reader the understanding of the work presented in this dissertation.

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CHAPTER II

CAPILLARY ISOELECTRIC FOCUSING WITH

CARRIER AMPHOLYTE pH GRADIENT

Introduction

The quality of capillary coatings is a key factor for achieving successful CIEF separations. This is because the purpose of coating is to eliminate or reduce EOF, which, if present, will move focused sample zones out of the capillary before they are focused. Generally speaking, columns covalently coated with polymeric layers on the inner walls showed better CIEF separation than dynamically coated columns [1]. For a coated column to yield highly efficient CIEF separations, the coatings must not only reduce the EOF, but it also must reduce the interactions between the capillary inner wall and the sample components. This can be achieved by multi-layers coatings [2-7]. As will be shown in this chapter, capillaries with covalent polymer coatings and zwitterions dynamic coatings are the most efficient approaches. Moreover, depending on the polymer properties and different zwitterions used, the coatings showed preference for different mobilization methods. Therefore, the aim of this study is to evaluate some coating methods with the objective to gain some improvement in CIEF separations.

Experimental

<u>Reagents and Materials</u>

Lysozyme from chicken egg white, cytochrome C from bovine heart, ribonuclease A and α-chymotrypsinogen A, both from bovine pancreas, myoglobin from horse muscle, carbonic anhydrase I from human erythrocytes, carbonic anhydrase II from bovine erythrocytes, β-lactoglobulin A and α-lactalbumin both from bovine milk, trypsin inhibitor from soybean and amyloglucosidase from *Aspergillus niger*, and conalbumin type I and type II both from chicken egg white, and type III and type IV (iron-complex), both from turkey egg white, were purchased from Sigma (St. Louis, MO, USA).

Hydroxypropyl cellulose (HPC) with an average molecular weight of 55-75 *k*Da and 100 *k*Da were purchased from TCI America (Portland, OR, USA). γ-Glycidoxypropyl trimethoxysilane (GPTMS) was purchased from United Chemical Technologies (Bristol, PA, USA). Sodium hydroxide, phosphoric acid, *N, N*-dimethylformamide (DMF), boron trifluoride etherate (BF_3), formaldehyde and formic acid were purchased from Aldrich (Miwaukee, WI, USA). The zwitterionic surfactants 3-(3-cholamidopropyl)dimethylammonio-1-propane sulfonate (CHAPS) and hexadecyl dimethyl(3-sulfopropyl)ammonium hydroxide (HDSPA), 3-(trimethoxysilyl)propyl methacrylate (MAPS), *N,N,N',N'*-tetramethylethylenediamine (TEMED), and ammonium persulphate were purchased from Sigma. Sodium phosphate monobasic,

phosphoric acid and ammonium hydroxide were purchased from Mallinckrodt (Paris, KY, USA.)

Pharmalyte with a pH range of 3–10 was purchased from Amersham Pharmacia (Uppsala, Sweden). Reagent-grade sodium phosphate monobasic, phosphoric acid, and sodium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA, USA). Untreated fused-silica capillaries of 50 μm ID and 365 μm OD were purchased from Polymicro Technology (Phoenix, AZ, USA).

Instruments

The instrument for this study was a P/ACE 5010 capillary electrophoresis system from Beckman Instruments Inc. (Fullerton, CA, USE) equipped with a UV detector and a data handling system comprised of an IBM personal computer and P/ACE Station software.

Capillary Coatings

HPC Coating Series: Fused-silica capillary (50 μm I.D.) of a desired length was first rinsed with 1.0 M sodium hydroxide for one hr, then rinsed with a few drops of water, then with 0.1 M hydrochloric acid for one hr and finally with deionized water for 20 min.

The HPC coating procedure was performed following the method outlined in Ref. [4] with some modifications as follows. HPC of 55-70 kDa and HPC of 100 kDa were

dissolved in water at room temperature at concentrations of 2.5% (w/w) and 1.5% (w/w), respectively. The prepared polymer solutions were left overnight to eliminate bubbles in the solution. Two pretreated capillary columns were filled with the polymer solutions using 100 μL syringes. The filled columns were then purged using N_2 to eject the solution and dried another 30 min at room temperature. Thecolumns were filled with the polymer solutions again and then heated from 60 °C to 140 °C at 5°C min^{-1} and held at 140 °C for 74 min in a GC oven with a stream of N_2. These two columns were designated as H1 and H2 with the respective 55-70 kDa and 100 kDa HPC coatings.

Another capillary column denoted as H3 was first filled with a solution of 50% v/v GPTMS in acetone and allowed to react at room temperature overnight [8]. After the column was rinsed with water and acetone on the second day, it was coated following the same coating steps for column H2 as described above.

GPTMS-HPC Column Series: Fused-silica capillary (50 μm I.D.) of a desired length was first pretreated in the same way as HPC series columns. γ-Glycidoxypropyl trimethoxysilane (GPTMS) was dissolved in different solutions: (1) 50% (v/v) in acetone solution, (2) 10% (v/v) in a 5 mM pH 9.0 Na_2HPO_4 solution, and (3) 10% (v/v) DMF solution. Three different capillaries were produced based on the different solvents used in the GPTMS step. They were coded as G1, G2, and G3, respectively. Since the solutions of GPTMS were prepared in three different solvents and reacted with each capillary under different conditions, the three capillaries would differ in the extent to which their inner surface is covered with the GPTMS sublayer. Column G1 was first filled with solution (1) and then allowed to react at room temperature overnight. When

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finished, the column was rinsed with acetone and water. Column G2 was first filled with solution (2) and then allowed to react in GC oven at 80 ºC for one hour. This treatment was repeated three times. When finished, the column was rinsed with Na_2HPO_4 buffer solution and enough water. Column G3 was first filled with solution (3) and then allowed to react at 100 °C for 3 hrs. When finished, the column was rinsed with DMF solution and water.

HPC (100 kDa) was dissolved in dried DMF at room temperature at a concentration of 1.5% (w/v). The dried DMF was obtained by treatment with activated molecular sieve (3Å). 1% (v/v) BF_3 was added to the DMF solution before using. All columns were filled with this solution and were left at room temperature for 3 hrs for reaction. After this treatment, columns G1, G2 and G3 were then coated following the coating of the column H3, that is, columns were filled with degassed HPC (100 kDa, 1.5%) solutions using a 100-µL syringe. Then the columns were purged with N_2 to eject the solution for 30 min at room temperature. The columns were then filled with the same polymer solutions for one hr and then heated from 60 °C to 140 °C at 5 °C min^{-1} and held at 140 °C for 74 min in a GC oven with N_2 purging. The last step was repeated twice. Since the three columns differed in the extent of surface coverage with the GPTMS sublayer, they should also differ in the surface coverage with the HPC top layer, even though the top layer is reacted with the capillary under the same conditions.

G-Columns Dynamically Coated with Surfactants: Two zwitterionic surfactants, namely CHAPS and HDSPA, were used in this study. Zwitteronic surfactants were previously reported in CIEF with bare fused silica capillaries. [9, 10] A segment of

fused-silica capillary was rinsed only with a saturated HDSPA solution, and it was denoted as S1 which was used for a reference. Another two columns were first coated with exactly the same procedures as column G1, and then, they were rinsed with 10 mM HDSPA and 0.04 mg/mL CHAPS solutions. Therefore, the two columns were designated as S2 and S3, respectively.

Column Evaluations

The columns were first evaluated in CZE with a neutral marker solution, e.g., acetone at 25%, v/v in water solution. The detection was made at 254 nm and the running buffer was 100 mM or 50 mM sodium phosphate (pH 7.0). The columns were then evaluated in CZE with a mixture of basic proteins. Lysozyme (0.32 mg/mL), cytochrome C (0.64 mg/mL), ribonuclease A (0.64 mg/mL) and α-chymotrypsinogen A (0.32 mg/mL) mixture were used in our studies. The protein mixture solution was introduced into the column by electromigration injection for 5 sec. Running buffer was 100 mM or 50 mM sodium phosphate, (pH 7.0). Between runs of proteins separations, the column was rinsed with running buffer for 2 min. All analyses were run at 25 °C, controlled by Beckman 5010 instrument. Detections were made at 210 nm.

For the dynamically coated column series, the magnitude of EOF in the presence of ampholyte (2% v/v) solution was determined from the migration time of a neutral compound (i.e., acetone) under a constant voltage of 19 kV. Before each run, the column was rinsed at 20 psi for 5 min with buffer containing various concentrations of surfactants. The buffers were prepared by mixing ampholyte (2%, v/v), acetic acid (5 mM), and

phosphoric acid (5 mM) and adjusted to the desired pH with ammonium hydroxide. Acetone (25%, v/v) was introduced as an EOF marker into the column at a low pressure for 5 sec. Detection of acetone was made at 254 nm.

CIEF Procedures

Phosphoric acid (20 mM, pH 2.75) was used as anolyte and sodium hydroxide (40 mM, pH 11.75) was used as catholyte. Pharmalyte™ (pH 3-10, 2% if not specified) was used to produce the pH gradient.

Protein samples were dissolved in the Pharmalyte-water solution with different concentrations. The sample and pharmalyte solution was used to fill the whole column. Focusing was performed at a field-strength of 333 v/cm for 10 min unless otherwise stated. Mobilization can be achieved by applying a low pressure (0.5 psi) with high voltage or by replacing the catholyte with a 50 mM NaCl solution with high voltage. The focusing and mobilization process were monitored using a UV detector at 280 nm. After each run, the capillary column was rinsed with 10 mM phosphoric acid and enough water.

For dynamically coated column series, the column was rinsed by the surfactant solution containing 2% ampholyte (10 min, 20 psi) to regenerate the coating before each separation, followed by the protein-surfactant-ampholyte mixture under high pressure. Focusing was performed at 19 kV. Acetic acid and ammonium hydroxide (both 1%, v/v) were respectively used as the anolyte and catholyte. Acetone was also added to the surfactant-ampholyte solution to identify the end of the mobilization step.

Results and Discussions

An evaluation of three categories of capillary coatings was performed by separations of some standard proteins in the CIEF mode. The various capillary coatings were first evaluated by running a neutral marker to measure the extent of EOF reduction and four basic proteins in the capillary zone electrophoresis (CZE) mode to assess the utility of the capillary columns in CIEF separations.

HPC Column Series

Column Evaluations: HPC coated capillary columns were first evaluated by running acetone as a neutral marker and then four basic proteins under CZE conditions. The difference between the coatings of column H1 and H2 is the average molecular weight of HPC and its concentrations used for coating. As can be seen from the structure of HPC polymer (Fig. 1), the bigger the number n, the higher the average molecular weight and the lower the solubility in water [11]. Therefore, 1.5% (w/w) was used for higher molecular weight HPC and 2.5% (w/w) was used for lower molecular weight HPC. Figure 2 shows a typical electropherogram for running acetone to measure the EOF. As can be seen from Table 1 and Figure 2, the column coated with HPC of molecular weight 100 kDa exhibited slightly less EOF and about the same separations of the four basic proteins than that obtained on the column coated with HPC of molecular weight 55-70 kDa. As can be seen in Table 1, the residual EOF measured were 0.24 mm/sec and 0.20 mm/sec for columns H1 and H2, respectively. By simply curing an HPC layer on the

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capillary inner surface was obviously insufficient to eliminate the interaction between the column surface and proteins, as shown in Figure 3 (A) and (B), where different adsorption problems with the coatings can be detected. For column H1, the last two proteins were not well separated, and for column H2 the first protein was poorly separated. A difference of about 0.04 mm/s in EOF between columns H1 and H2 yielded a large difference in migration times of separated proteins. As can be seen in Fig. 3, it took 15 min for column H1 to achieve the protein separation, while it took 20 min on column H2.

For column H3, the capillary surface contained a covalently attached hydrophilic sublayer, which in turn allowed the covalent attachment of the HPC top layer. This involved first the coating of the capillary inner surface with a GPTMS solution, which then provided a spacer arm for the covalent attachment of HPC to the capillary surface. Thus the effect of reducing EOF was much better than the columns with a simple HPC coating. As can be seen from Table 1, the EOF of column H3 was much more reduced than the other two columns, with an EOF velocity of 0.05 mm/sec, which was four times less than columns H1 and H2. As shown in Figure 3 (C), a good separation of four standard basic proteins was achieved with column H3.

