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Journal of Chromatography A, 735 (1996) 165–190

JOURNAL OF CHROMATOGRAPHY A

Review

Capillary isoelectric focusing as a tool in the examination of antibodies, peptides and proteins of pharmaceutical interest

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Abstract

This paper describes the recent history and development of capillary isoelectric focusing (cIEF), as it has evolved over the past 10 years forming a distinct mode of high-performance capillary electrophoresis (HPCE). The theory, equations, fundamentals and basics of cIEF are discussed and described, including modes of focusing and mobilization, coated vs uncoated capillaries, different detection schemes, resolutions possible, peak capacity possible and final commercialized approaches now available. Then, the applications of the technique are emphasized, as applied to smaller peptides, larger proteins and still larger antibodies and antibody–protein complexes. The emphasis has been on the application of capillary electromigration techniques in drug analysis. Throughout, attempts have been made to emphasize the potential applications and uses of cIEF methods, and how these might be successfully utilized in drug analysis and assays for larger biopolymers.

Keywords: Reviews; Capillary isolectric focusing; Antibodies; Peptides; Proteins; Haemoglobin; Transferrin

Contents

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SSDI 0021-9673(95)01357-1

1. Background to cIEF literature, theory and equations, current state-of-the-art, methodologies prevalent and commercial approaches available

1.1. General overview and introduction

There have been several reviews of capillary isoelectric focusing (cIEF), dealing with the background, current methodologies in high-performance capillary electrophoresis (HPCE), specific applications for proteins and peptides, and these have tended to cover the literature through 1992–94 [1–7]. This review is therefore a summary of what we and others have already described, perhaps in a different form, as well as an up-date on where cIEF stands today with regard to commercialization, optimizations possible and newer applications. There appears to be a growing and ever-increasing utilization of cIEF in the literature and through commercialization by various instrument vendors. Instrument firms are now offering commercial kits for performing cIEF with their instruments, as well as specific recipes and directions for success in newer applications. Application Notes from vendors have also appeared on the market, describing how to apply cIEF for various applications, and how it may be utilized in the future. It would therefore appear that just within the past few years, there has grown a widespread adoption of cIEF in the areas of proteins and peptides, with some important advantages realized, as described below. It also appears that cIEF will continue to grow in importance and utilization, despite some lingering problems, which may be resolvable in the long run.

1.2. Isoelectric focusing and capillary isoelectric focusing

The protein tissue plasminogen activator (t-PA) has been recombinantly manufactured in Chinese hamster ovary cells [8]. There exists a mutant form of the recombinantly produced t-PA which has a glutamic acid substituted for an arginine at amino acid position 275. This represents a difference of one amino acid out of 527. The substitution is easily detected by reversed-phase HPLC tryptic mapping. A new peptide peak appears in the tryptic map of the mutant form and another one present in the reference lot of rt-PA disappears.

Another analytical problem encountered with rt-PA is that the protein is composed of two different chains linked by a disulfide bond. During bioprocessing some proteolytic clipping of the protein may occur, leading to the production of some two-chain rt-PA, amino acids 1–276 and 277–527. The amount of two-chain form produced may be determined by size-exclusion chromatography (SEC), where the native, one-chain form has a higher-molecular-mass (M_r) and is retained less than the two-chain forms. rt-PA is also heavily glycosylated, since it is produced in mammalian cells. Several carbohydrate forms exist with different amounts of sialic acid. These different forms are resolved by isoelectric focusing, as recently reported by Yim [9], who used cIEF to separate the different carbohydrate forms.

The t-PA example clearly illustrates the need for many analytical techniques to solve protein characterization problems. We describe here the evolution of just one of these techniques, cIEF,

and why it appears to offer certain unique advantages for proteins and their analogs.

1.3. Isoelectric focusing (IEF) (non-capillary). Ampholytes

Ampholytes are crucial to the success of both IEF and cIEF, which is really the capillary analog of conventional, flat-bed IEF, but now in a fully automatable and instrumental approach. IEF separates ampholytes in a pH gradient by focusing all species to the same net charge (isolectric), that charge being zero. Ampholytes are compounds which can have a net negative or net positive charge, depending on the pH. Amino acids, which contain amino and carboxylic acid functionalities, are ampholytes, which in turn means that peptides and proteins are also ampholytes. All ampholytes have a pH at which they have no net charge, and this pH is defined as the isoelectric point, or pI. Hence, the basis of separation in IEF is pI differences between analytes. The pI of an ampholyte can be obtained from its titration curve, a plot of pH vs. net charge. In Fig. 1, the titration curve of glutamic acid, a simple ampholyte, is shown, and the structures of the different charge states of this amino acid are also shown [10].

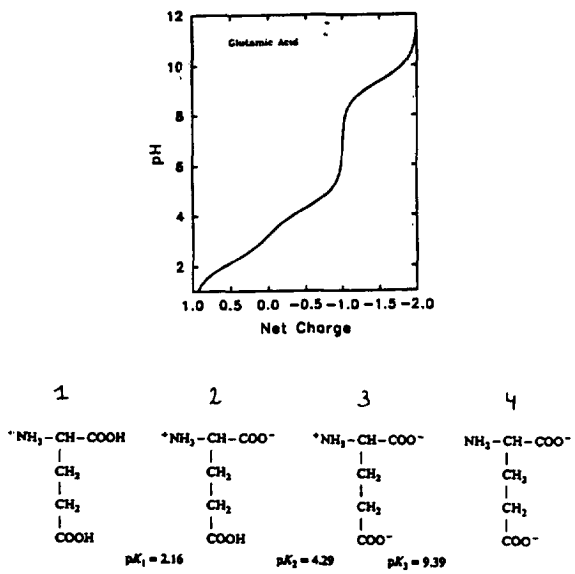

Fig. 1. Titration curve and structures of glutamic acid as a function of pH. (Reproduced with permission from Ref. [10]).

At pH values below 2, structure one is present, and the net charge on the amino acid is about +1. When the pH approaches and exceeds the pK_a of the α-carboxyl group, 2.16, structure two becomes more and more prevalent, and the net charge eventually becomes 0 at pH 3.22. As the pH approaches and passes the pK_a of the γ-carboxyl group, 4.29, structure three becomes more prominent and there is a net −1 charge. Finally, when the pK_a of the α-amino group is passed, structure four results, with a net negative charge of −2. At pH 3.22, glutamic acid has no net charge, and this pH is known as its isoelectric point, or pI.

The pI values of simple divalent amino acids, such as alanine, can be calculated by averaging the two pK values. For alanine, the pK_a of the carboxyl group is 2.3 and that of the amino group is 9.7, yielding a pI of 6.0. In the case of trivalent amino acids, such as glutamic acid, the pI can also be calculated by averaging the pK_a values of the two acidic or two basic groups, since the corresponding amino or carboxyl group will be 100% charged in the pH region near the pI and will play no role in determining the pI [10]. For glutamic acid, the pK_a values of the three ionizable groups are 2.13, 4.29 and 9.39. The pI (3.22) is calculated from the average of the two acidic pK_a values, since the amino functionality is completely protonated in this pH region. However, for large peptides and proteins, such simple rules cannot be applied, and the following equation must be used to calculate pI:

$$\text{net charge} = 0 = \sum n_i/(K_i/[H^+] + 1) - \sum n_j/([H^+]/K_j + 1) \quad (1)$$

where $i = 4$, arising from the N-terminus, lysine side-chain, arginine side-chain and histidine side-chain and $j = 5$, arising from the C-terminus, aspartic acid side-chain, glutamic acid side-chain, tyrosine side-chain and cysteine side-chain. For a net charge of zero, the equation is solved for hydrogen ion concentration, this pH being the pI.

This equation has the main assumption that a particular amino acid side-chain has the same pK value everywhere in the polypeptide chain, which is not always correct, especially in proteins, where three-dimensional effects can lead to differences. Specifically, the folded state of the protein can cause changes in the pK values of amino acid side-chains, depending upon whether or not the residues are buried, involved in hydrogen bonding or engaged in salt bridges with neighboring residues [11]. Nevertheless, the equation is reasonably accurate for small peptides and proteins.

It can easily be seen from Eq. 1 that the more lysines and arginines there are in a peptide or protein, the more basic is its pI (i.e., p$I > 7$), and the more glutamic and aspartic acids there are, the more acidic is its pI (i.e., p$I < 7$). Furthermore, it can also be seen why IEF is so powerful for detecting amino acid modifications, such as deamidation, where a neutral amino acid side-chain is converted into an acidic one, deletions and insertions occur, proteolytic clips ensue, N- or C-terminal modifications arise and carbohydrate content of proteins can vary greatly (e.g., antibodies).

1.4. Carrier ampholytes

Because the carrier ampholytes play such a crucial role in the final resolutions in both IEF and cIEF, we need to discuss how IEF resolves ampholytes based on pI differences. We should keep in mind at all times that the original ampholytes for IEF have been, for practical and commercial reasons, directly transferred into the cIEF format to obtain analogous but improved and easier to obtain separations. There have been advantages and disadvantages to their continued usage in cIEF, but until there are commercial sources of improved ampholytes, specific for cIEF requirements, users and vendors will continue to employ the older, IEF ampholyte mixtures.

The applied electric field and the pH gradient generated in IEF are responsible for the resolution based on pI. In IEF, an electric field is applied across a separation medium, with acid in the high-voltage reservoir (anode) and base in the low-voltage reservoir (cathode). The pH gradient is generated and maintained by carrier ampholytes, oligoamino-oligocarboxylic acids which have different pI values. These compounds were first obtained using a procedure developed by Vesterberg [12], where pentaethylenehexamine and acrylic acid were randomly polymerized together, generating thousands of ampholytes with different pI values. Today, there are several brands of commercially available ampholytes synthesized with proprietary procedures. The structure of a hypothetical ampholyte prepared by Pharmacia (Pharmalyte brand name) is shown in Fig. 2.

There are believed to be 50–1000 ampholytes per pH unit [13]. It has been calculated that in order to have an essentially stepless pH gradient, there should be about 30 ampholytes per pH unit [14]. Hence it can be assumed that the pH gradients established with commercially available carrier ampholytes are reasonably smooth, although not perfectly so [13].