TABLE 1

SUMMARY OF HPC COATED COLUMN SERIES

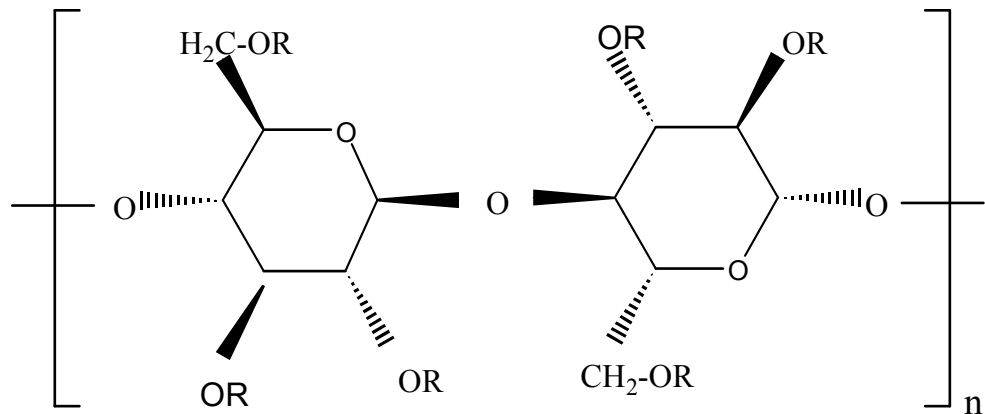| Capillary coding	GPTMS Pretreatment	HPC treatment	EOF (mm/sec)
H1	None	55-70 kDa, 2.5 %	0.24
H2	None	100 kDa, 1.5 %	0.20
H3	50 % in acetone	100 kDa, 1.5 %	0.05

$$R = -CH_2CH_2CH_2OH$$

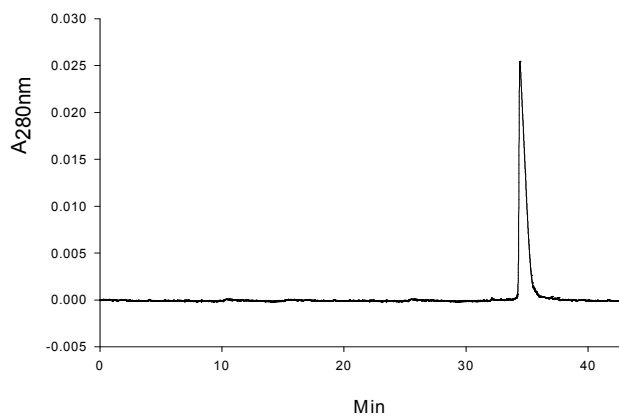**Figure 1**. *Representation of HPC polymer structure.*

Figure 2. *A typical electropherogram for EOF measurement. Sample: acetone (25%, v/v) water solution. Conditions: column: H1, 50 cm effective column length and 50 μm ID. Running buffer: 50 mM sodium phosphate, pH 7.0. Detection was made at 254 nm.*
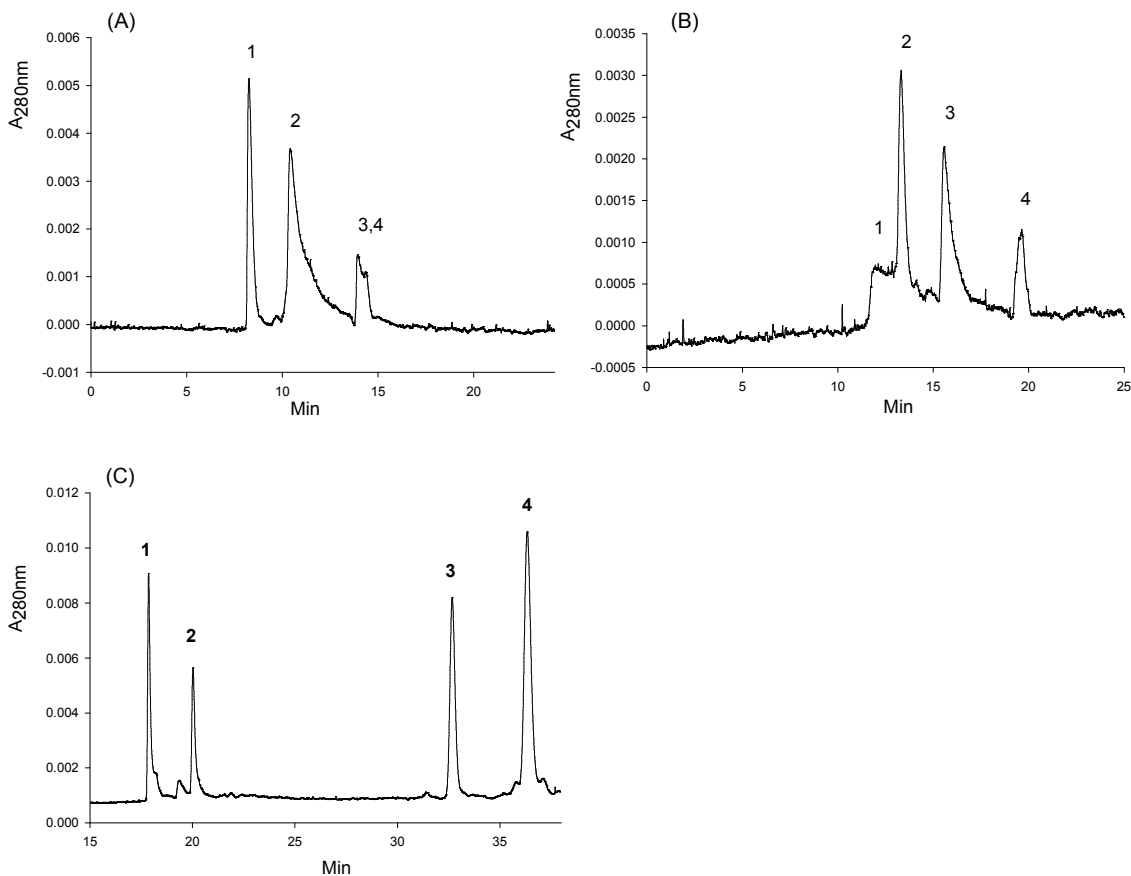
Figure 3. *Capillary zone electrophoresis separation of four basic proteins using HPC column series. (A) column H1, (B) column H2, (C) column H3. Separation conditions: 50 mM phosphate buffer at pH 7.0; 333 v/cm; column: 50 cm x 50 μm ID. Samples: 1. lysozyme, 0.32 mg/mL; 2. cytochrome C, 0.64 mg/mL; 3. ribonuclease A, 0.64 mg/mL; 4. α-chymotrypsinogen A, 0.32 mg/mL.*

CIEF Separations of Standard Proteins: The ability to form a linear, wide and stable pH gradient is essential to achieve good CIEF separations. This could be realized once the EOF was reduced to a negligible value. The formed pH gradient range is determined by the ampholytes used for focusing [12]. Pharmalyte pH 3-10 was used in our studies. As shown in Figure 4 (B), standard proteins with pI values ranging from 9.4 to 4.8 can be separated in the order of decreasing pI, while in Figure 4 (A), ribonuclease A with pI 9.4 cannot be detected. This would be due to the high pI of RNaseA so it

moved out of the column before the mobilization actually began. Also, the peak for RNaseA was not as sharp as that of other protein peaks. As proved later, in most cases, proteins with high pI values or low pI values cannot be separated well. Either the peak is too broad, or it cannot be detected at all. The linearity of the pH gradient was determined by plotting the pI *vs.* migration time as shown in Figure 5. While using pressure mobilization the separated protein peaks were not as sharp as the peaks obtained when using chemical mobilization, the corresponding pH gradient linearity was better ($R^2 =$ 0.9261>0.9003). In addition, the slope, which reflects the width of a certain pH gradient, was much smaller than the slope for chemical mobilization.
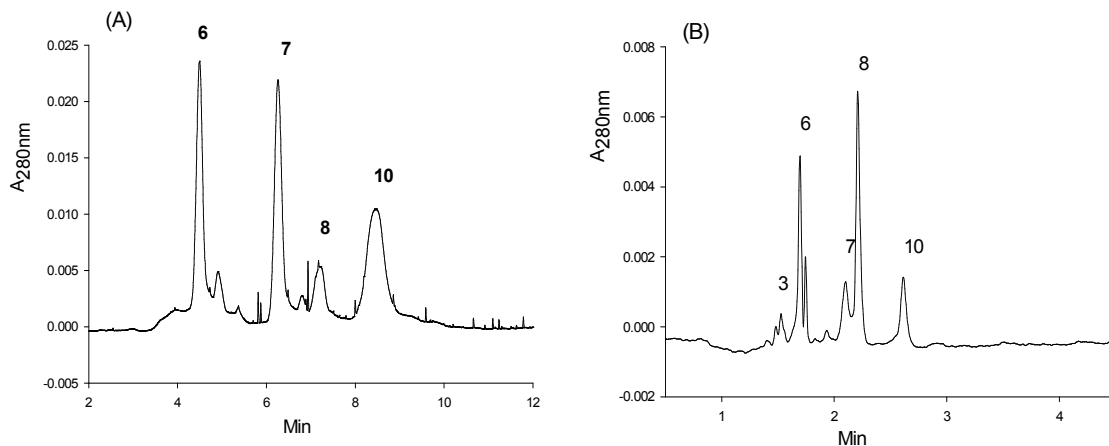
Figure 4. *CIEF of standard proteins using column H3. (A) Pressure mobilization; (B) Chemical mobilization. Conditions: column: 50 cm x 50 μm ID. Sample solution contained 0.2 mg/ml each protein, 2 % Pharmalyte (pH 3-10). Focusing was carried out at 333 v/cm for 15 min, with 20 mM H_3PO_4 and 40 mM NaOH. (A) pressure mobilization was achieved by applying 0.5 psi to the column with voltage maintained. (B) Chemical mobilization was achieved by replacing the catholyte with 50 mM NaCl solution at the end of focusing. Sample proteins as listed in Table 2.*
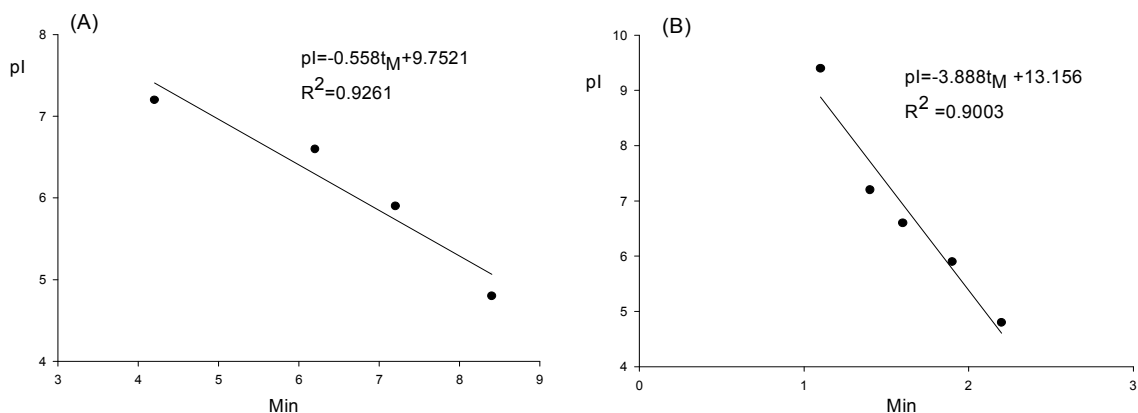
Figure 5. *Linearity of the pH gradient measured by plotting the pI of standard proteins against mobilization time. Concentrations and pIs of the proteins are listed in Table II. Plot (A) corresponds to CIEF with pressure mobilization and plot (B) corresponds to CIEF with chemical mobilization. The detailed CIEF conditions were the same as in Figure 4.*

TABLE 2

PROTEINS NAMES AND CONCENTRATIONS SEPARATED BY CIEF

No.	Protein	Source	Concentration (mg/ml)	pI
3	Ribonuclease A	Bovine heart	0.36	9.4
5	Trypsinogen A	Bovine pancreas	0.08	9.2
6	Myoglobin	Horse heart	0.06	7.2
7	Carbonic Anhydrase I	Human erythrocytes	0.03	6.6
8	Carbonic Anhydrase II	Bovine erythrocytes	0.06	5.9
9	β– Lactoglobulin A	Bovine milk	0.06	5.1
10	α– Lactalbumin	Bovine milk	0.05	4.8
11	Trypsin Inhibitor	Soybean	0.10	4.5

From the results of the HPC column series, it was concluded that HPC was a polymer effective in reducing EOF as was also reported by other researchers [4, 13, 14]. However, it is not very effective in the reduction of protein interactions with the capillary surface. As used in column H3, GPTMS provided a chance to improve the HPC coating performance. This observation led the way to the next column coating described in the following section.

GPTMS-HPC Column Series

Column Evaluations: To improve column performance, a GPTMS column series were first obtained by reacting GPTMS in different solvents including acetone (column G1), Na_2HPO_4 pH 9.0 (column G2) and DMF solution (column G3). The reaction between GPTMS and the capillary inner wall can be referred to as an epoxy activation of the capillary inner surface [15], [16]. The ideal reaction of GPTMS with the capillary inner surface involves the hydrolysis and bonding onto the silica surface by forming Si-O-Si bonds. The epoxy-activated surface is then reacted with HPC in the presence of a Lewis acid catalyst (BF_3), which will hydrolyze the oxirane ring to a free diol group followed by a condensation reaction with the HPC polymer. However, different solvents may induce hydrolysis to different extents and they can also influence the subsequent HPC coatings. GPTMS in acetone as the solvent worked better than with the other two solvents.

A comparative study among the three coatings was carried out. As shown in Table 3, the EOF could not be measured with the three columns since the peak of the

neutral marker could not be seen for up to 1.5 hrs. This proved that all the coatings showed the ability to suppress EOF to a negligible value and this should offer the possibility of good performance in CIEF separation.