It is not enough, however, that the ampholytes have certain pI values. If that were the case, there would be many possible ampholytes which could set up and maintain a pH gradient. The ampholytes used to set up pH gradients are called *carrier* ampholytes, as it is critical that they

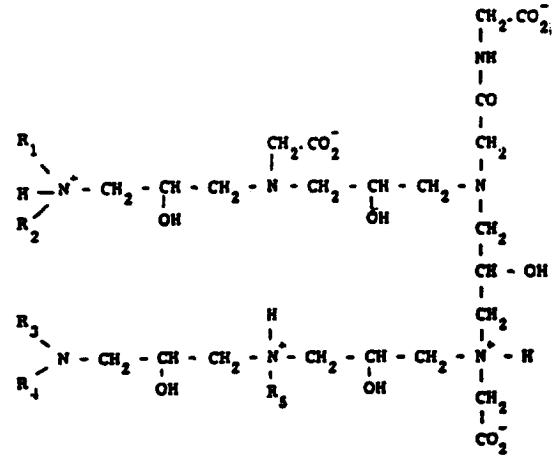

Fig. 2. Structure of a hypothetical ampholyte (Pharmalyte) prepared by Pharmacia [16]. (Reproduced with permission from Ref. [16]).

have good buffering capacity at their pI to "carry" the pH, and that they have good conductivity at their pI, to carry the current and maintain the pH gradient in a focused, continuous state. If the carrier ampholytes did not conduct appreciably in the focused state, large field drops would occur, leading to hot spots and poor resolution throughout the gradient. The ability to buffer at the pI, and also conduct, is determined by the degree of ionization at the pI, as discussed by Svensson [15]. The degree of ionization, α, is related to the total concentration of an ampholyte, C_T, and those of the cationic and anionic forms, C^+ and C^-, by the following equation:

$$\alpha = (C^+ + C^-)/C^T \quad (2)$$

The higher the value of α, the better are the conductivity and buffering capacity. In turn, the value of α is determined by the δpK value for a divalent ampholyte, where $\delta pK = pK_2 - pK_1$ [15]. The lower the δpK value, the higher is α and the better are the buffering capacity and conductivity of the ampholyte. There are very few naturally occurring ampholytes which satisfy all these criteria, which necessitated the procedure developed by Vesterberg to generate good carrier ampholytes. There are several types of commercially available carrier ampholytes covering wide (3–10) and narrow (6–8) pH gradients, with the trade names Pharmalyte (Pharmacia), Ampholine (LKB), Servalyte (Serva) and Bio-Lyte (Bio-Rad). Most commercial ampholyte mixtures span the ranges 3–10, 6–8, 9–11 and so forth.

Some other desirable properties of carrier ampholytes for IEF include (1) low M_r for ease of removal from proteins after separation, (2) minimal interaction with proteins, (3) good chemical stability, (4) solubility at the pI and (5) even buffering capacity and conductivity over the pH gradient range [13].

1.5. pH gradient formation and stability

In an electric field, a mixture of carrier ampholytes separate and form a pH gradient, with the most acidic ampholytes focusing near the anode and the most basic near the cathode. Fig. 3 shows the focusing and separation of three different ampholytes, A, B and C, in the beginning stages of focusing through the attainment of the steady state, where the most acidic ampholyte, C, has focused near the anode and the most basic, A, near the cathode. B has an intermediate pI. At the beginning of focusing, the three ampholytes are evenly distributed throughout the separation medium (a). As the focusing proceeds, A begins to stack near the anode and C near the cathode, with B in between (b). Finally, (c), the ampholytes have formed a steady state and are characterized by sharp, stationary concentration boundaries. During the initial stages of focusing, the current is highest, owing to the presence of many charge carriers. At the steady state, where complete focusing is achieved, the current is low. By following the current intensity and recognizing when it has become steady and decreased, one can identify the presumed focusing of all the ampholyte solution and resolution of the analytes present. This has been taken as a marker for complete focusing and a stage ready for mobilization and identification of species now resolved.

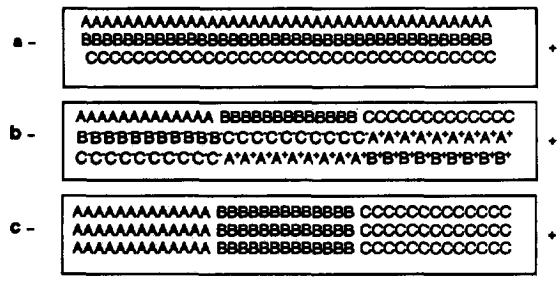

Fig. 3. Focusing and separation of three different ampholytes, A, B and C, in the beginning stages of focusing and through the attainment of the steady state, where the most acidic ampholyte, C, has focused near the anode, and the most basic, A, near the cathode [16]. The cathode is to the left and anode to the right. (Reproduced with permission from Ref. [16]).

Theoretically, once the pH gradient has formed, it is infinitely stable, as at their pI values ampholytes are not affected by the electric field. However, many researchers have shown that this

is not the case, there being certain drifts and non-linearity associated with ampholyte gradients. One well known effect is the plateau phenomenon, whereby the gradient tends to flatten out in the neutral region and become steeper in the acidic and basic regions [10]. This situation arises from the condition of electroneutrality. Consider a hypothetical ampholyte with pI = 10.0. The hydroxide ion concentration in the zone of this ampholyte is 100 μM. Thus, owing to electroneutrality, there must be an excess concentration of the positively charged form of the ampholyte over the negatively charged one of the order of 100 μM. This leads to a net migration of the ampholyte toward the cathode. For an acidic ampholyte, a similar argument can be made. Only neutral ampholytes will not have an excess of one charged form over the other. The further from neutrality an ampholyte's pI is, the greater is the difference between the two charged forms. In this discussion, a complex carrier ampholyte, with many ionizable groups, is assumed. At the pI, there is no net charge, but there are different charge forms. The net effect is that the resolution of analytes in the neutral region of the gradient will increase with time, and the resolution of analytes at the extremes of pH will decrease.

Other causes of the instability of pH gradients are known as cathodic and anodic drift, respectively. This refers to the successive loss of basic ampholytes into the cathode reservoir and acidic ampholytes to the anode. These drifts are isotachophoretic phenomena. Isotachophoresis (ITP) is based on ionic species assuming the same velocity and concentration of a leading ion of high mobility [10]. As discussed above with regard to the plateau phenomenon, ampholytes at the extreme acidic end of the pH gradient are net negatively charged, and those at the extreme basic end are net positively charged. Thus, in anodic drift, the anion in the anolyte acts as the isotachophoretic leader for the negatively charged ampholytes in the acidic region, while the cation in the catholyte does the same for the cationic ampholytes in the basic region.

Fig. 4 is a computer simulation of the focusing of fifteen ampholytes with 30 mM phosphoric acid and 30 mM sodium hydroxide as anolyte and catholyte, respectively [10]. In these simulations, the anolyte is to the left. At time zero, before application of the field, the ampholytes are evenly distributed between the phosphoric acid on the left and sodium hydroxide on the right. At 20 mins, the ampholytes have focused as sharp, Gaussian zones. Note, however, that the sodium ion boundary has begun to migrate towards the cathode, and that the most basic ampholyte is beginning to form a flat-topped ITP band. At 80 mins, the pI 10 ampholyte has formed a complete ITP zone, and the pI 9.5 ampholytes are beginning to do the same. The sodium ion boundary is almost completely out of the chamber.

At 100 mins, more basic ampholytes are forming ITP zones, and some of the acidic ampholytes are doing the same. As shown in Fig. 4, when equal concentrations of phosphoric acid and sodium hydroxide are used, the drift is more pronounced towards the cathode. This is due to the fact that the sodium ion boundary has a larger velocity than the phosphate one under these conditions.

The elegant work of Mosher and Thormann [10,17] has shown that, depending on the concentrations of anolyte and catholyte, primarily anodic, cathodic or symmetrical drifts can be obtained. When the concentration of sodium hydroxide is 2.25 times that of the phosphoric acid, symmetrical drifts are obtained. If the concentration of sodium hydroxide exceeds 2.25 times the phosphoric acid concentration, the drift will be anodic. If the ratio is less than 2.25, then the drift will be primarily cathodic.

1.6. Resolution in isoelectric focusing

Resolution in IEF is defined in terms of the smallest pI difference which can be resolved, with the following equation applicable [18]:

$$\Delta(\mathrm{p}I) = 3[D(\mathrm{dpH}/\mathrm{d}x)/E(-\mathrm{d}\mu/\mathrm{dpH})]^{1/2} \qquad (3)$$

where D is the diffusion coefficient of the analyte, dpH/dx is the rate of change of pH with

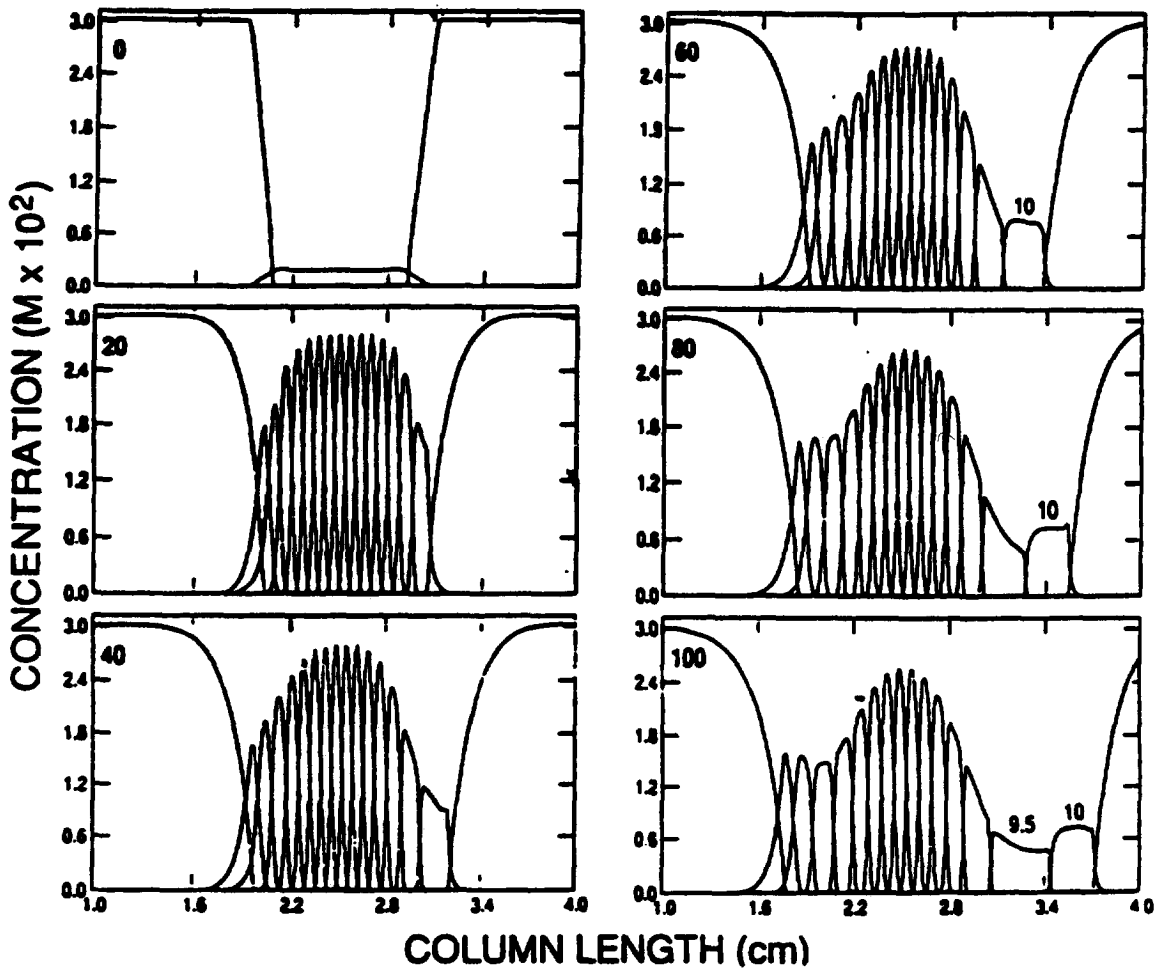

Fig. 4. Computer simulation of the focusing of fifteen ampholytes with 30 m*M* phosphoric acid and 30 m*M* sodium hydroxide as anolyte and catholyte, respectively. Numbers in the upper left of each panel are the focusing time in minutes. The anolyte is to the left. (Reproduced with permission from Ref. [10]).

distance x, E is the applied electric field and $-d\mu/dpH$ is the change in mobility with pH. Experimentally, the variables to be controlled are E, with higher fields leading to higher resolution, and dpH/dx, with narrower pH gradients leading to higher resolution. Thus, when separating a mixture containing a wide range of p*I* values, one would use a wide range pH gradient, such as pH 3–10. For high resolution of a narrower p*I* range, a narrow pH gradient would be used, such as pH 6–8, commonly used for hemoglobin analysis. A maximum resolving power of 0.02 pH units has been calculated for IEF with carrier ampholytes [13].

The two variables intrinsic to the analyte are the diffusion coefficient and the rate of change of mobility with pH near the isoelectric point. It is desirable that the analyte has a low diffusion coefficient and large rate of change of mobility with pH. In general, proteins satisfy these requirements fairly well. Smaller peptides do not, since they have larger diffusion coefficients and smaller changes in mobility with pH. It is desirable that the analyte's diffusion coefficient is

small, because once it is focused, there is an equilibrium occurring between electrophoretic and diffusional mass transport, described by the differential equation of isoelectric focusing [19]:

$$C\mu E = -D\mathrm{d}C/\mathrm{d}x \quad (4)$$

where C is concentration, μ mobility, E field and $\mathrm{d}C/\mathrm{d}x$ the concentration gradient. Integration of this equation leads to concentration profiles which are Gaussian. Thus, low diffusion coefficients lead to sharper bends. Furthermore, it is desirable that the analyte shows markedly different mobility with pH near the pI, since if diffusion does occur, the analyte will be moving into a region of different pH, acquiring charge. If the mobility changes greatly with a small change in pH, the analyte will quickly refocus at the pH = pI. If the analyte diffuses towards the anode, or into a lower pH region, it will attain a positive charge and will migrate toward the cathode and refocus at pH = pI, and vice versa. To obtain sharp bands, the rate of refocusing must be faster than the rate of diffusion. This can be accomplished by performing the separations in high-viscosity media to minimize diffusion, or by working with high fields.

1.7. Peak capacity in IEF

The peak capacity of a separation method is defined as the minimum number of peaks which can be fitted into a given time frame with a minimum resolution of 1 between adjacent peaks [20]. As described by Giddings [20], the peak capacity in IEF is calculated with the following equation:

$$n_c = (afL^2/2)^{1/2}(1/8RT)^{1/2} \quad (5)$$

where L is the separation length, R is the gas constant and T is absolute temperature. The quantity a is defined as:

$$a = -E(\mathrm{d}\mu/\mathrm{dpH})(\mathrm{dpH}/\mathrm{d}x) \quad (6)$$

Several important conclusions can be drawn from Eqs. 3, 5 and 6. First, larger fields lead to both higher resolution and peak capacity, as does a greater change in mobility with pH. Longer separation length leads to higher peak capacity. However, a larger pH gradient, which leads to higher peak capacity, also leads to lower resolution, because peak capacity encompasses the whole range of pH, whereas resolution focuses on only two peaks.

It is important to realize that the equations for resolution and peak capacity in IEF apply to experimental formats, where the system is allowed to come to a steady state, i.e., the pH gradient is completely focused. In dynamic IEF systems, as described below, the steady state is never attained, so the equations do not entirely hold, especially in terms of the field.

1.8. Problems in IEF and cIEF

The two most recognized problems in IEF are precipitation of proteins at the pI and intolerance to salt. Precipitation occurs at the pI since protein molecules have no net charge and do not electrostatically repel each other. Salt is a problem because it contributes to the conductivity of the medium and interferes with gradient formation.

Instability of the pH gradient has been discussed above. Carrier ampholytes do, in some cases, interact with proteins. These and other problems have led to the development of immobilized pH gradients [13], where the carrier ampholytes are chemically attached to a gel matrix. With immobilized pH gradients, resolution of species differing in pI by 0.001 pH units is obtainable, because extremely narrow pH gradients can be generated. Precipitation problems are also minimized in this approach, owing to the high ionic strength possible in the focused gradient.

1.9. Capillary isoelectric focusing. Introduction

Conventional IEF has been performed in a number of formats, including slab gels, ultra-thin gels, polyacrylamide rods, density gradient columns and quartz tubes [13]. The motivation for performing IEF, and also other forms of electrophoresis, in microcapillaries is the ability to apply very high fields without problems of Joule heat-

ing and convection [11]. Joule heating refers to the temperature increase in a separation medium due to the heat generated during application of the electric field. The heat generation rate, or power density Q (W/cm^3), is calculated with the following equation [21]:

$$Q = EI/(\pi r^2) \quad (7)$$

where E is the applied electric field, I is the current and r is the radius of the electrophoresis chamber. In the case of a tube, r is the internal bore radius [21]. A more important measure of the effect of heat generation is the temperature difference between the middle of the tube and the wall, DT_c:

$$DT_c = Qr^2/4k_b \quad (8)$$

where k_b is the thermal conductivity of the buffer medium in W/cm · K [21]. Thus, going to a smaller diameter tube, such as a microcapillary (I.D. less than 100 μm), greatly reduces the current level at a given field and the temperature difference between the wall and the center of the tube, allowing the application of much higher electric fields than in gels or large-diameter tubes (4 mm I.D.). Temperature differences between the wall and the center of the tube lead to band dispersion. This can either be due to convection because of density differences between the solution near the wall and the center, or due to the fact that the mobility of an analyte, μ, changes by approximately 2%/°C [21].

Eq. 4 demonstrated that in order to attain sharp bands in IEF, diffusion must be minimized and/or the electric field maximized. In gel IEF, the high viscosity of the gel matrix minimizes diffusion, but band dispersion due to Joule heating limits the field to about 1–10 V/cm [11]. In tubes of 2–4 mm I.D., fields are also limited to values less than 100 V/cm owing to convective band dispersion at higher fields [13]. IEF in μm I.D. capillaries should allow the application of fields in the 100–500 V/cm range [22].

The performance of IEF in capillary columns of 200 μm I.D. or less began with the work of Hjerten in 1985 [22,23]. His approach of using linear polyacrylamide-coated capillaries to eliminate electroosmotic flow (EOF) and salt mobilization for the detection of focused zones has been extensively developed and since commercialized [24–31]. Another group also reported using polyethylene glycol-coated capillaries and salt mobilization [32]. The rest of this review will be concerned with capillary isoelectric focusing (cIEF).

1.10. Coated capillaries and salt mobilization. Background

Hjerten and Zhu [22] believed that it was necessary to eliminate EOF for performing isoelectric focusing in silica capillaries. EOF arises in bare fused-silica capillaries due to the presence of ionized silanol groups, Si–O$^-$. These negative charges are fixed, while the positively charged counter ions are free to move in solution. When an electric field is applied, the positively charged counter ions are driven towards the cathode, dragging solvated water molecules along and generating a bulk flow moving from the anode to the cathode. Thus, the initial work used capillaries coated with methylcellulose [22] or bonded with linear polyacrylamide [22–26], which essentially eliminated any EOF. The coated capillary was loaded with the sample–ampholyte mixture and the focusing voltage applied, with acid at the anode (10 or 20 mM phosphoric acid) and base at the cathode (20 mM sodium hydroxide). The pH gradient was formed with Pharmalyte 3–10 as the ampholytes.

Initially, when the focusing voltage was applied, the current was at a maximum value, then immediately began to decay in an exponential fashion. This decrease in the current was due to the depletion of charge carriers in the capillary as the focusing proceeded. Theoretically, when the zones were completely focused, they would have no net movement in the presence of the electric field, since they would have no net charge and since there was no EOF or other bulk flow to move them. Thus, a means of pushing the focused zones past a stationary detection point in the capillary was required.

In the first study [22], two means of mobilization were investigated. One involved applying a

mechanical pump to the capillary and generating hydrodynamic flow. In this case, the focusing voltage was on during the pumping, to avoid any zone broadening due to the hydrodynamic flow. The disadvantage of this method of mobilization is the additional hardware required and the difficulty of applying a pump to a small diameter capillary. Such approaches are now commercialized, with some obvious improvements and advantages. The advantage is that protein zones can be recovered from the capillary quite easily in fractions.

The second means of mobilization involved turning the focusing voltage off when focusing had been achieved, followed by replacement of the base at the cathode with acid or replacement of the acid at the anode with base. Next, the voltage was turned on, leading to mobilization towards the cathode if the base was replaced, or towards the anode if the acid was replaced. To explain this phenomenon, we shall only discuss the case where replacement of the base at the cathode with acid is performed, but a similar argument would apply for replacement of the acid at the anode with base.

When both the anode and cathode contain acid (phosphoric acid) solution and the voltage is reapplied to the focused pH gradient, protons enter the capillary at the anode end and phosphate anions enter at the cathode end. The incoming protons begin to titrate the ampholytes and focused proteins until they are net positively charged, causing them to move towards the cathode, past the detection point. During the mobilization, the conductivity in the capillary increases owing to the incoming protons and phosphate ions, as evidenced by the increased current.

In Fig. 5, typical cIEF separations of hemoglobin and transferrin are shown using both anode mobilization and cathode mobilization. Note that during the mobilization steps, the electric field was 250 V/cm [22]. In Fig. 5a, where base is present at both the anode and cathode, mobilization is towards the anode, and the most acidic protein, transferrin, migrates first, since it focuses closest to the anode. In Fig. 5b, where acid is present at both the anode and cathode, mobilization is towards the cathode, and the most basic protein, hemoglobin, migrates first.