TABLE 3

SUMMARY OF GPTMS-HPC COATED COLUMN SERIES

Column	γ- GPTMS		Second layer	Third layer	EOF Evaluation	
Coding	%	Solvent	Of coatings	Of coatings	U_{eo} (mm/sec)	Time (h)
G1	50	Acetone	HPC, 100 kDa,	HPC, 100 kDa,	None	2
G2	10	Na$_2$HPO$_4$	1.5 %, with 1 %	2.0 %,	None	2
		5 mM, pH9	BF$_3$ in DMF	GC oven		
G3	10	DMF	solution	1.5 Hours	None	1.5

In order to better assess the performance of the coated columns, four basic proteins were separated by CZE on the different GPMTS-HPC columns. As shown in Figure 6, column G1 did the best separation under CZE conditions while the other two columns have good separation for the last two proteins but not the first two. In the first few initial runs, the performance of column G2 was better than column G3 in terms of peak shape, but, after several runs, these two columns behaved similarly.

The GPTMS column series can be used under CZE conditions at pH 7.0 for about one week. Unlike the results reported by other researchers [17], even our best columns could not last for over several hundreds runs, and the migration time kept decreasing after 3 days, especially for the last resolved peaks. RSDs for run-to-run and column-to-

column and separation efficiency are listed in Table 4, using our best performance column G1.

TABLE 4

SEPARATION EFFICIENCY AND PERCENT RELATIVE STANDARD DEVIATION (% RSD) OF THE MIGRATION TIMES OF SOME BASIC PROTEINS WITH G1

| | Efficiency | R.S.D % (n = 3, for 5 columns) | |
Proteins	(plates/m)	Run-to-run	Column-to-column
Lysozyme	337,120	1.46	3.77
Cytochrome C	342,720	1.34	5.91
Ribonuclease A	273,812	2.59	9.30
α-Chymotrypsinogen A	156,822	2.51	12.78

TABLE 5

MIGRATION TIMES OF SOME BASIC PROTEINS USING DIFFERENT COATING COLUMNS AND THEIR AVERAGE SEPARATION EFFICIENCY

| Proteins | Average Migration Time (min) | | | | | |
| | (n = 4 for H columns and 8 for G columns) | | | | | |
	H1	H2	H3	G1	G2	G3
Lysozyme	11.0	13.0	23.6	24.0	23.2	23.4
Cytochrome C	12.4	15.2	27.5	28.0	27.0	27.0
Ribonuclease A	20.5	33.3	31.6	64.0	54.4	57.2
α-ChymotrypsinogenA	22.8	40.0	62.7	78.8	63.2	64.4
Average Efficiency	92,605	125,070	260,586	328,676	189,630	219,150

Table 5 lists the average migration time for several runs for different columns between HPC series and GPTMS-HPC series columns. It could be concluded that column G1 was the most satisfactory one in terms of separation efficiency and migration time reproducibility. The separation efficiency is as high as 328,676 and RSD for run-to-run < 2.59%, and for column-to-column < 12.78%. Column G1 good performance may indicate that acetone provided a proper environment for the first reaction step to happen and for the HPC reaction to continue.
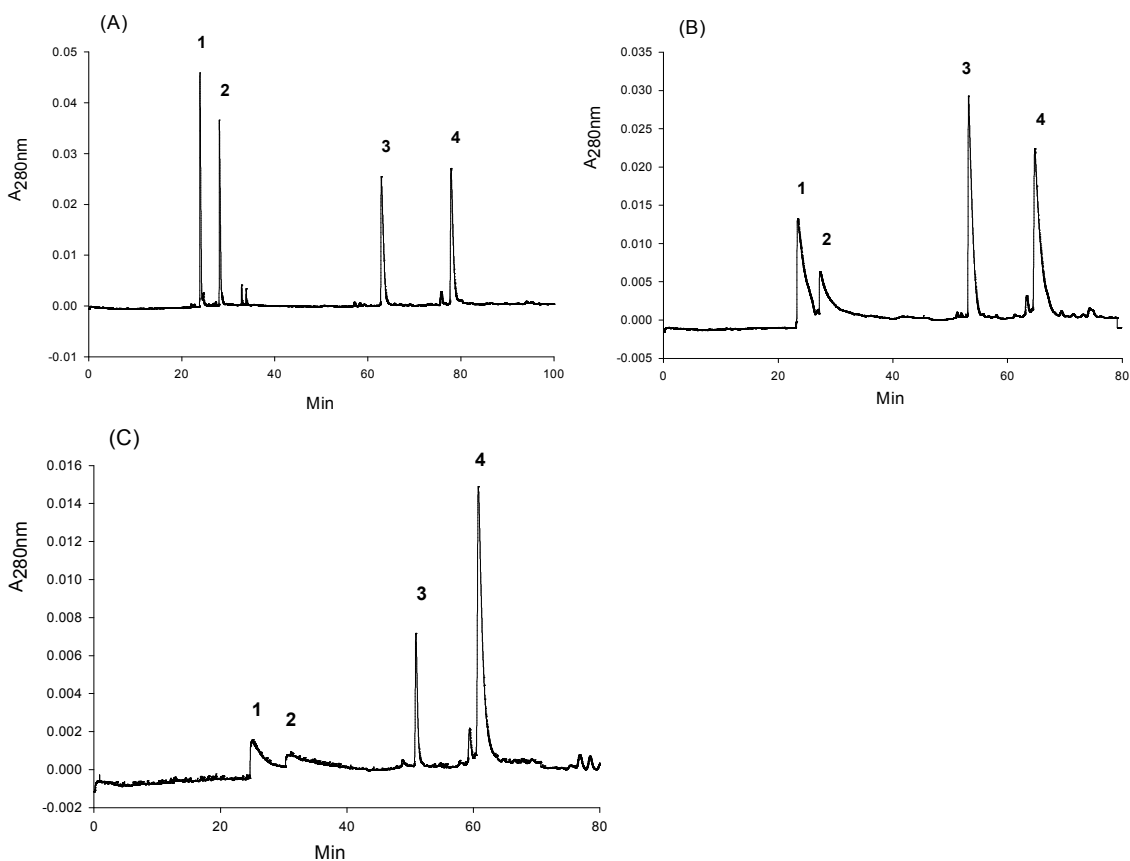

Figure 6. *CZE of four basic proteins using HPC column series. (A) column G1, (B) column G2, (C) column G3. Other separation conditions are the same as in Figure 2.*

CIEF Separations by GPTMS-HPC Column Series: A test protein mixture containing seven proteins: RNase A (pI 9.4), myoglobin (pI 7.2), carbonic anhydrase I (pI 6.6) and II (pI 5.9), β-lactoglobulin A (pI 5.1), α-lactalbumin (pI 4.8), and trypsin inhibitor (pI 4.5) was used to investigate the property of GPTMS-HPC column series. The results are shown in Figures 7-9, which display the possibility and high quality of CIEF separations. Most importantly, they illustrate the influence of the nature of capillary coatings on the quality of the CIEF separations.

Figure 7 shows the CIEF separations of the standard protein mixture using column G1, which was pretreated by 50% GPTMS in acetone solution. Figure 7A illustrates the electropherogram of CIEF separation followed by pressure mobilization and its corresponding linear plot of pI vs. mobilization time (Figure 7C). Relatively sharp peaks were observed and a good linear relationship was obtained ($R^2 = 0.9543$). The only exception was the last peak obtained at the end of separation and this can be attributed to the distortion of pH gradient at the end of separation due to the movement of large amount of anolyte into the column. Figure 7B shows the same CIEF separation but followed by chemical mobilization. Compared with pressure mobilization, the peaks were not so sharp but not as broad as the last peak using pressure mobilization. During the process of salt mobilization, the pH gradient was moved evenly and it resulted in a better linear relationship ($R^2 = 0.9693$) than using pressure mobilization for the plot of pI vs. mobilization time. The disadvantage was that the whole mobilization process was finished within two minutes, which was evidenced by the rapid rise of current through the column. This resulted in a steep pH gradient as can be seen from Figure 7D, the slope of

the linear equation was much higher than using pressure mobilization (5.216 versus 1.6076).
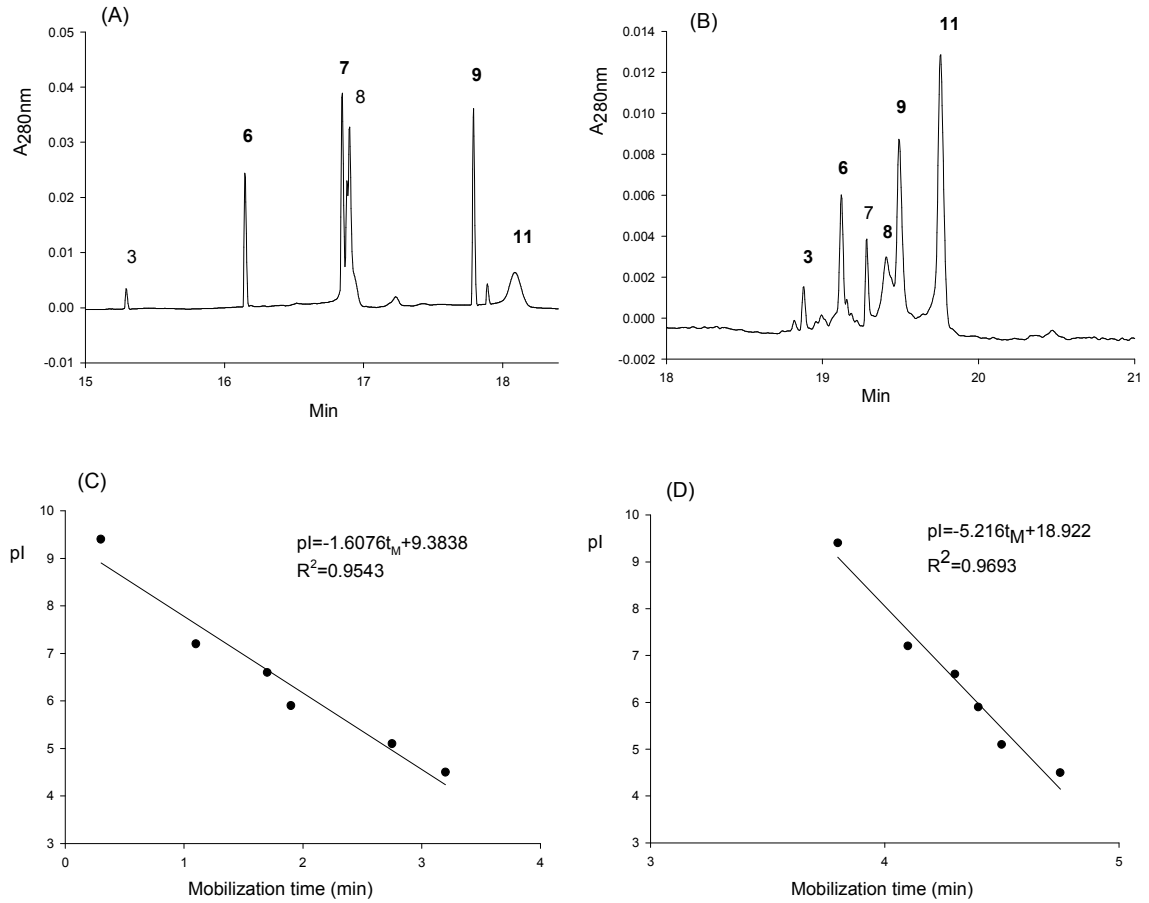

Figure 7. *CIEF of standard proteins using column G1. (A,B) CIEF electropherogram and (C,D) linearity of the pH gradient measured by plotting the pI of proteins against mobilization time. The proteins denotations, concentrations and pIs are listed in Table II. (A,C) CIEF with pressure mobilization and (B,D) CIEF with chemical mobilization. Other conditions are the same as listed in Figure 3.*

Figures 8 and 9 show the CIEF separations using column G2 and G3, respectively. As expected from column evaluations by running basic proteins under CZE conditions, columns G2 and G3 were not as good as column G1.
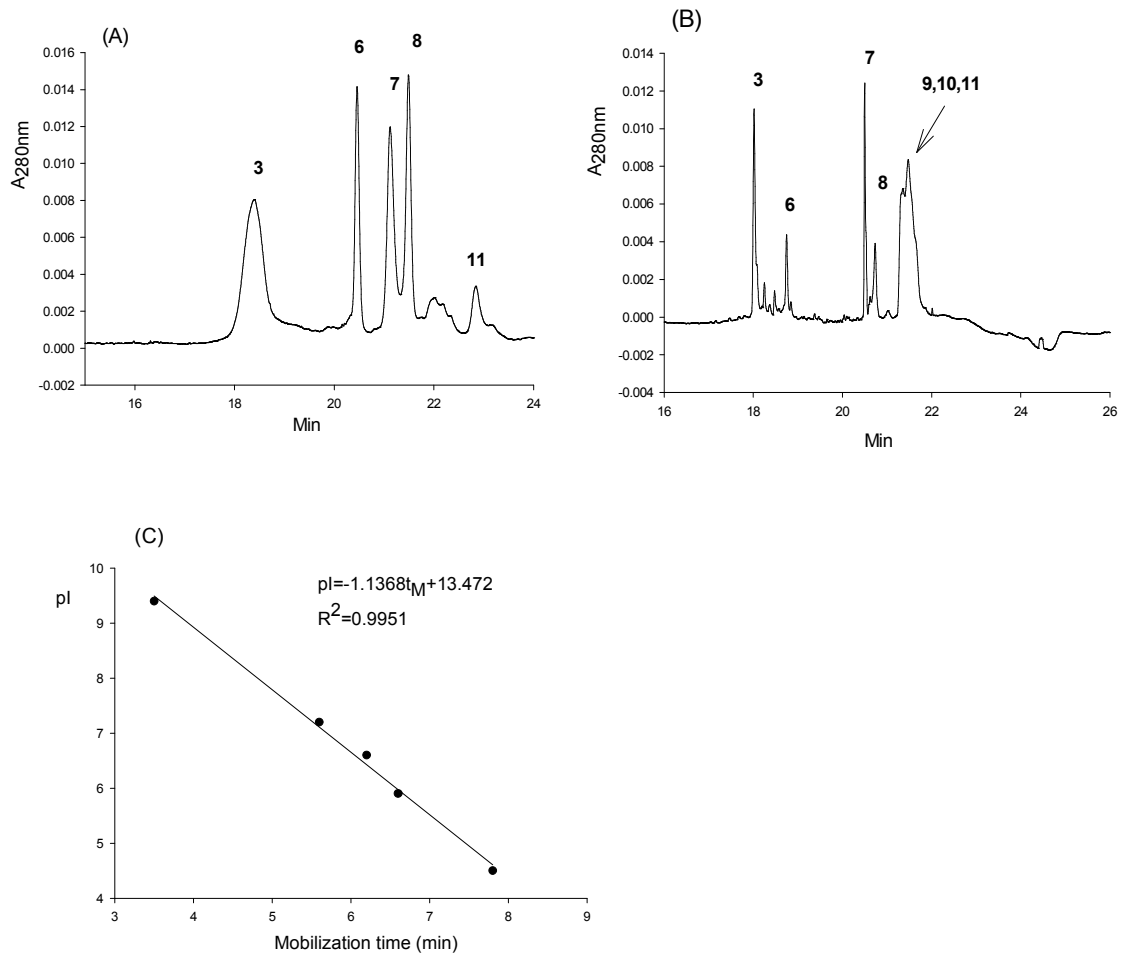

Figure 8. *CIEF separation of standard proteins using column G2. Experimental and plot conditions are the same as in Figure 7. In (B) chemical mobilization was not achieved there is no corresponding pI vs.mobilization time plot.*

Figures 8 and 9 show the CIEF separations followed by pressure mobilization and chemical mobilization for columns G2 and G3, respectively. As far as pressure mobilization is concerned, columns G2 and G3 achieved good separations for most of the proteins studied. The limitation is that the resolved peaks are broad, especially for the first peak corresponding to a higher pI protein. The plots of pI *vs.* mobilization time indicated good linearity relationship (R^2 = 0.9951 and 0.9798, for G2 and G3 respectively). The slopes of the plots are low (1.1368 and 0.4403, respectively), indicating good pH gradient formed in the process of focusing.

However, using column G2 with chemical mobilization, the basic proteins were well separated but not the acidic proteins, as seen from Figure 8 (B). The first four peaks were sharp, but the last three peaks were overlapped. This may be explained by the closeness of the pIs of the three acidic peaks (5.1, 4.8 and 4.5) as opposed to the pIs of the four other proteins that are actually widely separated (9.4, 7.2, 6.6 and 5.9). In practice, the rapid rise of current was observed at the same time as the mixed broad peak for acidic proteins exited from the column. In the case of column G3, the separation was even worse, [see Figure 9 (B)]. All the acidic proteins eluted out of the column within a narrow range of time window, which resulted in a very broad peak. The same separation pattern occured to the neutral proteins. Therefore, it is impossible to make the plot of pI versus mobilization time with columns G2 and G3.

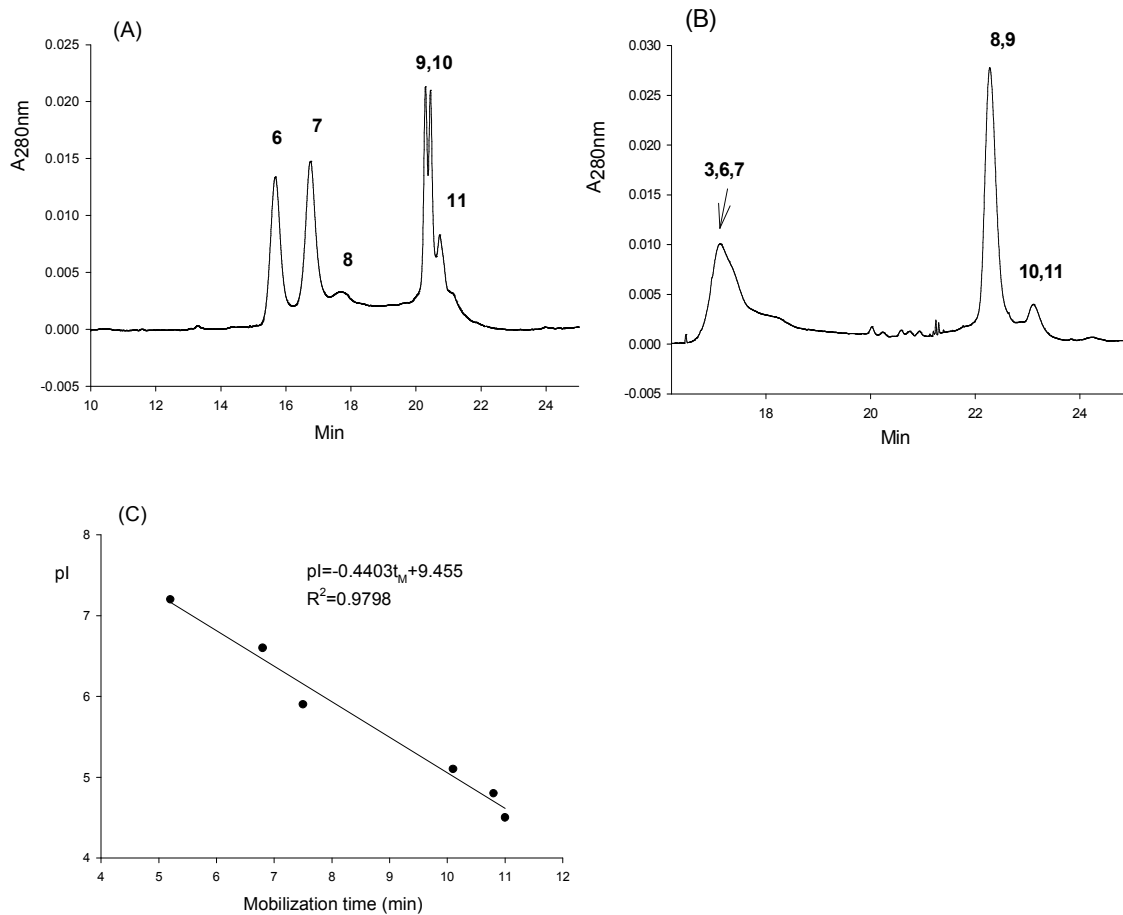**Figure 9**. *CIEF separation of standard proteins using column G3. Experimental and plot conditions are the same as in Figure 7. In (B) chemical mobilization was not achieved there is no corresponding pI vs. mobilization time plot.*

For CIEF separation of proteins, the major factor influencing the reproducibility is the chemical stability of the coatings on the capillary inner wall. Our results showed that consecutive runs on the same day (%RSD = 8.2) produced more reproducible results than for runs from different days (%RSD = 14.8). The differences observed are probably due to the changes in the capillary inner wall. As far as column G1 is concerned (which was the best coated column in the study), the separation performance of a capillary typically lasted for about 25 runs and, after that, a rapid increase in EOF was observed by running acetone in the CZE mode, and the capillary was abandoned. (%RSD was not calculated for columns G2 and G3 since not all sample components can be separated.)

The improvement of the GPTMS-HPC column series over the HPC column series is that the former column series performed much better in CIEF separations with the pressure mobilization technique. On the other hand, the limitation of GPTMS-HPC column series is that it cannot achieve good CIEF separations with the chemical mobilization technique. Ken *et al.* [9] introduced zwitterions and this helped solving this problem as shown in the next section.

G-Columns Dynamically Coated with Surfactant

Dynamic coatings are an alternative to covalent coatings. The formation of dynamic coatings is based on non-covalent forces such as electrostatic and/or hydrophobic effects [9, 18]. Ken *et al.* first demonstrated the use of surfactants as additives in CIEF to dynamically modify the surfaces of bare fused silica capillaries. The surfactants they used were zwitterionic sulfobetaines. Zwitterionic surfactants consist of

a hydrophobic tail and a hydrophilic head group possessing both cationic and anionic functionalities.

Usually, zwitterionic surfactants display critical micelle concentrations (cmc) higher than nonionic surfactants and lower than ionic surfactants of similar hydrophobic chain length. This is a benefit when zwitterionic surfactants are used in capillary column coatings for EOF reduction. In our study, we used concentrations of 5 mM for HDSPA, considering its cmc is 0.028-0.07 mM [9]. According to Ken *et al.*, the alkyl chain length of the zwitterionic surfactants has little effect on the degree of EOF suppression. This may explain why using CHAPS and HDSPA the EOF was reduced almost to the same level, although their concentrations and structures were different.

TABLE 6

SUMMARY OF SURFACTANT COATED COLUMN SERIES

Column Symbol	First layer	Second layer	Third layer	Surfactant		EOF Mm/sec
				Name	Concentration	
S1	None	None	None	HDSPA	5 mM	1.02
S2	50 %	HPC,1.5 %,	HPC,	HDSPA	5 mM	>1.5 h
S3	GPTMS in	1 % BF_3 in	2.0 %,	CHAPS	0.4 mg/ml	>1.5 h
	Acetone	DMF	oven			(can not be
	solution	solution	1.5 hrs			measured)

Table 6 listed the different coatings for the S column series. For comparison, we made a column coated simply with surfactant, designated as S1. This column offered no

advantages over any other column. Therefore, the column was not considered further. The other two columns, denoted as S2 and S3, were dynamically coated with the zwitterionic surfactants HDSPA and CHAPS, respectively, after coating them as column G1 (i.e., GPTMS-HPC).

In our studies, the S column series involved two kinds of coating: first, covalent coating of the capillary surface with polymer HPC, and second, dynamic coating with a zwitterionic surfactant. Thus, theoretically, the S column series (except for S1) should provide better CIEF separations than the GPTMS/HPC column series. Figure 10 shows the separation of basic proteins with column S2 under CZE conditions. Compared with the same CZE separation using column G1, the analysis time increased, which meant that the EOF was more reduced. The average separation efficiency (316,850 plates/meter, n = 3) was about the same as column G1 (328,676 plates/ meter, n = 8). However, the stability of column S2 and S3 was not very good (average %RSD = 14.9, n = 3) as compared with column G1 (average %RSD = 7.94, n = 5). The dynamic coatings could be easily regenerated by rinsing the columns with the surfactant solutions after some runs, but this resulted in different separation patterns after several times of regenerations.
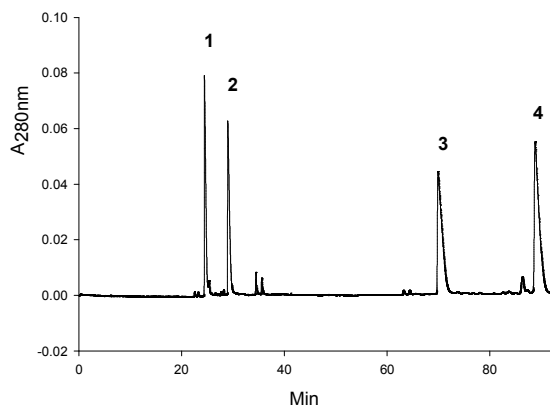

Figure 10. *CZE of proteins with column S2. Buffer contains 1% zwitterionic surfactant (HDSPA) solution. Other separation conditions are the same as in Figure 3.*

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CIEF Separations of Standard Proteins: Using the zwitterionic surfactants as coatings and additives, CIEF was performed on a mixture of seven standard proteins with pIs ranging from 9.4 to 4.5 (see Table 2). Figure 11 shows the CIEF separation of the proteins in the HDSPA–modified capillaries. The change of baseline at the end of separation was caused by the mobilization solution marked with acetone and the height of the acetone signal was proportional to its concentration in the mixture. This concentration was different from run-to-run, which can be attributed to the volatilization of acetone that resulted in the variation in concentration. As can be seen from Figure 11 (A), almost all of the peaks came out in a 5-min-window and it was crowded at the acidic end, which was evidenced from the pH gradient profile again. The linear relationship of pI vs. mobilization time was not as good as column G1 ($R^2 = 0.8913 < 0.9693$), and the separation was not improved compared with the separation performance for column G1.
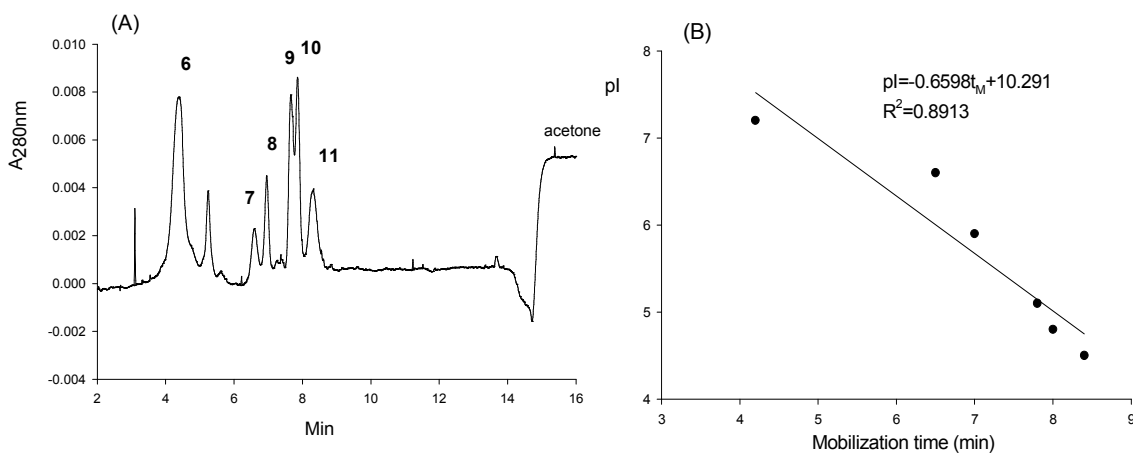