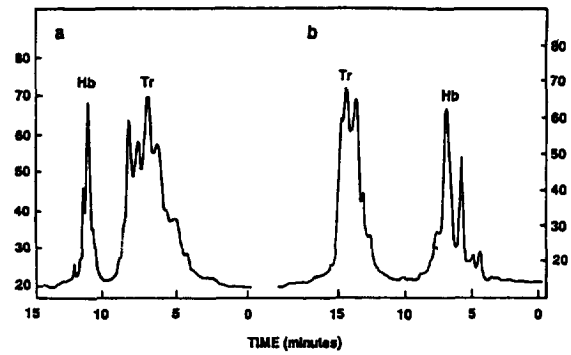

Fig. 5. Typical cIEF separations of hemoglobin (Hb) and transferrin (Tr) using both (a) anode and (b) cathode mobilizations. (Reproduced with permission from Ref. [22]).

As can be seen in Fig. 5, different separation patterns were achieved for each protein in the two different mobilizations. In both cases, resolution was highest for the protein which migrated first, indicative of loss of resolution for the later migrating proteins. Thus, using this type of mobilization, it appears that in order to obtain optimum resolution for both acidic and basic proteins, both anodic and cathodic mobilization must be performed.

In a later paper, Hjerten et al. [24] described the now popular salt mobilization technique. Rather than having acid or base at both the anode and cathode, salt, i.e., sodium chloride, was added to either the anolyte or catholyte. Owing to electroneutrality, if salt was added to the anolyte, mobilization would be towards the anode, and if added to the catholyte, mobilization would be towards the cathode. We shall consider the case where salt is added to the catholyte, called cathodic mobilization, but similar arguments can be made for addition of salt to the anode.

The concept of electroneutrality in IEF is that for every anion which enters the capillary, a corresponding cation must also enter or an anion leave. As a rough approximation, when acid is at the anode and base at the cathode, for every proton entering at the anode there must be a hydroxyl ion entering at the cathode. If sodium

chloride is added to the base at the cathode and voltage applied, for every proton entering at the anode, there will be an anion entering at the cathode. This anion can be either hydroxyl or chloride ion. Let us consider that five protons have entered the capillary at the anode. Owing to electroneutrality, five anions must enter at the cathode. Depending on the relative mobilities of the chloride ion and hydroxyl ion, it may be that two chloride ions and three hydroxyl ions will enter. In any case, the net effect is a reduction in the amount of hydroxide in the capillary, leading to titration of the pH gradient, generating a net positive charge on the ampholytes and bulk movement towards the cathode.

The salt mobilization technique suffers from the fact that the mobilization efficiency is not the same throughout the pH gradient [31]. When cathodic mobilization is performed, the acidic region of the pH gradient is mobilized with less efficiency than the basic region. This has been overcome by using a zwitterion instead of sodium chloride for the mobilization [31]. In Fig. 6a, cIEF of a standard protein mixture is shown with sodium chloride added to the cathode for salt mobilization. There was no peak seen for the acidic protein phycocyanin, p*I* 4.65. In Fig. 6b, a p*I* 3.22 zwitterion was added to the cathode instead of sodium chloride, and phycocyanin was detected. It was believed that the zwitterion entered the capillary and stopped in the pH gradient at its p*I*, forming an ever increasing zone width, thereby pushing the pH gradient above the zwitterion's p*I* out of the capillary.

The concept of using a zwitterion for mobilization can be used to mobilize portions of the gradient selectively. In Fig. 7a, a p*I* 3.22 zwitterion was used for the mobilization, whereas in Fig. 7b, a p*I* 6.90 zwitterion was used, leading to mobilization of all species with p*I* values above 3.22 in Fig. 7a, and only those species with p*I* values above 6.90 in Fig. 7b. The clear disadvantage of the zwitterion mobilization scheme is the different resolution generated in different regions of the gradient, as seen in Fig. 7a. Here, the resolution between the two hemoglobins, p*I* 7.5 and 7.1, was much larger than the resolution between the p*I* 7.1 hemoglobin and the phyco-

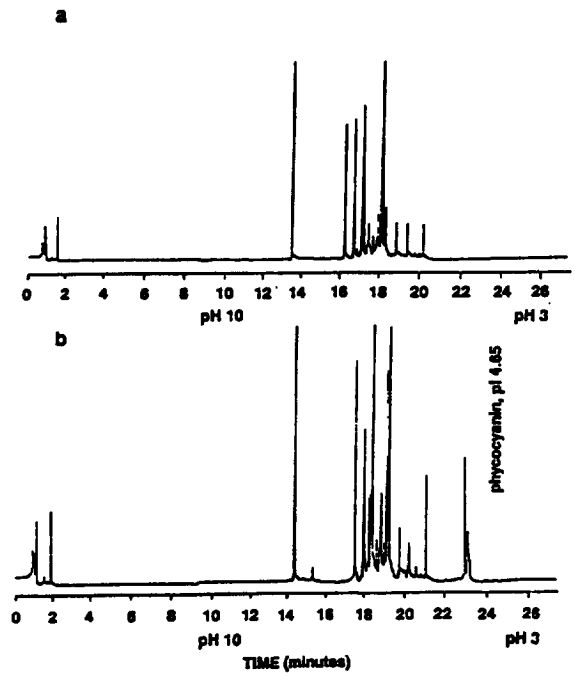

Fig. 6. Mobilization of standard protein mix (a) with sodium chloride in the cathode (phycocyanin is not detected) and (b) with a p*I* 3.22 zwitterion in the cathode. (Reproduced with permission from Ref. [31]).

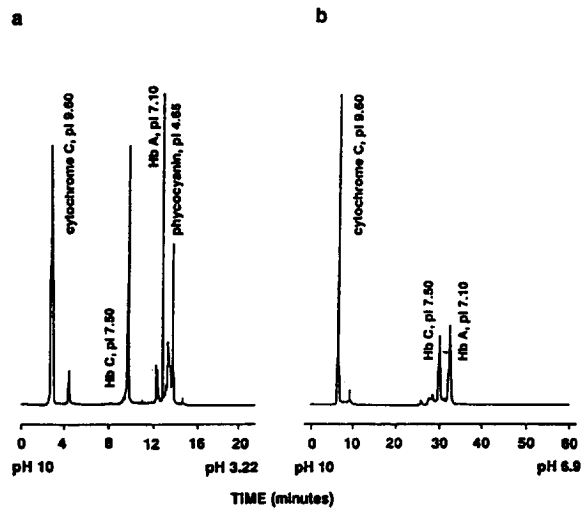

Fig. 7. Mobilization of proteins with (a) p*I* 3.22 zwitterion and (b) p*I* 6.90 zwitterion added to the cathode compartment. (Reproduced with permission from Ref. [31]).

cyanin, p*I* 4.65. It would seem that phycocyanin was mobilized with better efficiency than hemoglobin, leading to the poor resolution.

It appears at this point that no means of mobilization, whether salt, zwitterion or having acid or base at both electrode reservoirs, can provide a linear pH gradient. This has to be considered disadvantageous, since one would ideally like to use cIEF to determine the p*I* values of proteins quickly. Theoretically, this would be done by making a plot of migration time vs. p*I* over the pH gradient, then estimating the p*I* of an unknown based on its migration time. This plot can be used over the entire pH gradient if the gradient is linear but, as mentioned, this has yet to be achieved with the salt mobilization approach.

This problem of non-linear pH gradient seems to arise from the salt mobilization step. Specifically, those analytes which focus closest to the non-salted buffer are mobilized with poor efficiency. Since the salt mobilization approach essentially titrates the pH gradient, the poor mobilization of species at the extremes of the gradient may arise from differences in buffering capacity towards protons and hydroxides.

Another means of estimating p*I*s by cIEF has been described by Kilar [30]. As discussed above, during the mobilization step the current increases. In Kilar's approach, a plot of observed current at the migration time of a peak vs. its p*I* was constructed. However, the curve was not linear over even one pH unit portion of the gradient, and it is not clear that this approach could be used over a wide range of pH.

1.11. Recent improvements of cIEF over earlier approaches

Chen and Wiktorowicz [33] described the use of commercially available, dimethylpolysiloxane-coated capillaries (DB-1, J&W Scientific) and vacuum mobilization for performing IEF in capillaries. First, the capillary was filled with catholyte, 20 m*M* NaOH in 0.4% methylcellulose. Next, 0.5% Servalyte 3–10 in 0.4% methylcellulose was drawn into the capillary from the anode end to a position just before the detector, by applying vacuum at the cathode. A small amount of sample was then introduced by vacuum into the capillary at the anode, followed by a small amount of Servalyte solution. Focusing was then performed at 30 kV for ca. 6 min, followed by application of vacuum at the cathode, with the voltage maintained, to mobilize the focused zones past the detector. Resolution of some RNase T1 mutant peptides, differing in p*I* by 0.2 pH units, was achieved in less than 30 min total run time.

There are two main advantages of the Chen and Wiktorowicz approach. First, a linear pH gradient was achieved, as evidenced by a linear plot of migration times vs. p*I*, covering the pH range 2.75–9.5. This is due to the fact that the force of mobilization, vacuum, shows no discrimination in mobilizing different regions of the gradient, as with salt mobilization. Second, the coating used, dimethylpolysiloxane, seems to be much more stable than linear polyacrylamide, as linear plots of migration time vs. p*I* were obtained on one capillary after 2 months of continuous usage. This stability is attributed to the fact that the methylcellulose added to the ampholytes can coat any region of the capillary wall which may have been hydrolyzed [33]. The disadvantage is the fact that the vacuum mobilization produces a laminar flow profile, which could lead to band broadening and loss in resolution. This may be minimized by applying high fields. Furthermore, detectability is compromised in this format, since much less sample is loaded into the capillary at the start.

All of the above-discussed methods for performing cIEF have used experimental approaches which eliminate EOF. Mazzeo, Krull and co-workers described the performance of cIEF in uncoated capillaries, which maintains some EOF to allow for mobilization past the detection point [1–6,34–36]. Thormann et al [37] also developed a very similar method. An uncoated capillary was first filled with 20 m*M* NaOH containing 0.06–0.3% hydroxypropylcellulose, which was added to decrease EOF and minimize adsorption of proteins to the negatively charged wall. A small amount of sample in ampholytes (2.5 or 5% Ampholine 3–10) was then introduced by vacuum at the anode end. Application of voltage caused the protein and

ampholyte plug to migrate towards the cathode with EOF, focusing as it moved.

This represents an entirely different approach to performing cIEF, in the sense that focusing and mobilization are occurring at the same time. The key point is that EOF is slow enough to allow the bands time to focus, but fast enough to elute them past the detector. As with the method of Chen and Wiktorowicz [33], this method compromised detectability due to the lower load. Furthermore, resolution should be compromised, since the pH gradient is extended over a much smaller length of the capillary. Finally, performing cIEF in uncoated capillaries with EOF mobilization suffers from the pH dependence of the EOF.

The analogous, combined focusing with mobilization (residual EOF) approaches, introduced by Mazzeo, Krull and co-workers, follow along the lines of Thormann et al., but now filling the entire capillary (from anode or cathode end to detector window) with the sample and ampholyte mixture, as well as other additives [tetramethylethylenediamine (TEMED, methylcellulose, etc.] [1–6, 34–36]. Because the approaches of Thormann et al. and Mazzeo, Krull and co-workers, are so similar, and because they have been described several times in various reviews, they will not be described further in this particular overview, which is applications oriented. These basic approaches, especially that of Mazzeo, Krull and co-workers, have also been commercialized by several instrument vendors. Commercial kits are now available with coated capillaries (ABI, Beckman, Hewlett-Packard, etc.) to perform these methods, using protocols and procedures that have become almost standardized.

2. Existing/lingering problems inherent in current cIEF methods and possible future solutions

2.1. Advantages of cIEF

The general advantages of performing IEF in capillaries include the potential for quantification and automation, on-line detection, fast analysis times and, as described above, the ability to use high fields. CE has been referred to many times as the instrumental format of electrophoresis, and the same can be said of cIEF. In comparison with CZE, the advantage of IEF is that much more sample is loaded into the capillary, since no injection is made. Thus, detection limits should be lower and the preparative capabilities of cIEF should be greater than those of CZE. The specific advantages of the coated capillary and salt mobilization approach of performing cIEF are fast run times (due to high fields and short column lengths, 400–500 V/cm and 10–20 cm, respectively), high resolution and commercial availability. In terms of run times, the focusing step is usually of the order of 5–10 min and the mobilization step about 10–20 min, leading to total run times of 30 min or less. High resolution has been obtained, as evidenced by the separation of hemoglobin C, S, F and A in Fig. 8. These proteins differ in p*I* by as little as 0.05 pH units (A and F). Finally, the entire approach has been commercialized by Bio-Rad (Richmond, CA, USA) [29,31]).

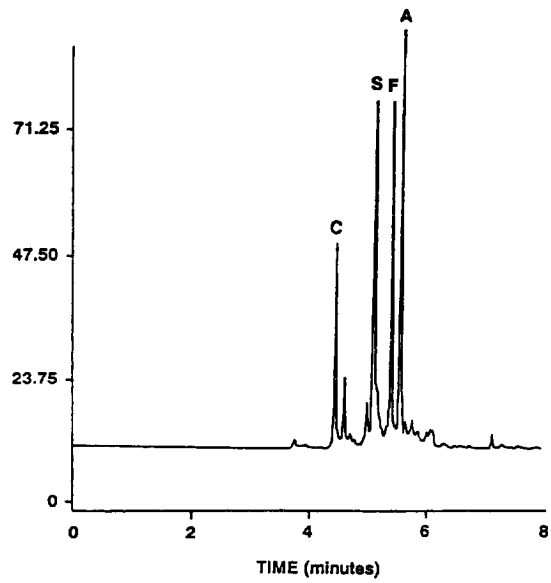

Fig. 8. cIEF separation of hemoglobins C (p*I* 7.50), S (p*I* 7.25), F (p*I* 7.15), and A (p*I* 7.10). (Reproduced with permission from Ref. [31]).

2.2. Limitations and existing problems

During the development of cIEF, several problems and limitations were discovered and then minimized. One is that proteins tend to precipitate at their p*I* values, a well known phenomenon in IEF, leading to sharp spikes in the separation pattern [25,29,31]. Addition of ethylene glycol or a suitable surfactant (e.g., Brij 35) to the separation media will minimize this problem. The method is very intolerant to salt in the protein sample, and all proteins must be desalted to a maximum of 10 m*M* prior to separation [31]. Intolerance to salt is well known in all forms of IEF [13], but seems to be especially problematic in cIEF, especially where the entire capillary is loaded with a mixture of sample and ampholyte. The presence of salt is easily noticed, as the initial focusing current will be much higher than usual and the separation will be marked by very broad peaks and sharp, inconsistent spikes from precipitation. Sample dependent, the removal of most salt content can be problematic, requiring careful dialysis or filtration and transfer to another, more compatible cIEF buffer. This would seem to be an insurmountable problem in all current modes of cIEF, as it has been in conventional IEF for many years.

Another problem is that the very basic portion of the gradient focuses past the detection point in the capillary, near the cathode, and cannot be detected unless mobilization is directed toward the anode. For instance, if a 14 cm capillary is used with a pH 3–10 gradient, there are 2 cm of capillary per pH unit. Thus, if the detection window is 10 cm away from the anode, the pH 8–10 region of the pH gradient will focus in the 4 cm of capillary past the detection point. This can be overcome by supplementation of the ampholytes with TEMED, a compound which extends the basic region of the pH gradient [38,39]. This causes the pH at the detection window to increase, forcing the pH 8–10 region of the gradient to focus before the window, allowing detection.

One issue with this approach is the stability of the coating with the higher pH in the region past the window. There are certain more pH-stable coatings, recently commercially introduced, that would appear to overcome these problems [1,2,6,7,40]. Even polyacrylamide-coated capillaries can now be made pH stable, by utilizing different linkage (immobilization) chemistries [7]. It is a matter of the linkage of the polymeric coating to the underlying silica support that provides pH stability, as well as the specific polymer (e.g., PEG, FC, PVP) itself. There are now several commercially available, permanently coated capillaries that are pH stable through even extreme pH values.

Detection is another point to consider. Theoretically, because cIEF is a concentration technique, detectability should be improved over free solution capillary electrophoresis (FSCE) with no preconcentration. However, because the ampholytes used to establish the pH gradient absorb below 280 nm, detectability is compromised in comparison with CZE, where wavelengths as low as 185 nm can be used. This is a serious and potentially fatal limitation of all cIEF approaches, no matter what the modes of focusing/mobilization. If it remains impossible to guarantee reasonable detection limits and/or even qualitative detection of all peptides in a typical peptide map, then the overall cIEF techniques will be limited in their final applications.

Recent reports of concentration gradient detection for cIEF may overcome this problem, as described by Wu and Pawliszyn [38]. In this case, an He–Ne laser is used as a probe beam, focused on a fixed position of the capillary, and a position-sensing photodiode measures the deflection of the beam. The deflection signal, q, is directly proportional to the concentration gradient, dC/dx, of an analyte according to the following equation:

$$q = \frac{L}{n} \cdot \frac{dn}{dC} \cdot \frac{dC}{dx} \tag{9}$$

where L is the I.D. of the capillary and n is the refractive index of the solution in the capillary [38]. Note that this is not a refractive index detector, but a refractive index gradient detector, such that the resulting chromatograms show derivative peaks. This detector is ideally suited to cIEF, since the sensitivity is directly proportional

to the concentration gradient of the analyte zone, and we have already discussed the ability to use high electric fields to generate large concentration gradients in cIEF.

The fundamental property of an analyte which this detector measures is dn/dC, and this value generally increases with M_r [38]. Concentration sensitivity, calculated to be 10^{-8}–10^{-7} M, is also limited by the fact that the ampholytes show signals, although not as sharp as protein zones since they do no exhibit large concentration gradients. As reported, the detector shows sensitivity similar to that of commercial UV detectors, but dual-beam designs may improve this situation [38]. Non-UV-absorbing ampholyte mixtures are not yet commercially available, and have not even been described in the open literature. There was no valid commercial reason for producing such novel ampholyte mixtures, since the market entailed flat-bed IEF approaches, without UV detection. It has only been since the introduction of cIEF, several years ago, that there has become apparent the interest in and perhaps the need to have fully transparent, non-UV-absorbing ampholyte mixtures of various pH ranges.

As discussed above, a major limitation of the coated capillary and salt mobilization approach to cIEF is the inability to obtain a linear pH gradient, owing to the lack of a mobilization scheme which can mobilize the entire pH gradient as a train. The question also arises of when to stop the focusing voltage and begin mobilization. It has been said that it is best to stop at some minimum current value, or to stop when a plot of current vs. time reaches a certain slope. It has also been reported that the migration time during the mobilization step is dependent on the focusing time [30]. One can imagine that it is critical that the focusing time be rigorously controlled to lead to reproducible migration times. Ideally, one would prefer not to have to perform mobilization in a separate step.

Rather than mobilize the gradient past a stationary detection point, the capillary can be scanned with the detector [41], or physically moved through a stationary detector [42]. As an extension of their work with a concentration gradient detector for cIEF, Wu and Pawliszyn have also described scanning the pH gradient by moving the probe beam and photodiode along the length of the capillary [41]. Alternatively, Wang and Hartwick [42] pulled the capillary through a stationary UV detector by attaching it to a motor. Both of these approaches fall into the category of whole column detection, and offer substantial advantages for cIEF. First, linear pH gradients should be obtainable. Second, the analysis time is decreased, since no mobilization step need be performed subsequent to focusing. Third, by using signal averaging, the signal-to-noise ratio and concentration detectability can be improved. However, they require removal of the outer polymide coating from along the capillary, which can lead to breakage, currently limiting their practical usefulness. The recently introduced, totally transparent, yet flexible capillaries for CE introduced by Supelco should be ideally applicable in these detection modes [43].

Finally, we come to the issue of the coating used to eliminate EOF, namely the stability of the coating, especially at alkaline pH [44,45]. In the case of a linear polyacrylamide-coated capillary, anchoring to the silica wall is through a siloxane linkage, Si–O–Si, a bond which is known to be susceptible to base hydrolysis [45]. The catholyte solution in cIEF is 20 mM sodium hydroxide, a condition basic enough to cause hydrolysis of the coating. A recent paper reported that when using the polyacrylamide-coated capillary for cIEF of antibodies in a pH 5–8 gradient, the column lifetime was five runs or less [44]. Clearly, the stability of the linear polyacrylamide-coated capillary, when used in cIEF, is an area of concern. Cobb et al. [45] described a linear polyacrylamide-coated capillary which is anchored to the silica wall by a Si–C–Si bond, which is much more pH stable than the siloxane linkage. This capillary was shown to have good stability at pH 2 and 11 when run in the CZE mode for protein separations. So far, there have been no reports using this coating for cIEF.