Figure 11. *CIEF separation of standard proteins using column S2. (A) electropherogram of CIEF separation with pressure mobilization and (B) Linearity of the pH gradient profile. The sample proteins are listed in Table II. Buffer: 2% ampholyte with 4 mM zwitterionic surfactant (HDSPA) solution. Other conditions are the same as in Figure 4.*

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Figures 12 and 13 demonstrate the CIEF separations in HDSPA- or CHAPS-modified columns with full-voltage (A) and half-voltage (B). The chemical mobilization was achieved by replacing the NaOH solution by 50 mM NaCl solution at the end of focusing.
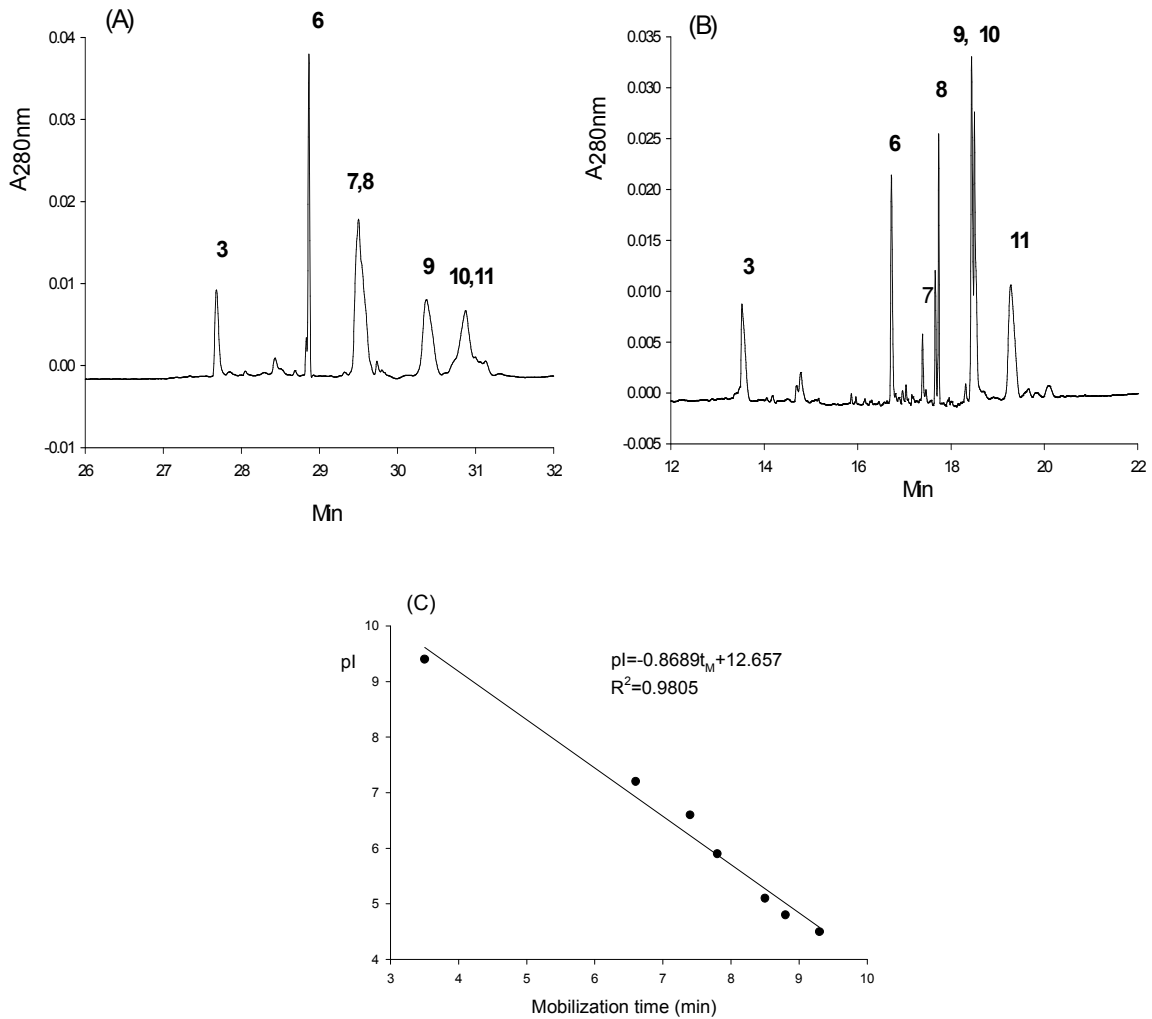

Figure 12. *CIEF separation with chemical mobilization using column S2. The sample proteins are listed in Table II. Buffer: 2% ampholyte with 2.5 mM zwitterionic surfactant (HDSPA) solution. Focusing was carried out at 333 V/cm for 15 min, with 20 mM H_3PO_4 as anolyte and 40 mM NaOH as catholyte. Chemical mobilization was achieved by replacing the catholyte with 50 mM NaCl solution at the end of focusing and applying 19 kV (A) or 10 kV (B) voltage. Other conditions are the same as in Figure 3. (C) Profile of linearity of the pH gradient for separation corresponding to (B).*

As stated in Chapter I, during the mobilization step, the application of high voltage should be kept to maintain the sharpness of peaks even though it causes high current and overheating problems. More often, the same voltage as used in the focusing step is applied for mobilization, as shown in Figure 12 (A) where 19 kV was applied for 57 cm long column with 50 cm effective separation length. Recently, it was observed that with only half of the voltage used for focusing step, the separation was even better and it helped solve the overheating problems as well. As can be seen from Figure 12 (A) and (B), peaks 7, 8 and 10, 11 were overlapped (in later runs, peaks 9, 10 and 11 appeared as one single peak) when using the same applied voltage (19 kV), but they were resolved when using only half of the applied voltage in the focusing step (10 kV). Another advantage, which cannot be neglected, was the sharpness of the peaks, which corresponds to high separation efficiency. As in Figure 12 (C), the profile of the pH gradient showed very good linear relationship (R^2 was up to 0.9805).

The same tendency was observed in Figure 13 (A) and (B) with the CHAPS-modified column. The difference between this column and the HDSPA-modified column was that the former column could not provide as good separation in the acidic region as HDSPA-modified column. As shown in Figure 13 (B), peaks 10 and 11 were not totally resolved. This may be due to the spatial effect (steric hindrance) of the structure of CHAPS, which made the bonding sites less available when interacting with the coated capillary surface.

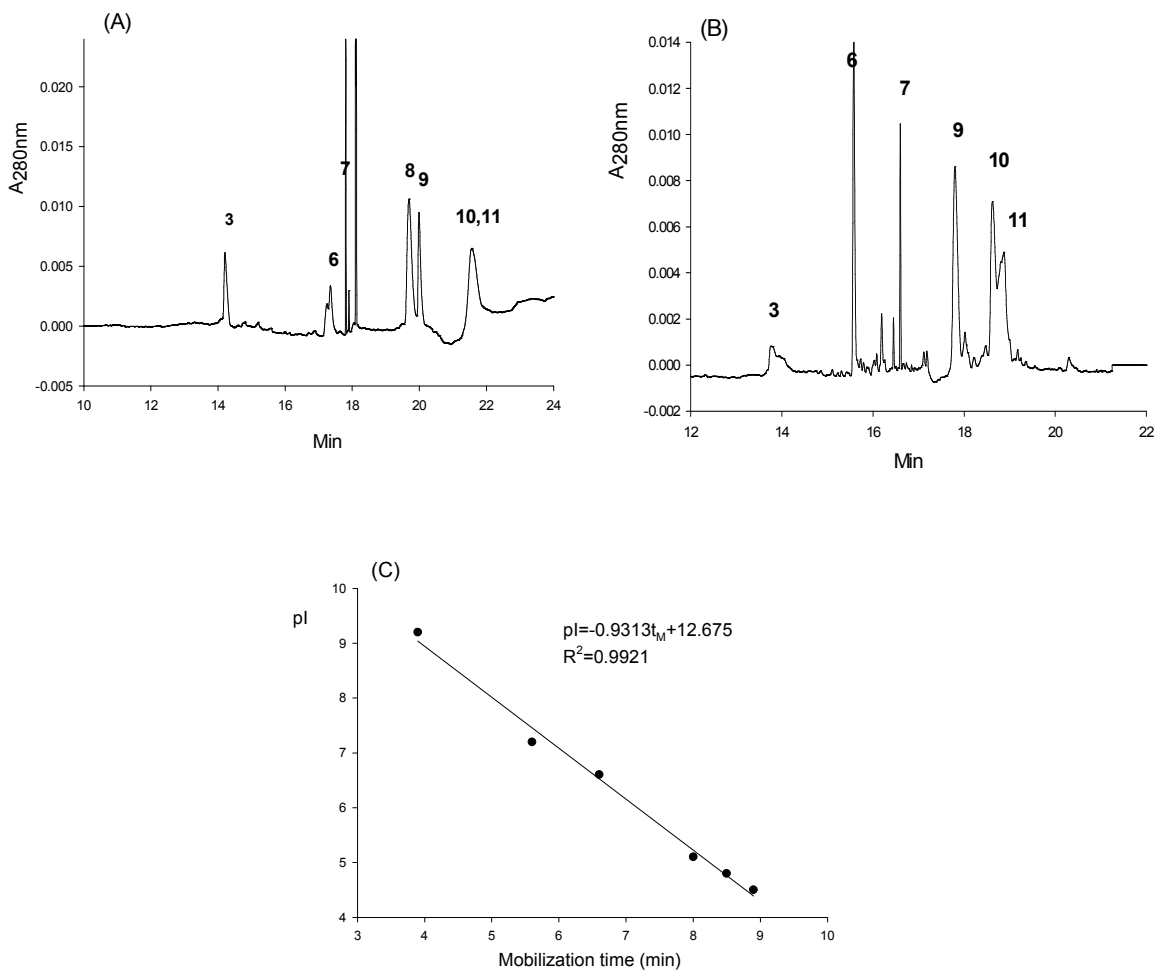**Figure 13**. *CIEF separation with chemical mobilization using column S3. Buffer: 2% ampholyte with 0.2 mg/mL zwitterionic surfactant (CHAPS) solution. Chemical mobilization was achieved by replacing the catholyte with 50 mM NaCl solution at the end of focusing and applying 19 kV (A) or 10 kV (B) voltage. Other conditions are the same as in Figure 13. (C) Profile of linearity of the pH gradient for separation corresponding to (B).*

Conclusions

This research has evaluated three series of capillary column coatings, namely HPC, GPTMS-HPC, and surfactants-HPC in the CIEF of standard protein mixtures. Among these coatings, the GPTMS-HPC capillary columns achieved good CIEF separations with pressure mobilization. However, the column stability was only slightly improved. On the other hand, the GPTMS-HPC coating provided a method to effectively reduce EOF. The HDSPA-modified column exhibited good CIEF separations with chemical mobilization, especially with half of the voltage as in the focusing step. Overall, the study has, for the first time, assessed the usefulness and limitation of each coating technique by performing step-by-step systematic evaluations.

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CHAPTER III

CAPILLARY ISOELECTRIC FOCUSING WITH IMMOBILIZED pH GRADIENT

Introduction

In principle, immobilized pH gradient (IpG) is more convenient than the carrier ampholyte pH gradient. Carrier ampholytes are expensive and their presences in sample mixtures reduce the detection sensitivity due to the high absorbance of ampholyte components at low wavelengths (e.g., 214 nm) where the detection of proteins is usually performed. Moreover, the presence of a large amount of carrier ampholytes increases the current and then induces the problem of Joule heating during focusing. In this regard, IpG columns used for CIEF separations provide the possibility of using low wavelengths without interference and a good chance for pre-concentration when CIEF is coupled with other analytical procedures.

Righetti *et al.* were the first to describe the concept of producing IpG for traditional electrophoresis, based on the principle that the buffering groups are covalently linked to the matrix used as the anticonvective medium [1, 2]. The pH gradient gels were cast in the same way as traditional pore gradient gels, but instead of varying the acrylamide content, the acryloyl monomer buffering solutions were adjusted to different pH values with the Immobiline buffers [3]. IpG in gel electrophoresis offered high

resolution and loading capacity, and it has uniform conductivity and buffering capacity [3-6].

To make IpG capillary columns, the most important step is immobilizing a pH gradient by predetermined compositions of ampholytes. In other words, the aim of the research is to find the optimized ampholyte components and their ratio used for immobilization. As will be demonstrated in this Chapter, the 25:75 Amphline/Biolyte ratio achieved the best separation pattern, and adding five different amino acids (pIs distributed from acidic to basic) into the immobilized stock solution yielded good linearity for the pH gradient.