It seems that a greater issue is the stability of polyacrylamide itself. Specifically, it is well known that at high pH acrylamide is converted

into acrylic acid [13]. Even though the coating may be anchored to the silica wall in a pH-stable manner, as described by Cobb et al. [45], the acrylamide may be hydrolyzed to acrylic acid, leading to fixed negative charges on the wall and EOF in the cathode direction. This problem would become magnified in cIEF, where the pH in the capillary is not constant. This would lead to a non-homogeneous coating along the capillary. The answer may be to use vinyl acetate as the monomer for attachment to the coupling reagent of Cobb et al. This would give a linear poly(vinyl acetate)-coated capillary, which, when base hydrolyzed, would generate a poly(vinyl alcohol) coating. A poly(vinyl alcohol) coating should be pH stable over a wider range than the polyacrylamide coating.

There remain the lingering issues of detectability and universal detection of all proteins and peptides in cIEF modes. Unless a totally UV-transparent ampholyte mixture can be produced that would also be commercialized, future cIEF applications will be limited to those proteins, peptides and antibodies that are UV absorbing at or above 280 nm. Even the refractive index gradient detector of Pawliszyn is not totally universal, and it is very insensitive, depending on differences in the refractive indices of the analyte/protein peaks vs. ampholytes. There are some potential solutions to this major limitation, at least in terms of detectability. One might be to pre-injection tag all species in a homogeneous manner, generating single species (perhaps with multiple tags), which would then have improved UV and/or fluorescence (FL) detection properties [46,47]. This is not as easy or simple as in CZE or HPLC modes, since in cIEF protein/peptide derivatives would also have different or non-existent p*I* values, reaction/reagent dependent. If the reactions took place at free amino groups, the most likely sites of derivatization, this would alter final p*I* values and perhaps make these unusable for cIEF separations. It is also true that solution derivatizations of proteins and peptides, pre-injection, almost always leads to multiple products, which is counter to what is needed in CE and cIEF, in general [46,47].

Another potential solution is to use laser-induced fluorescence (LIF) detection, at least for those native fluorescing proteins and peptides, which would exclude a large number of other interesting analytes. This would solve the problem of UV detectability, but would introduce other problems of non-FL proteins, of which there are a very large number. Indirect UV/LIF detection, perhaps using a UV/FL-absorbing buffer component, in addition to the ampholyte, might be another possible solution. Also, if the additive had a very strong absorbance in the UV–Vis region, beyond 280 nm, this might provide improved detectability and universal detection for all proteins/peptides.

Clearly, there are lingering problems in applying cIEF in a more general manner than heretofore. The above problems are not insurmountable, just problematic. Since current commercial instrumentation is now able to run cIEF in a totally automated or automatable manner, it remains for both academic and commercial researchers to tackle the few, as above, lingering problems of salt content, concentration detectability, universal detectability and/or precipitation of the sample during the cIEF process.

3. Application of cIEF to antibodies, Fab fragments, affinity cIEF, immunoaffinity cIEF, etc.

Ever since the first report on the generation of monoclonal antibodies (Mab) in 1975, they have been widely investigated as purification and diagnostic agents and as therapeutic drugs [48]. According to a 1993 survey by the Pharmaceutical Manufacturers Association, 35% of biotechnology medicines in clinical trials in the USA were Mabs. In the synthesis and secretion process the antibody (Ab) molecule may be modified, or some artifacts or variants may be formed mainly through translation errors, improper folding, etc. Hence monitoring the purity of the product is of great importance.

IEF is one of the assays suggested by the FDA to characterize Mab products. Slab gel IEF has been widely used in biotechnology laboratories for the analysis of such proteins. In recent years,

cIEF has increasingly gained interest in this kind of application. Compared with conventional IEF, cIEF is much less labor-intensive or time consuming, it is easy to quantitate and automate and less sample is required. Indeed, of all the possible HPCE approaches, cIEF appears to offer the greatest advantages in terms of resolution of isoforms according to p*I* differences, in addition to identification and purity assessment. It also provides perhaps the best quantitative approach in terms of relative quantification of individual species (percentage area) or absolute levels, if suitable, individual Ab standards are available. In comparison with CZE or CGE, two other possible modes applicable to Abs, cIEF still appears to be the dominant and most valuable/suitable method for this class of proteins. Examples of individual applications of cIEF techniques to different Ab samples now follow.

Silverman et al. [44] reported the separation of a recombinant Ab, maB b72.3, by cIEF into five major and one minor band in a pH 5–8 gradient (Fig. 9). In their study, a direct comparison was made between slab gel IEF, cIEF and ion-exchange chromatography. In comparison with the other two techniques, cIEF was found to give the same resolution as slab gel IEF, but better than ion-exchange chromatography. cIEF was much faster than gel IEF, and the reproducibility of peak areas was much better by cIEF than gel IEF. However, the major limitations of cIEF were found to be the poor reproducibility of migration time, as high as 33% R.S.D., and the instability of the capillary coating, with capillaries being useful for only 3–5 runs. This has long been one of the major problems in using certain, coated capillaries (e.g., polyacrylamide) for cIEF with basic ampholyte mixtures. Certain coatings are generally unstable to highly basic conditions, as at the pH extremes of most ampholyte mixtures. However, certain newer bonded coatings have been developed, such as Hewlett-Packard's poly(vinylalcohol) (PVA) or polyethylene glycol (PEG), which appear to be much more durable and stable to pH extremes.

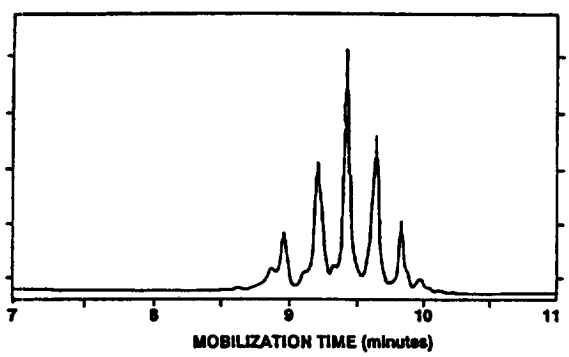

Fig. 9. cIEF separation of a monoclonal antibody. (Reproduced with permission from Ref. [44]).

Another application has been reported by Costello et al. [49]. They used cIEF and other traditional separation techniques, including sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), free zone capillary electrophoresis (FSCE = CZE) and gel IEF, to characterize humanized anti-tac Mab. In SDS-PAGE and CZE, fewer peaks were resolved. A similar, quantitative profile of five peaks was obtained in cIEF and gel IEF, but the quantitative profile of the five peaks was different. This might have been due to the differences in detection methods (280 nm UV detection for cIEF vs. organic dye and densitiometric scanning for gel IEF). No stability studies of the capillary coating and reproducibility results were reported.

A recent paper in this area was reported by Pritchett at Beckman Instruments [50]. In contrast to Silverman and Costello's work, in which two-step cIEF was employed with salt mobilization after focusing, one-step cIEF was used, so that separation and detection could be achieved at the same time. This particular approach, now successfully commercialized by Beckman Instruments, utilizes a pressure with applied potential mobilization step, after complete focusing of proteins, together with a coated capillary to eliminate EOF. Six peaks were recovered for the particular Ab (anti-CEA). The Ab was run together with internal standard p*I* markers, and the p*I* values of the Ab peaks were calculated by using a fitted, linear calibration graph.

The relative percentage area of each peak was determined by peak-area measurements. The R.S.D. of migration times was ca. 0.5% and the R.S.D. of peak areas was in the range 0.6–4.7%.

A study of Ab concentration vs. peak area was also performed, and good linearity was achieved in the Ab concentration range 25–250 μg/ml.

Another application, reported by Kundu and Fenters [51], showed different results. They found that the p*I* values of Mabs determined by the one-step cIEF method and fitted to a linear calibration graph showed appreciable deviations from the p*I* values separately determined by a slab gel method. They modified the one-step cIEF method by using non-overlapping internal standards with p*I* values below and above the p*I* ranges of Mabs and calculating p*I* values from a fitted, non-linear calibration graph. Differences between the calculated and literature p*I* values of the tested proteins were less than 0.04 p*I* unit when fitted, non-linear calibration graphs were used, compared with 0.3 p*I* unit when fitted, linear graphs were employed.

The above result was consistent with Conti et al.'s observation [52]. They found in the separation of hemoglobin variants Hb F (p*I* 7.05), A (p*I* 6.98) and F_{AC} (p*I* 6.95) that baseline separation was achieved between A and F_{AC}, but not between F and A, although the p*I* difference between F and A is about twice that between A and F_{AC}. This suggested that the pH gradient in the capillary was not linear. They attributed this to the uncovered silanol groups inside the capillary. A better separation was achieved by adding 3% short-chain polyacrylamide to the buffer, to form a dynamic coating, and 50 m*M* β-alanine to flatten the pH gradient around pH 7.

In addition to the characterization of Mabs, cIEF has been used to perform immunoassays of proteins. In Shimura and Karger's work [53], fluorescence (FL) tagged Fab′ fragments of the Mab was used instead of the intact Ab, because of the microheterogeniety of Fc portion of the Ab. The Fab′ fragment was in excess, it was mixed with the antigen (Ag) sample of interest, and incubated before they were introduced into the capillary. As the Ab and the Ag–Ab complex have different p*I* values, the excess Ab fragment was separated from the complex (Fig. 10). The Ag sample was determined by measuring either the Ab or the complex peak or both. Owing to the focusing effect of cIEF and the great sensitivity of LIF, a detection limit of 0.1 ng/ml of hGH was achieved. This is significantly lower than normally possible for direct protein detection with either UV or LIF, and approaches that which should be possible by selective (if possible) UV/FL, pre-capillary tagging methods [46,54].

In summary, cIEF is an excellent method for the characterization of Mabs. Its high resolving power makes it possible to discriminate the subtle changes in Mab isoforms. With the development of capillary coating techniques, both quantitative and qualitative analysis may now be achieved. The focusing effect of cIEF is a significant advantage when performing immunoaffinity CE [54].

4. Application of cIEF to peptides and proteins

cIEF is a high-resolution technique that has been used to separate proteins and peptides, as above, based entirely on their p*I* differences. In cIEF, a coated or an uncoated capillary with polymer additive in the buffer is generally employed. Single-point, on-column UV detection at 280 nm is most commonly used. Wu and Pawliszyn [38,41,55–57] developed a whole-column concentration gradient detector and imaging system. In a similar approach, Wang and Hartwick [42] designed whole-column absorbance detection. FL detection in cIEF has also been reported [58–60]. Beale and Sudmeier [59,61] recently described the application of FL detection in cIEF for standard proteins, but without any applications to actual samples. As few proteins or peptides are naturally fluorescent, proteins and peptides have first to be labeled with a FL tag before analysis. In most cases, multiple products were obtained, because more than one reaction site was available for the derivatization reaction. This problem has to be solved before FL detection can be routinely used for protein/peptide analysis in all of HPCE, not just for cIEF [46,54,62]. In HPCE, as opposed to HPLC, postcolumn tagging methods are problematic and non-commercialized, and also non-automatable, as yet.

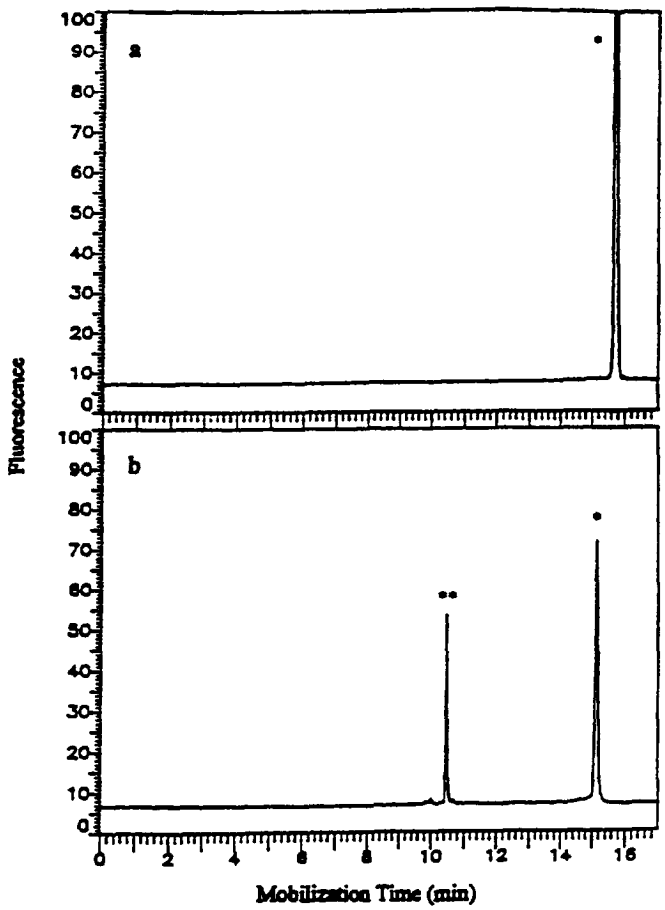

Fig. 10. Example of an approach to immunoaffinity recognition in cIEF using a FL probe Fab' fragment of the Ab [53]. cIEF analysis of TR-Fab'^{2} and its complexes with met-rhGH. APCE was carried out with Pharmalyte 3–10 and using TR-FAB'^{2} (700 ng/ml) as an affinity probe with (a) TTA–BSA buffer as a blank and (b) met-rhGH (100 ng/ml) as a sample. The free TR-Fab'^{2} and the complexes are marked with * and **, respectively. (Reproduced with permission from Ref. [53]).

Proteins such as cytochrome *c*, ribonuclease A, myoglobin, carbonic anhydrase, bovine serum albumin and trypsin inhibitor have been extensively studied in cIEF [33,37,63,64]. They were used as internal standard p*I* markers or model proteins to evaluate the cIEF methods being developed and applied. As RNase T1 and RNase ba are two proteins that have p*I* values at the two extremes of a pH 3–10 gradient, these two proteins, along with site-directed mutants of RNase T1, were sometimes used to demonstrate that the reported methods worked well in the entire pH range (pH 3–10) [33]. The cIEF study of proteins and peptides of clinical or pharmaceutical interest is reviewed below.

4.1. Hemoglobins

Hemoglobins (Hb), the main protein components in red blood cells, are polypeptide tetramers consisting of two pairs of unlike globin chains (e.g., α, β, γ, δ), each of which is covalently linked to four different heme groups [65]. Many congenital and acquired hematological disorders exist, which lead to both abnormal product of normal globin chains and/or the

production of abnormal or variant globin chains [39]. Thus, accurate identification and quantification of normal and abnormal Hb variants are of great clinical importance.

Four hemoglobin variants (A, F, S and C) have very close p*I* values, from 7 to 7.40; the p*I* difference between A and F is only 0.05 pH. These four hemoglobin variants have often been used as model proteins to demonstrate the high resolving power of cIEF. The potential utility of cIEF for clinical analysis of human Hb variants has been discussed by Zhu and co-workers [39,66], Molteni et al. [67], Hempe and Craver [68], Conti et al. [52] and others.

In Molteni et al.'s work [67], a high-resolution separation of Hb variants, including Hb A_{1c}, A, F, D, S, E and A_2, was obtained, thus permitting unambiguous characterization of Hb patterns of normal adults, newborns and patients with diabetes. Also identified were different hemoglobinopathies and thalassemia syndromes. This work compared cIEF data with two currently used diagnostic methods, viz., HPLC and gel IEF. The results were consistent qualitatively, which proved the feasibility of using cIEF for the analysis of Hb variants in red blood cell lysates. No quantitative data were reported.

Hempe and Craver [68] reported validation of the use of cIEF for routine clinical analysis of Hb variants in human blood. They compared the relative migration times and quantitative analysis of Hb variants in normal blood and in blood obtained from subjects with various hemoglobinopathies, shown in Fig. 11. Their results showed that cIEF estimates of p*I* values were very consistent, with intra- and inter-assay R.S.D.s <0.05% for all Hb variants tested. The detection limit for any variant was at least 0.5% of total Hb, which was more than enough for most of the diagnostic applications. Assay precision determined with commercial controls gave R.S.D. <0.2% for Hb A and S and 1–11% for minor variants A_2, F and A_{1c}. The results obtained by cIEF for patients' samples correlated well with values determined by conventional assays ($r^2 > 0.95$). These results demonstrated that cIEF is a rapid, sensitive and high-resolution automated method for the routine, quantitative clinical analysis of Hb variants. Conti et al. [52] reported the separation and quantification of the

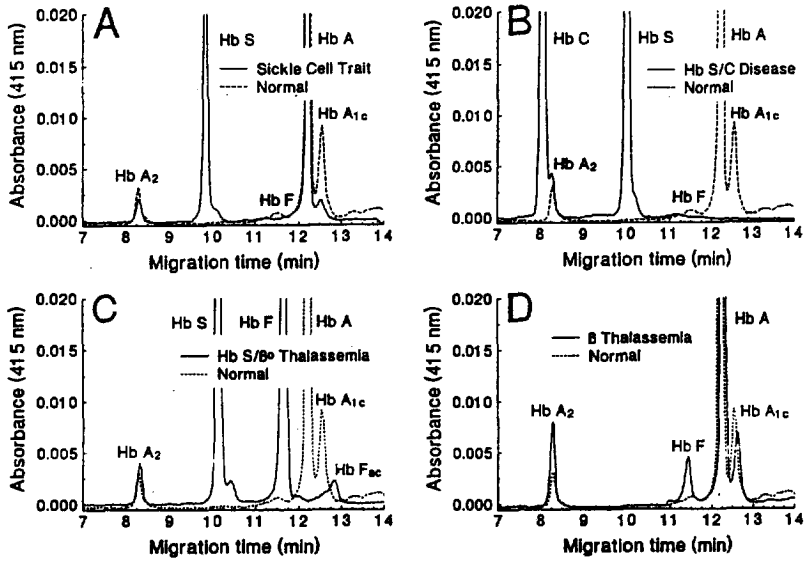

Fig. 11. Comparison of hemoglobin variants in normal blood (same sample, A–D) and blood from a subject with sickle cell trait (A), Hb S/C disease (B), Hb S/bo-thalassemia (C), or beta-thalassemia minor (D). (Reproduced with permission from the copyright holder and Clinical Chemistry, Ref. [68]).

three main Hb components of umbilical cord blood. The best separation was achieved by adding 3% polyacrylamide and 50 m*M* β-alanine to the ampholyte buffers.

4.2. Glycoproteins

cIEF has been used to separate the isoforms of glycoproteins, named glycoforms. Glycoforms are glycoproteins sharing an identical polypeptide backbone, but differing in the structural location and incidence of individual oligosaccharides. It is becoming increasingly obvious that protein glycosylation patterns are or can be very important, if not essential, to their function and/or therapeutic efficacy.

Kilar and Hjerten [26,27] reported cIEF separations of human transferin isoforms. The isoforms were different from each other by a single negative charge (mono-, di-, tri-, tetra-, penta-, hexa- and heptasialo). In one case, cIEF was used to separate transferrin bound to different numbers of iron atoms [26]. When iron-free transferrin was subject to cIEF separation, several peaks were obtained, which was believed to arise from different amounts of sialic acid bound to the transferrin [27]. When the iron-free transferrin was incubated with neuraminidase and the sample separated by cIEF over time, a clear pattern arose in terms of a decreasing number of peaks (Fig. 12), presumably owing to release of sialic acids. Wu and Pawliszyn [69] studied the interaction of iron and transferrin by cIEF with a concentration gradient imaging detection system. Transferrin was first focused inside the capillary by IEF. A plug of iron(III) was introduced into the capillary. Since the iron-free and iron-binding transferrins had different p*I* values, they were focused at different positions in the capillary. The whole process was monitored, and it was found that iron-binding and dissociation rates were different for different isoforms. They claimed that the binding kinetics could be studied in this way.

Another application was reported by Yim [9]. The glycoforms of rt-PA were separated in a pH 6–8 gradient. Several peaks were observed, but incubation with neuraminidase led to only two major bands of higher p*I*, strongly suggesting that the different peaks corresponded to proteins with different amounts of sialic acid. This case, and the transferrins above, indicate that separating glycoforms with varying sialic acid content is a strong point of cIEF. The fact that cIEF could discriminate such subtle differences between two complex and yet similar mixtures suggests that this technique could be used to study subtle changes in protein molecules.

4.3. Miscellaneous applications

Yowell et al. [70] reported the analysis of a recombinant granulocyte macrophage colony stimulating factor (GM-CSF) dosage form by cIEF. GM-CSF was easily separated from human serum albumin. A linear range for GM-CSF was observed from 10 to 40 μg/ml using a 47-cm capillary. When the protein concentration was over 250 μg/ml, no peak was observed owing to protein precipitation. They pointed out that the linear dynamic concentration range could be extended by shortening the length of the capillary, and this range was protein dependent.