Experimental

Reagents and Materials

Glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EDMA), 2, 2'-azobisisobutyronitrile (AIBN), 3-(trimethoxysilyl)propyl methacrylate (MAPS), and 1-dodecanol were purchased from Aldrich Co. (Milwaukee, WI, USA). Cyclohexanol, HPLC grade acetonitrile and methanol, analytical grade acetone, ammonium acetate and glacial acetic acid were all from Fisher Scientific (Fair Lawn, NJ, USA). Glutaraldehyde (25 %, w/w, in water), sodium cyanoborohydride and L-lysine, L-arginine, L-histidine, L-aspartic acid and L-glutamic acid were purchased from Sigma (St. Louis, MO, USA). 1, 4-Butanediol was from Baxter (McGaw Park, IL, USA). Ampholine with a pH range

of 3.5-9.5 was purchased from Amersham Pharmacia (Uppsala, Sweden). Biolyte with a pH range of 3-10, was purchased from Bio-rad Laboratory (CA, USA).

Fused-silica capillaries with an internal diameter of 75 µm and 100 µm and an outer diameter of 365 µm were from Polymicro Technology (Phoenix, AZ, U.S.A.). All other chemicals used for this study were the same as in Chapter II.

Instruments

The in-house assembled CE instrument consisted of two 30 kV DC power supplies of positive and negative polarity, Model MJ30P400 and MJ30N400, respectively, from Glassman High Voltage (Whitehouse Station, NJ, USA) and a UV-Vis variable wavelength detector, Model 200, from Linear Instrument (Reno, NV, USA) equipped with a cell for on-column detection. Detection was performed at 280 nm for all proteins. The electropherograms were recorded with a Shimadzu data processor Model C-R4A (Kyoto, Japan).

In experiments involving pressure mobilization, a syringe pump (74900 series) from Cole-Parmer Instrument Co. (Vernon Hills, IL, USA) was used.

Monolithic Capillary Column Preparations

Column Pretreatment: A segment of 75 µm or 100 µm ID fused-silica capillary of a desired length was treated with 1.0 M sodium hydroxide for 60 min, followed by few drops of water and the column was then flushed with 0.10 M hydrochloric acid for 60

min and was finally rinsed with water for 30 min. After this pretreatment, the capillary was then allowed to react with a 50% (v/v) solution of 3-(trimethoxysilyl)propyl methacrylate in acetone for 24 hrs at room temperature to vinylize the inner wall of the capillary. Thereafter, the capillary was successively rinsed with methanol and water and then dried under a stream of nitrogen.

Polymerization: Polymerization solutions weighing 2 g each were prepared from the monomers GMA and EDMA and a porogenic solvent in the ratio of 30:70 (w/w) monomers/solvents. The mixture of monomers was dissolved in a porogenic solvent consisting of 85 wt. % cyclohexanol, 15 wt. % dodecanol AIBN (1.0 % (w/w) with respect to monomers) was added to the solution as initiator [7-9]. The polymerization solution was then degassed by a stream of nitrogen for 15 min.

A 45 cm length of the pretreated capillary was then filled with the polymerization solution up to 35 cm by immersing the inlet of the capillary in the solution vial and applying vacuum to the outlet. The capillary ends were then plugged with a GC septum, and the capillary was submerged in a 50 °C water bath for 24 hrs. The resulting monolithic column was successively washed with acetonitrile and water using an HPLC pump. Columns made by this method were denoted as S-columns.

On the other hand, while keeping the same monomers, but changing porogen, we produced monolithic columns with low permeability (low flow rate). A segment of a 75 µm ID column was pretreated in the same way as for the above monolithic columns. A different stock solution was used to prepare this monolithic column. AIBN (7.2 mg) was added to a mixture of 160 µL of EDMA, 200 µL of GMA, 400 µL of n-propanol, and 800

µL of 1, 4-butanediol [10]. The solution was degassed by a stream of nitrogen and then sonicated for 5 min. The pretreated column was filled with this solution and plugged with a GC septum and then submerged in a 55 °C water bath for 12 hrs. The resulting monolithic column was successfully washed with acetonitrile and water using an HPLC pump. Columns made by this method were denoted as F-columns.

Immobilization of Ampholine: The immobilization of the pH gradient was performed as previously reported by Yang et al., with some modifications [10]. Ammonia (1 M dissolved in a mixture of 1:1 v/v water/acetonitrile) was passed through the monolithic column to react with the epoxy functions of the monolith for 4 hrs. A solution of 10% glutaraldehyde (dissolved in a mixture of 1:1 v/v water/acetonitrile) was pumped through the monolithic column, which was kept at room temperature for 12 hrs.

Solutions of different percentages of Ampholine and Biolyte mixed and dissolved in a mixture of 1:1 v/v water/acetonitrile were used to immobilize the pH gradient. The different percentages and corresponding column symbols are listed in Table 1. Table 2 lists another series of columns made by adding five different amino acids (L-lysine, L-arginine, L-histidine, L-aspartic acid and L-glutamic acid) into the immobilization solution.

The resulting columns were filled with the different immobilization solutions. The whole injection process was controlled under low temperature (about 0 ºC) by placing the coated capillary in a large glass tube (5 cm Outer diameter), which was filled with ice water. A gradually increased voltage (0-12 kV during 5 min) was applied to focus the carrier ampholytes. It is important to keep the current lower than 45 µA to

avoid air bubbles in the column. The focusing lasted about 25 min and then the capillary was kept at room temperature for 12 hrs in an attempt to allow the immobilization of the pH gradient onto the aldehyde activated monolithic surface.

TABLE 1

COMPOSTITION OF THE POLYMERIZATION SOLUTIONS USED IN THE PREPARATION OF THE DIFFERENT MONOLITHIC CAPILLARY COLUMNS

Monolithic Column Coding.	Percentage of Ampholine	Percentage of Biolyte
M1	100	0
M2	75	25
M3	50	50
M4	35	65
M5	25	75
M6	0	100

Scavenging: The resulting column was then rinsed for 4 hrs at room temperature with a solution of 0.4 M Tris/HCl (pH 7.2) solution containing 50 mM sodium cyanoborohydride to react with any unreacted aldehyde group [11]. The resulting column was then rinsed with water and cut to desired length, usually 32 cm total and 25 cm from the injection end to detection window.

TABLE 2

FIVE AMINO ACIDS AND THEIR CONCENTRATIONS IN THE
IMMOBILIZATION SOLUTION WHICH CONTAINED 25 % AMPHOLINE AND 75
% BIOLYTE (COLUMN M5)

Monolithic Column No.	Lysine (mg/mL)	Arginine (mg/mL)	Aspartic acid (mg/mL)	Glutamic acid (mg/mL)	Histidine (mg/mL)
A	1.0	1.0	0.8	1.2	2.0
B	----	----	0.8	1.2	2.0
C	1.0	1.0	----	----	----

Feasibility Evaluations

The column was filled with 50 mM ammonium acetate (pH 4.1) and the two columns ends were immersed in 20 mM H_3PO_4 and 40 mM NaOH, which were used as anolyte and catholyte, respectively. Subsequently, a focusing voltage of 12.5 kV was applied. If the current gradually decreased from its maximum value of ~ 40 μA to a stabilized low value of ~ 1.5 μA, it was taken as an indication that the carrier ampholyte was immobilized on the column surface. If the current dropped to zero within a very short time, it was taken as an indication that the coating was not successful [10].

CIEF Procedures

Protein samples were dissolved in 25 mM ammonium acetate buffer (pH 3.9) at different concentrations as listed in Table 3 and then filled into the column with a syringe pump. 20 mM H_3PO_4 and 40 mM NaOH were used as anolyte and catholyte,

respectively. Then a focusing voltage of 12.5 kV was applied, which lasted for 10 min. After focusing was finished (the current drop to about 25% of the original value), the protein zones were pushed to pass the detection window by a syringe pump with a flow rate of 0.25 mL/h (unless specified). Chemical mobilization was carried out by replacing the catholyte with a 5 mM NaCl solution at the end of focusing, however, the process was not successful and it usually resulted in a blocked column at the end of mobilization.

TABLE 3

PROTEINS USED FOR IpG COLUMN CIEF SEPARATIONS

No.	Protein	Source	Concentration (mg/ml)	pI
6	Myoglobin	Horse heart	0.16	7.2
7	Carbonic Anhydrase I	Human erythrocytes	0.08	6.6
8	Carbonic Anhydrase II	Bovine erythrocytes	0.16	5.9
10	α-Lactalbumin	Bovine milk	0.16	4.8
11	Trypsin Inhibitor	Soybean	0.22	4.5
12	β-Casein	Bovine milk	0.07	4.0
13	Amyloglucosidase	A. *niger pepsinogen*	0.12	3.6

Results and Discussions

Basic Characteristics of IpG Columns

The ampholytes used for immobilization in our studies were mixtures of Ampholine pH (3.5-9.5) and Biolyte (pH 3-10). As any ampholytes, they are a complex mixture containing many different oligoamino and oligocarboxylic acids with molecular weights from about 300 to more than 1000 [10, 12]. Ampholine (or Biolyte) reacts with glutaraldehyde and produces Schiff bases, as shown in Figure 1, which is the basis of forming immobilized pH gradient. [10] The last step is to reduce the C=N double bond to stabilize the covalent attachment. Upon applying high voltage, a process called focusing, locates carrier ampholytes in different positions according to their pI. After scavenging the unreacted aldehyde groups, the columns were ready for CIEF separations.

When the coating procedures was finished, the IpG monolithic capillary columns were first run with an ammonium acetate (50 mM, pH 4.1) solution at an applied voltage of 12.5 kV. The current value gradually decreased from a maximum of ~ 50 μA to a minimum of ~ 2 μA and remained stable. It worked well with the S-column series. This may be explained that after applying the high voltage between anolyte and catholyte, the acetate buffer in the column was gradually consumed and the amino and carboxyl functional groups immobilized on the monolithic surface maintained a low current along the column. However, it did not work for F-columns. The current value dropped to zero very quickly. This may be due to the very low pressure-driven flow resulting from the porogens used and their ratio. The porogens used (1-propanol and 1,4-butanediol) resulted in poor permeability and no continuous monolith [13]. Therefore, our research focused on optimization of the ratio of the two components Ampholine and Biolyte in the stock solution for immobilization. Pharmalyte was proved to be not useful for immobilization since no stable current could be established.
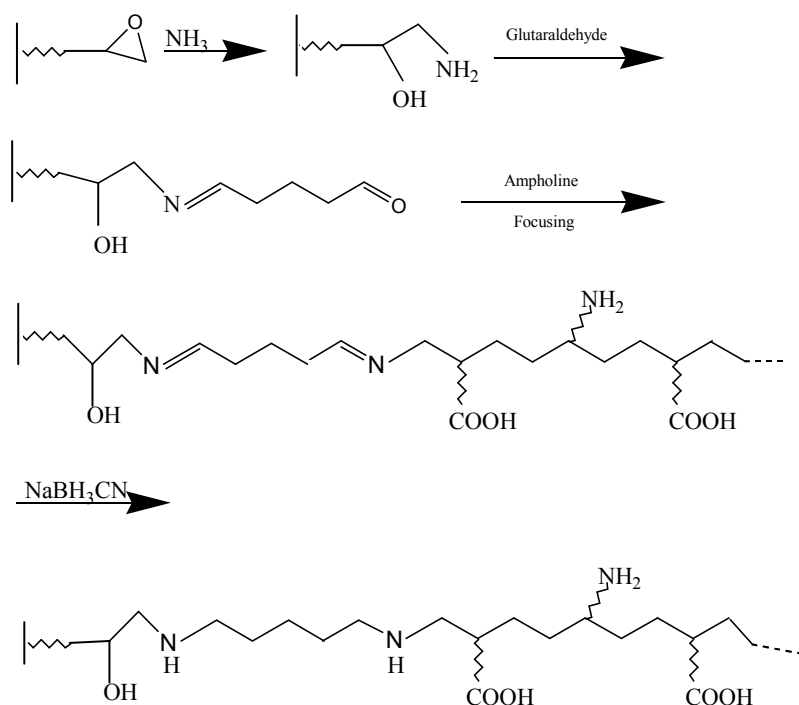
Figure 1. *Illustration of the process of preparation of immobilized pH gradient in a monolithic column. (These steps are the same for S-columns and F-columns.)*

Optimization of CIEF Separation Conditions with IpG Monolithic Columns

Unlike CIEF with carrier ampholytes, which has been extensively exploited, CIEF with IpG columns is relatively new although some similar research have been done (i.e., making use of the electrolysis of water [14], autofocusing [15] etc.). To achieve good CIEF separations with IpG monolithic columns, the experimental conditions still need to be improved substantially. Yang *et al*. [10] used 75 µm ID columns when they developed the method, but later we found out that a larger ID column (100 µm I.D.) was superior to a 75 µm ID column.