Another potential application area of cIEF is for monitoring deamidation and proteolytic clipping of proteins. This has been reported for human growth hormone (hGH), wherein cIEF was used to monitor the degradation of hGH to the deamidated and clipped forms [29]. Application of cIEF to differentiation of lipoprotein was reported by Hu et al. [71]. Using narrow-range ampholytes, multiple peaks were resolved from HDL, LDL and VLDL samples isolated from human plasma.

cIEF analysis of metalloprotein was reported by Richards and Huang [72]. Chicken conalbumin, an iron-binding protein, was studied. Iron-free conalbumin separated into three major components with p*I* values of 7.18, 6.64 and 6.17. When the protein was saturated with iron (2 Fe/mol protein), a shift to lower p*I* was detected with a major peak at p*I* 6.07 and lesser peaks at p*I* 6.23 and 5.93. Hence, cIEF can provide additional information about the metal-binding properties of specific metalloproteins.

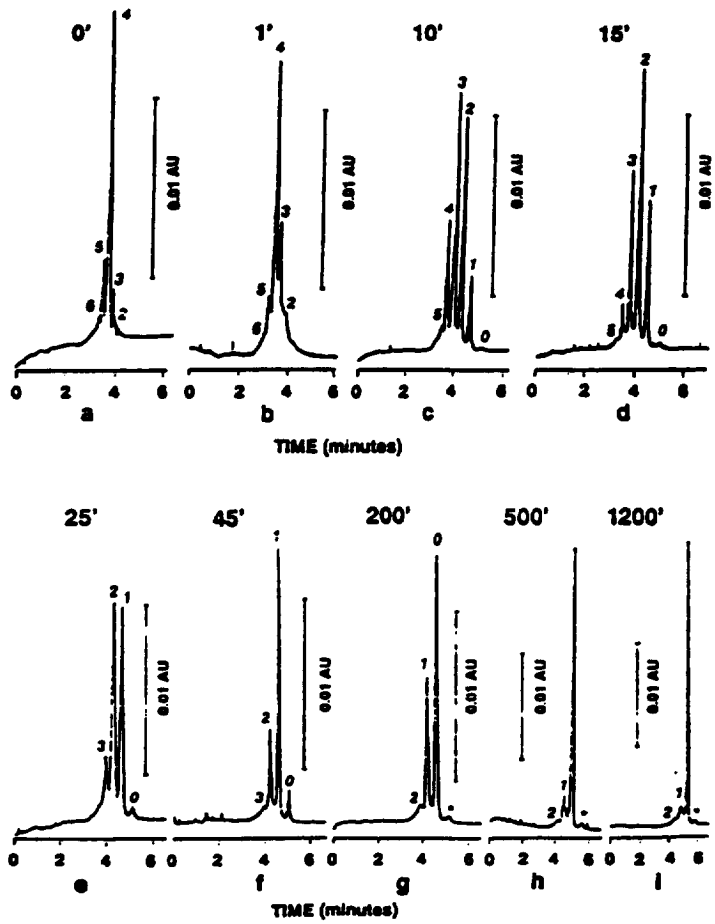

Fig. 12. cIEF separation of transferrin incubated with neuraminidase from 0 to 1200 min. (Reproduced with permission from Ref. [27]).

5. Summary and future developmental prospects

cIEF has now become a commonly used technique, in just under a decade of active research and development in various academic and industrial groups. In may slowly but surely replace conventional, flat-bed isoelectric focusing, especially when it is possible to utilize bundles of capillaries to run several different samples at the same time. It will also prove entirely feasible to run the same sample in different ampholyte gradients, again using an HPCE instrument with multiple capillaries. This could provide a great deal of information about the sample composition, p*I* value, purity of sample, nature of impurities and even peptide mapping by running several different ampholyte gradients at the same time. Applications to proteins, antibodies, peptides, tagged zwitterions (amino acids) and larger molecules, perhaps even viruses, all suggest a growing trend of using cIEF in more and more applications in bioanalytical chemistry. It has become clear that almost all major instrument manufacturers have now commercialized their own variety or brand of cIEF operations for their specific instrumentation. Application notes and technical literature on cIEF abound. The ability to automate cIEF methods fully is now commonplace, which makes future applications even

more likely and plentiful. cIEF has taken its place alongside CZE, CGE and MECC as one of the more popular methods for separating biopolymers, assuming that they have unique p*I* values.

However, for those species without a p*I*, such as neutral or small molecules, or uncharged polymers, the technique has little potential. Future advances in using cIEF for such materials will require tagging those neutral molecules in such a way as to produce a derivative having a unique p*I* value. This is clearly possible, requiring the development of reagents unique for cIEF applications, so that we can then impart to the original, uncharged analyte a p*I* of our choosing, reagent dependent. This is yet another area now under development.

The continued use of cIEF for affinity or immunoaffinity electrophoresis, perhaps even for immunoassays in CE, remains under development, and will provide improved methods for identifying and determining antigens for which unique antibodies are available. These techniques are not simple to operate, and they may always require Fab or Fab′ fragments of the original antibodies. They may also always require tagging of these fragments or the original antigenic protein with specific reagents to improve detection and/or alter p*I* values and therefore resolutions. There is significant interest at present in developing newer immunoassays using cIEF, and also immunoaffinity or affinity methods to alter resolutions via complexation, p*I* changes and so forth.

The ability to vary the p*I* values of the original protein is of interest, because in so doing we can then alter the resolutions obtained. We should, at some time, be able to select a given reagent for tagging, and predict changes in the final p*I* value of the tagged protein species. This could provide a unique set of reagents to force the protein to migrate in one electrode direction or the other compared with the untagged species, depending on how the final p*I* values change. Such reagents, having one or more charges on the tag, would also improve detectability by possessing a chromophore, fluorophore and/or electrophore somewhere in its structure. We could thus accomplish two worthwhile goals in cIEF, improved detectability and altered resolution with a single tagging species.

Finally, we come to the important goal of improving detectability, in general, for all species amenable to cIEF, including lower-molecular-mass peptides. This too will require either precolumn tagging with a suitable UV/FL-absorbing reagent, which will still leave the final derivative with a unique p*I* value, perhaps different from the starting analyte, as above. At the same time, such reagents will provide a general way to detect all species in cIEF, and not just those which absorb naturally (UV) above the ampholyte background. The problem in cIEF remains the nature of the ampholyte mixtures, materials that were never first developed for cIEF.

In conventional flat-bed IEF, because the methods of final detection did not involve direct UV absorbance, the nature of the ampholyte mixtures was relatively unimportant from a detection perspective. However, when we simply take such commercial ampholytes and apply them in cIEF, we introduce UV-absorbing materials that block absorbance by the analytes at wavelengths lower than 280 nm. This has been and remains a most serious disadvantage in the continued usage of such UV-absorbing ampholytes. There simply must be a better material/additive found if cIEF is to be useful for non-UV-absorbing species and offer a guaranteed universal detection for all species once separated. This is especially crucial for peptide mapping approaches in cIEF, where smaller and smaller peptides will not possess significant UV absorbance at 280 nm or above. Unless we can devise tagging methods that still permit resolutions based on p*I* differences, and/or non-UV-absorbing ampholytes, we shall never be able to provide low-level detection of all species present in a sample.

The continued interfacing of cIEF with MS is also an important area. cIEF provides sample preconcentration during the separation process, which means that eluting species entering the mass spectrometer have much higher concentrations and therefore detectability. The major

problem in using cIEF–MS techniques is that the ampholyte buffer entering the ionization source also provides a high background noise level that can, at times, interfere with the direct detection of more interesting sample species. The presence, at times, of high-M_r ampholyte species in the mass spectrometer only complicates identification of a given species from the sample, especially if this is of a relatively low M_r and at trace levels. The commercial ampholytes now being used in cIEF were never developed with the idea of eventually using them in the cIEF–MS mode. They are not only UV absorbing, but also produce very strong ions in the mass spectrometer. The continued development of newer ampholyte mixtures, having narrow and broad pH ranges, non-UV-absorbing, and not producing high-m/z ions in the mass spectrometer, remains a worthwhile goal. The problem, of course, is that almost all ampholytes are broad distributions of high-M_r polymers, zwitterionically charged, providing a broad distribution of pI values, just what is needed for cIEF. The use of alternative buffers, such as Good's buffers, has not yet yielded as good cIEF resolutions as conventional ampholyte mixtures, and they may never.

6. Abbreviations

Ab	antibody
Abs	antibodies
Ag	antigen
anti-CEA	antibody to carcinoembryonic antigen (cancer marker)
cIEF	capillary isoelectric focusing
CE	capillary electrophoresis
CZE	capillary zone electrophoresis
ED	electrochemical detection
EOF	electroosmotic flow
FL	fluorescence (detection)
Fab	antibody fragments formed by enzymatic digestion of intact antibody
Fab′	antibody fragments formed by enzymatic digestion of intact antibody
FC	fluorocarbon polymer
FSCE	free solution capillary electrophoresis
FDA	US Food and Drug Administration
GM-CSF	granulocyte macrophage colony stimulating factor
HPCE	high-performance capillary electrophoresis
hGH	human growth hormone
Hb	hemoglobin
IEF	isoelectric focusing
ITP	isotachophoresis
I.D.	internal diameter
LIF	laser-induced fluorescence (detection)
Mab	monoclonal antibody (antibodies)
MS	mass spectrometry
M_r	molecular mass
pI	isoelectric point of a zwitterion
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PVP	polyvinylpyrrolidone
PVA	poly(vinyl alcohol)
PEG	polyethylene glycol
RP-HPLC	reversed-phase high-performance liquid chromatography
rt-PA	recombinant tissue plasminogen activator
R.S.D.	relative standard deviation
SEC	size-exclusion chromatography
SDS	sodium dodecyl sulfate
TEMED	tetramethylethylenediamine
UV	ultraviolet (detection)

Acknowledgements

Our own research in cIEF areas was initially supported by a grant and CE instrument donation from Isco. We thank M. Gurkin, J. Algaier, K. Patt, J. Tehrani and B. Allington at Isco for their continued support. In more recent years, our work in cIEF areas has been supported by instrumentation donations from Thermo Separation Products (TSP) through the collaboration of B. Howe, H. Kenny and P. Jandik. Graduate student support has also been forthcoming from TSP, for which we are most grateful. In addition, there have been several methods development

awards from Upjohn Pharmaceutical and Biogen. We are grateful to J. Nappier, M. Staples and K. Kobiyashi of these companies for their support and collaboration. Finally, the Society of Analytical Chemists of Pittsburgh is gratefully acknowledged for supporting J. Mazzeo via an ACS summer research fellowship. Certain parts of Dr. Mazzeo's Ph.D. Thesis have been condensed and/or combined to serve as the introductory or background sections to this review paper, with permission from and acknowledgement to the original author and copyright holder. We thank Dr. Z. Deyl, Editor of the Symposium and Special Topics Volumes of Journal of Chromatography, for the original invitation to prepare this contribution.

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