Effect of Column IDs: A mixed solution containing six proteins: myoglobin (pI 7.2), carbonic anhydrase I (pI 6.6) and II (pI 5.9), trypsin inhibitor (pI 4.5), β-casein (pI 4.0) and amyloglucosidase (pI 3.6) was used to investigate the effect of the inner diameter of the IpG monolithic column on CIEF separation. The immobilized pH gradient was provided by 100% Ampholine solution. As can be seen from Figure 2 (A) and (B), using a 75 μm ID column, carbonic anhydrase A I and II cannot be separated. The three acidic proteins with the lowest pI values were pushed out (i.e., mobilized) of the column at the same time. With a 100-μm ID column, carbonic anhydrase A I and II were well separated and there was a little improvement on the separation of the three acidic proteins. This effect may be attributed to the effectiveness of forming IpG caused by the extent of the immobilization reaction, that is, how many effective functional groups in the stock solution were attached to the column surface. In addition, larger ID columns provide larger monolithic surfaces than smaller ID columns, which can increase the surface density of the immobilized gradient.

Effect of Pressure-Driven Mobilization Flow Rate: Since only pressure mobilization is accessible for IpG monolithic columns and in practice, the flow rate used for mobilization is an important parameter that influences CIEF separations. In this study, three different flow rates were used and the separation results are shown as in Figure 2 (B),and Figure 3 (A) and (B) using 0.50 mL/h, 0.25 mL/h and 0.15 mL/h, respectively. As the electropherograms show, and under the same focusing conditions, using a lower flow rate yielded a better separation than using a higher flow rate, especially in the acidic region. In our studies, 0.25 mL/h was used for pressure-driven mobilization.
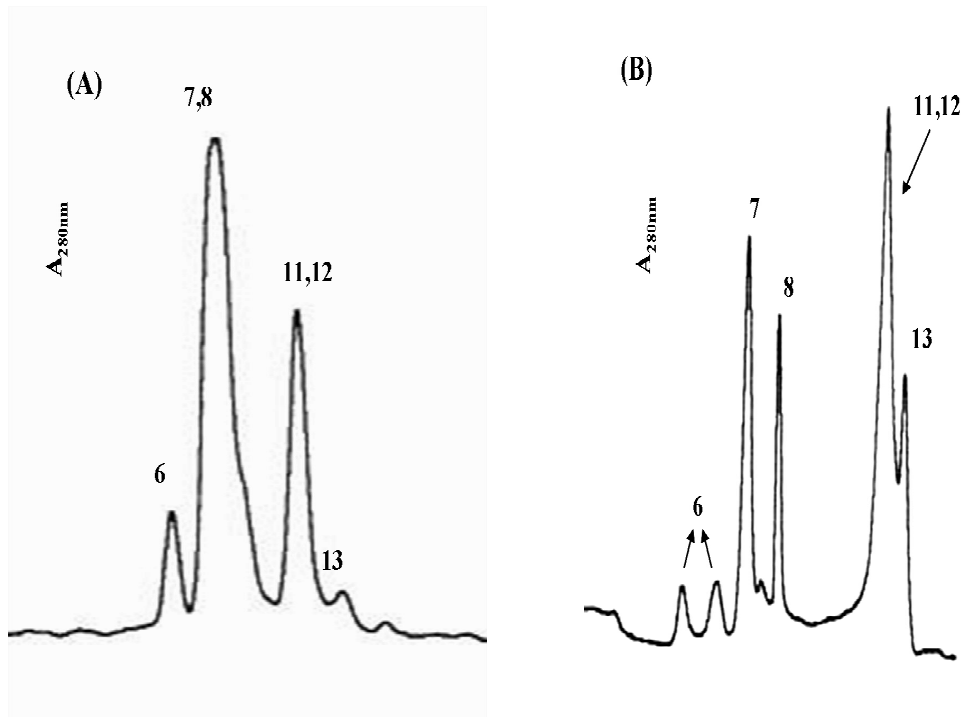
Figure 2. *Electropherogram of CIEF separation using monolithic IpG columns with 75 μm ID (A) and 100 μm ID (B). Immobilization stock solution used: 100% ampholine. Column: L= 35 cm and l= 27 cm. The sample proteins pIs and concentrations are listed in Table I. Anolyte: 20 mM H_3PO_4 pH 2.25 and catholyte: 40 mM NaOH pH 11.75. Focusing was achieved at 12.5 kV for 10 min and flow rate used for CIEF mobilization was 0.50 mL/h.*
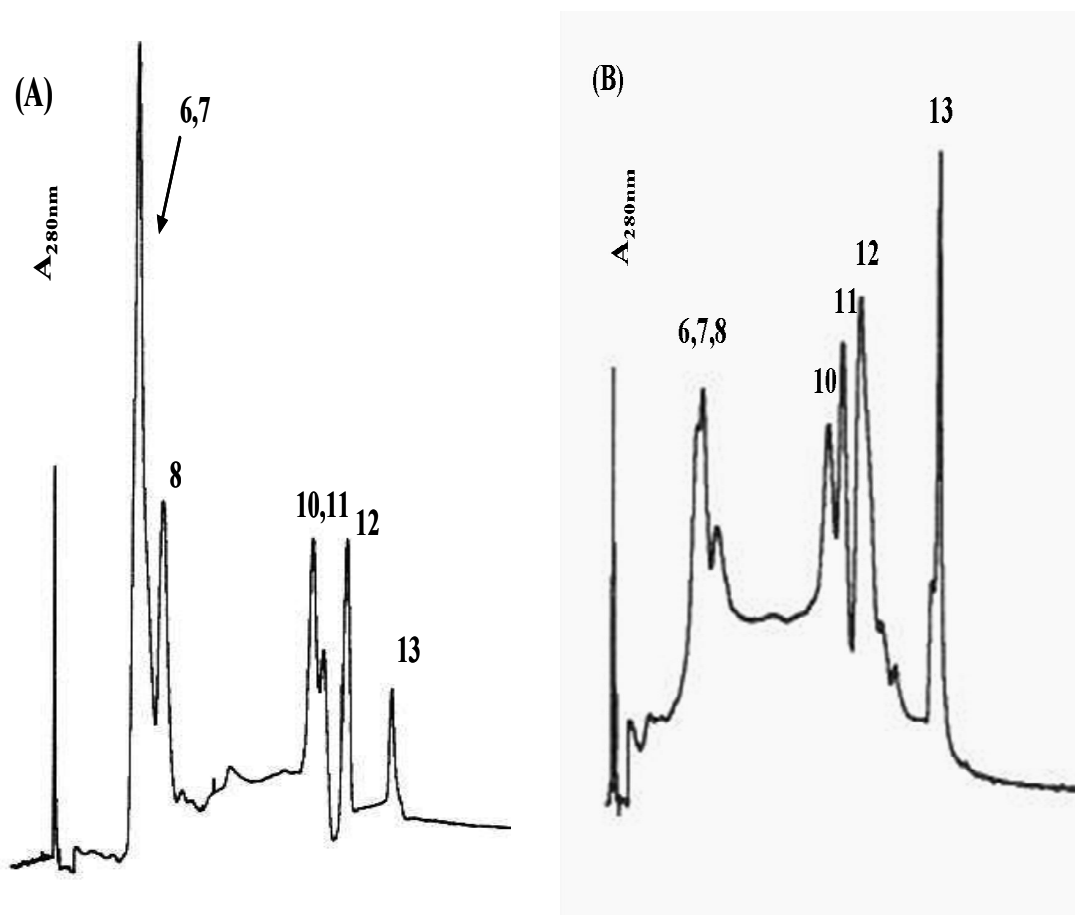
Figure 3. *Electropherograms of CIEF separation with monolithic IpG columns with different flow rates for mobilization: (A) 0.25 mL/h; (B) 0.15 mL/h. Immobilization stock solution used: 100% ampholine. Column: 100 μm ID, L= 35 cm and l= 27 cm. The sample proteins pIs and concentrations are listed in Table I. Other separation conditions are the same as in Figure 2.*

Effect of Ampholytes Compositions: To achieve a good CIEF separation, the formation of pH gradient is important too. As mentioned previously, ampholytes are a complex mixture containing many different oligoamino and oligocarboxylic acids with different molecular weights. The most essential property for a good carrier ampholyte molecule is a good buffering capacity at its isoelectric point. This requires many pK values close to the buffering capacity at its isoelectric point for each molecular species,

making most ampholytic substance especially most amino acids useful as carrier ampholytes [16]. Based on this theory, immobilized pH gradient produced by pure Ampholine and pure Biolyte were tested. The separation results of using 100% Ampholine (Column denoted as M1) is shown in Figure 3 (A). In general, most proteins can be separated by this column, but as far as the separation resolving power and pH gradient linearity are concerned, the column still needs to be improved. As to the column whose pH gradient is provided by 100% Biolyte (Column denoted as M6), it cannot achieve good separation at all. However, its failure is different from that of the F-columns. The M6 column is readily to be blocked in the process of preparation and its running current ($\sim$ 75 μA) is much higher than the other working columns if it can be tested. In the case of the F-columns, the current was very low and dropped to zero very quickly, which means there is no effective pH gradient formed on the monolithic surface. This should be attributed to the different compositions of ampholytes. The average pH range of Ampholine and Biolyte are almost the same, 3.5 to 9.5 and 3 to 10, respectively. However, the individual components and their ratio which made up this range must be very different. Ampholine and Biolyte are commercially available and their exact compositions are proprietary and not well disclosed. Thus, we mixed these two ampholytes and adjusted their ratio to achieve better separations.

Effect of Ampholine/Biolyte Ratio: In this investigation, the effect of the Ampholine:Biolyte ratio in solutions used to produce IpG was studied. Figure 5 shows the different separations corresponding to different Ampholine:Biolyte ratios. It was found that the higher the ratio of Biolyte, the greater the separation between the focused

protein zones. However, too much Biolyte (100%) in the stock solution did not yield satisfactory IpG columns. Column M6 was always blocked in the middle of preparation. In addition, Figure 4 shows the plot of pI vs. mobilization time, which reflects the linearity of the pH gradient. As can be seen, the plots were almost parallel except for M1, whose pH gradient was produced by 100% Ampholine. In other words, the properties of the columns whose pH gradient was produced only by Ampholine or only by Biolyte are different from those columns made by a mixture of the two ampholytes. This indicated that the components of the two ampholytes were much different. The slopes of the linear plots decrease as the percentage of Biolyte increased, which proved that in Biolyte, the pIs of the compositions were distributed in a wider range than the compositions in Ampholine. In addition, the linearity coefficients increased as the percentage of Biolyte increased with column M2 as an exception. Considering all the factors in this investigation, the Ampholine/Biolyte mixture with a 25/75 ratio was found to be the best to make IpG monolithic columns, as seen from Figure 4 (M5). Another detail must be noticed is the detection wavelength for column M4 and M5, usually 280 nm instead of 210 nm has to be used. There is too much noise at 210 nm and thus it is impossible to make peak identifications.

M1: $pI=-0.9416t_M+12.163$, $R^2=0.9153$

M2: $pI=-0.7541t_M+7.7404$, $R^2=0.9852$

M3: $pI=-0.6773t_M+8.2155$, $R^2=0.9718$

M4: $pI=-0.6232t_M+8.6847$, $R^2=0.9780$
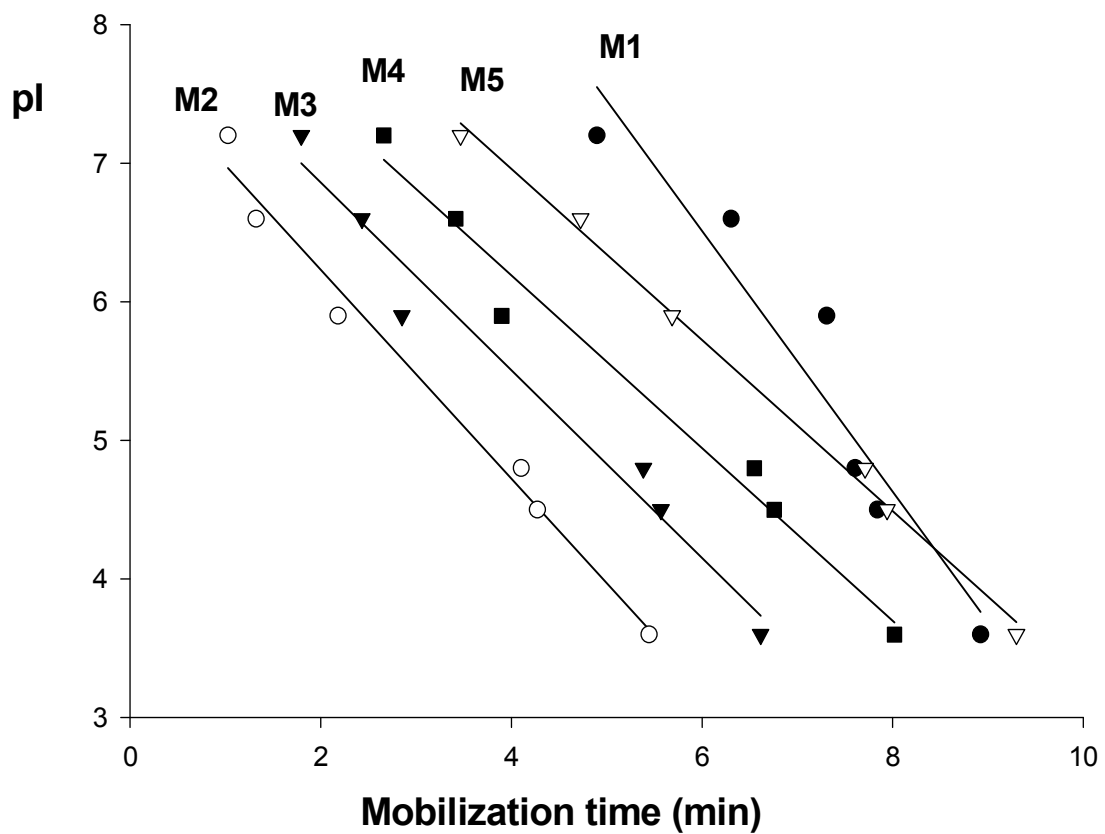
M5: $pI=-0.6175t_M+9.4291$, $R^2=0.9955$

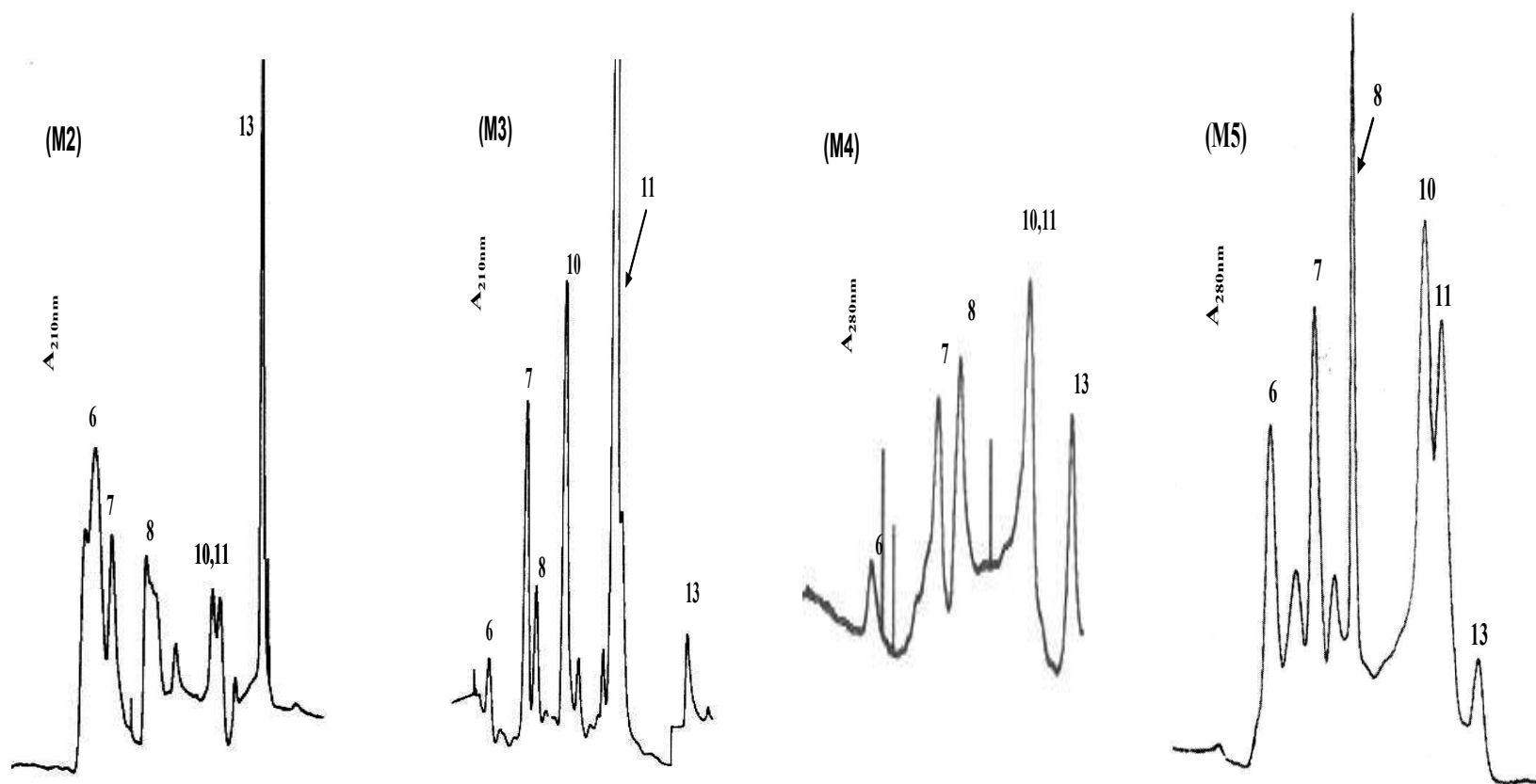**Figure 4.** *Linearity of pH gradient. Experimental conditions are the same as in Figure 5.*

Figure 5. *Electropherograms of CIEF separations using monolithic IpG columns. Column: 100 μm ID, L= 33 cm and l = 25 cm. Separation samples are the same protein mixture solutions, as listed in Table II. Column coatings: (M2) Column M2, 75% ampholine + 25% Biolyte; (M3) Column M3, 50% ampholine + 50% Biolyte; (M4) Column M4, 35% ampholine + 65% Biolyte; (M5) Column M5, 25% ampholine + 75% Biolyte. Anolyte. 20 mM H₃PO₄ pH 2.25 and Catholyte: 40 mM NaOH pH 11.75. Focusing: 12.5 kV for 10 min and mobilization was achieved with a pressure applied by a syringe pump at 0.25 mL/h.*

As mentioned above, ampholytes are made up of many amino and carboxylic acid functions with different pI values. Therefore, similar to mixing two kinds of ampholytes, adding amino acids into the ampholytes mixture should improve CIEF separation performance too. Five amino acids with pIs distributed from acidic (aspartic acid with pI= 3.0, glutamic acid with pI= 3.2) to basic (histidine with pI= 7.6, lysine with pI= 9.8 and arginine with pI= 10.8) were studied. In principle, the presence of some amino acids of specific pI values should increase the possibility of focusing proteins with closer pI values. Thus the aim of adding amino acids was to extend the immobilized pH gradient in the monolithic columns.

Effect of Amino Acids: The effect of amino acids was studied by adding different amino acids into the solutions of ampholytes used in the immobilization of pH gradient on the basis of column M5 (25/75 Ampholine/Biolyte ratio). The experimental conditions were the same as the conditions used for column M5. As shown in Figure 6, the electropherograms of the separation with 5, 3, and 2 amino acids were slightly different among each other as far as the separation resolving power and separation patterns are concerned. However, when compared with column M5, the resolving power increased as was evidenced by the sharpness of peaks. Figure 7 shows the corresponding plots of pI *vs.* mobilization time for each column. In this investigation, it was found that adding amino acids improved the peak shapes in the basic and neutral regions but very little extension of the pH gradient range.
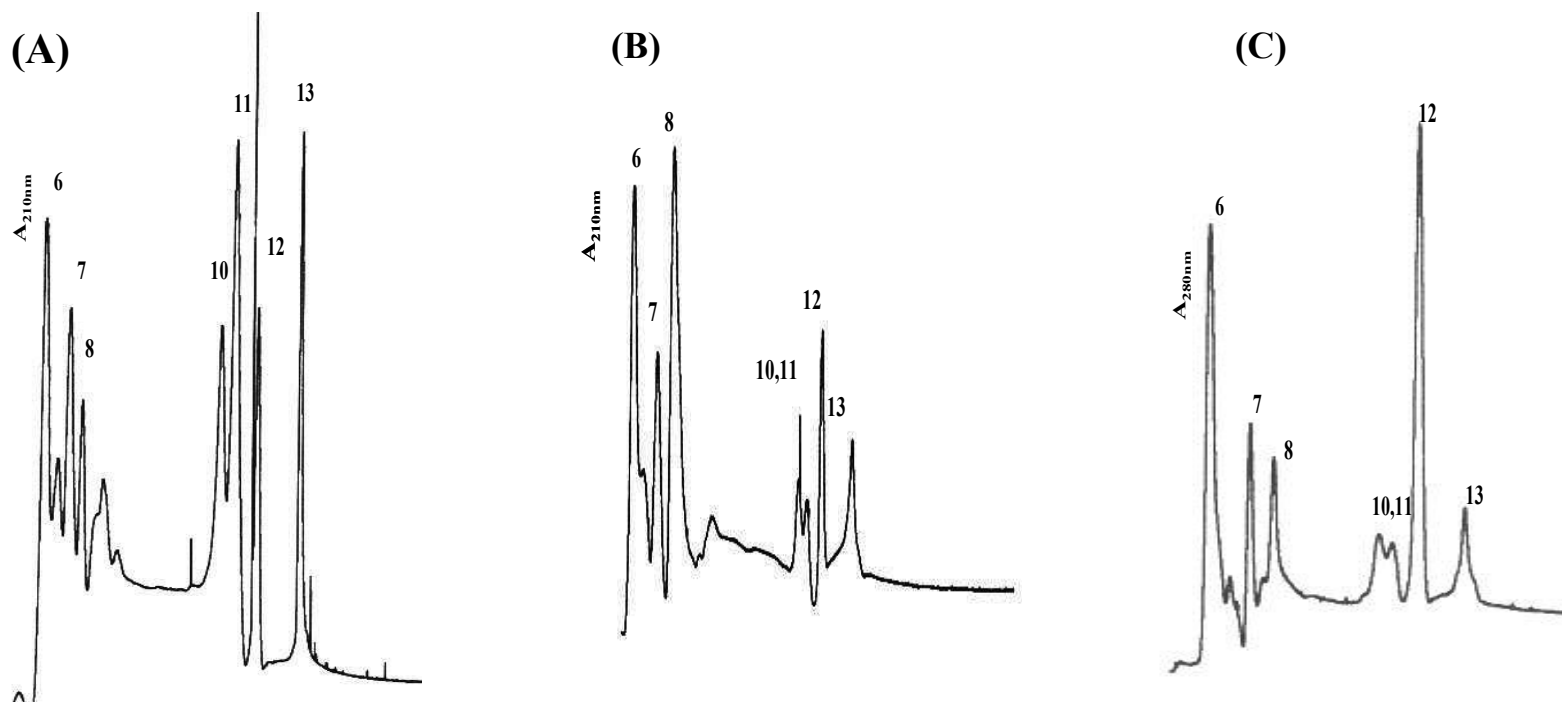
Figure 6. *Electropherograms of CIEF separation using monolithic IpG columns. Column: 100 μm ID, L= 33 cm and l = 25 cm. Separation samples are the same protein mixture solutions, as listed in Table II. Column coatings: (A) 25% ampholine + 75% Biolyte with 5 amino acids; (B)25% ampholine + 75% Biolyte with 3 amino acids (Aspartic acid, Glutamic acid and Histidine); (C) 25% ampholine + 75% Biolyte with 2 amino acids (Arginine and Lysine). Anolyte: 20 mM H_3PO_4 pH 2.25 and Catholyte: 40 mM NaOH pH 11.75. Focusing: 12.5 kV for 10 min and mobilization was achieved with a pressure applied by a syringe pump at 0.25 mL/h.*
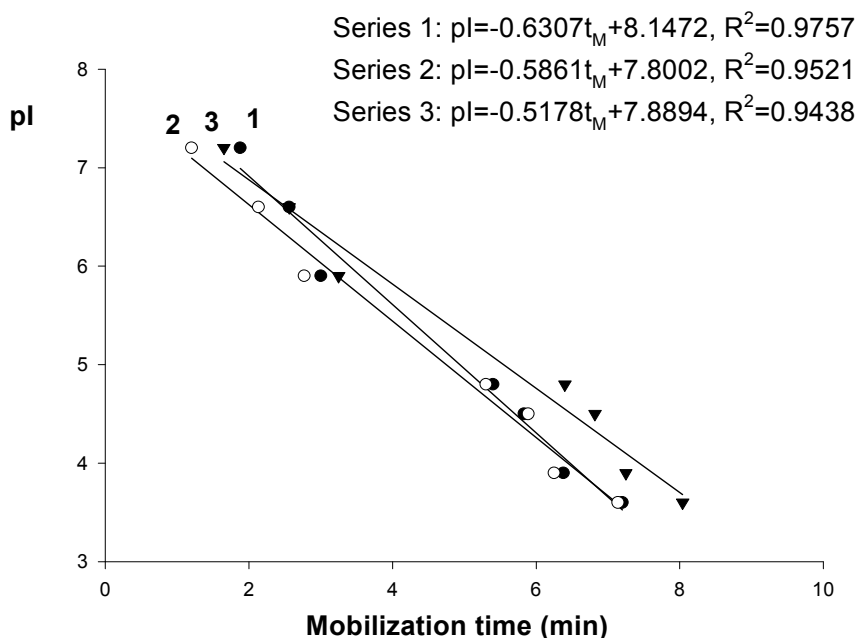
Series 1: $pI=-0.6307t_M+8.1472$, $R^2=0.9757$
Series 2: $pI=-0.5861t_M+7.8002$, $R^2=0.9521$
Series 3: $pI=-0.5178t_M+7.8894$, $R^2=0.9438$

Figure 7. *Plot of the linearity of the pH gradient. Experimental conditions are the same as listed in Figure 6.*

Conclusions

In this chapter, a novel technology to immobilize the pH gradient used in CIEF separations was performed as previously described but with some modifications [10] and its basic features were discussed. Moreover, a series of parameters that influence the separation performances were investigated. These include the effect of column IDs, mobilization flow rates and ampholyte compositions. Different Ampholine/Biolyte ratio mixtures as well as adding a few amino acids into these mixtures were evaluated in the aim of improving the resolving power and linearity of the immobilized pH gradient. In this investigation, the ratio 25/75 Ampholine/Biolyte with 5 five amino acids was found to be superior.

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