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Capillary electrophoresis of peptides in isoelectric buffers

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Abstract

Peptide maps in capillary zone electrophoresis are typically obtained in 80 m*M* phosphate buffer, pH 2.0, since at this pH value essentially all surface silanols of silica are protonated and thus one obviates to the need of chemically treating the inner capillary surface. When developing a peptide map of a tryptic digest of bovine β-casein under these conditions, good separations were obtained, but at the expense of a rather long running time (80 min) due to the low maximum voltages applicable (only 110 V/cm) under experimental conditions utilizing relatively large bore capillaries (100 μm I.D.) for increased sensitivity. When resorting to isoelectric aspartic acid as the sole buffering ion [pH=isoelectric point (p*I*)=2.77 at 25°C] much higher voltages could be applied (up to 800 V/cm) with a concomitant reduction of total running time (down to only 6–7 min). However, at this operative pH, strong adsorption of larger peptides to the capillary wall was noticed. This inconvenience was fully eliminated when finally adopting a buffer of the following composition: 50 m*M* Asp, pH 2.77, added with 0.5% hydroxyethylcellulose number-average molecular mass (M_n) of 27 000 and with 5% 2,2,2-trifluoroethanol. In this buffer, very high resolution could be achieved, due to the very low band spread at such greatly reduced running times and to the absence of wall adsorption. The most important parameter, when selecting amphoteric buffers, is their buffering power, which is a function of their p*I*−p*K* values. The buffering power, for isoelectric amino acids, is very strong for Lys and Asp (27 and 26 mequiv. l^{-1} pH^{-1}, respectively, for 50 m*M* solutions), intermediate in the case of Glu (20), and rather minute for His and Arg (6 and 5.5, respectively). It is suggested that the minimum buffering power for a background electrolyte in CZE should be kept in the order of ≥10 mequiv. l^{-1} pH^{-1}.

Keywords: Buffer composition; Peptides; Caseins

1. Introduction

Capillary zone electrophoresis (CZE) is becoming a technique of widespread use for analysis of small polar peptides [1]. There are fundamentally two basic variants: for peptides which have similar structures and the same electric charge, micellar electrokinetic chromatography (MEKC) is adopted [2]; for peptides of widely differing composition, in terms of mass and charge (such as typically found in proteolytic fragments of proteins), plain, free solution CZE is adopted, typically in acidic phosphate buffers at or close to pH 2 [3]. In the first case, for instance, Liu et al. [4] and Wainright [5] separated angiotensin II derivatives and other small peptides in the presence of CTAB, DTAB or SDS micelles. The same micelles were also adopted for separation of larger peptides, such as motilisins and insulins [6]. Reduced and oxidized forms of glutathione were separated by SDS [7], gonadorelin and its analogues by CTAB and CHAPS [8] and oxytocin and vasopressin by SDS, Triton X-100 and CHAPS [9]. Sodium taurodeoxycholate was also used as the micelle forming agent for separation of di-, tri- and tetrapeptides at pH 3 [10].

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In the second case (free solution CZE), the technique has been widely adopted for peptide mapping. For instance, Ross et al. [11] have optimized CZE separations for the tryptic digest mapping of normal whole hemoglobin and of separated globin chains. Cobb and Novotny [12] and Hogan and Yeung [13] reported CZE maps of tryptic digests of β-casein. Nashabeh and El Rassi [14] reported CZE maps of α_1-acidic glycoprotein and Chang et al. [15] published tryptic maps of ruthenium-modified cythochrome *c*. Adamson et al. [16] utilized CZE for separation and detection of multiple phosphoseryl-containing peptides from casein.

We have recently undertaken a project for the continuous enzymatic production of peptides from bovine β-casein. This protein has the peculiarity of containing in its sequence some peptides liable to interfere in mineral nutrition [e.g., peptide β-CN(1–25)], some peptides with opioid activities [peptide β-CN(60–66) and derivatives], antihypertensive [peptide β-CN(177–183)] and immunomodulatory activities [peptide β-CN(63–68)] [17–19]. Trypsin digestion of β-casein releases such peptides either directly or indirectly as precursors (e.g., peptide β-CN(49–97)]. In principle, isoelectric focusing, both in soluble amphoteric buffers [20] or in immobilized pH gradients [21], could be a very good method for following kinetically such enzymatic digestion, due to its very high resolving power. In practice, only peptides longer than 8–10 amino acids can be precipitated and stained in a dye solution consisting of Coomassie Brilliant Blue G-250 as a micellar, leuco-form in trichloroacetic acid [22]. We have thus adopted CZE, in the standard 80 m*M* phosphate buffer, pH 2.0, for a rapid and sensitive method for displaying all the peptides released by tryptic digestion of β-casein. This buffer offers the unique advantage of allowing separations of even large peptides without resorting to coating of the inner capillary wall, since at pH 2.0 the entire population of silanols should be fully protonated (in fact Bello et al. [23] have proven that the point of zero silanol dissociation occurs at precisely pH 2.3). We have in fact adopted this procedure and obtained very good results. However, one major problem still remains: the analysis time. Due to the intrinsic high conductivity of this buffer, and to the fact that we use a 100 μm I.D. capillary for high sensitivity, we could perform separations only at 110 V/cm, which required, in a 37 cm long capillary, a total running time of 80 min. In an attempt at drastically shortening this analysis time, we report here the use of isoelectric buffers, namely aspartic acid as the sole buffering ion in the background electrolyte. Due to its extremely low conductivity, much higher voltage gradients could be applied, with a reduction of analysis time of almost one order of magnitude.

2. Materials and methods

2.1. Reagents

Trypsin (TPCK treated, type XIII) and β-casein were obtained from Sigma (St. Louis, MO, USA). Aspartic acid, CAPS [3-(cyclohexylamino)-1 propanesulphonic acid], Immobilines p*K* values 3.6, 4.6, 6.2, 7.0, 8.5 and 9.6 and 2,2,2,-trifluoroethanol were from Fluka, Buchs, CH. Ampholine pH 3–10 were from Pharmacia-Upjohn, Uppsala, Sweden. Acrylamide, Tris(hydroxymethylaminomethane), ammonium peroxodisulphate and N,N,N′,N′-tetramethylethylene diamine (TEMED) were obtained from Bio-Rad Labs. (Hercules, CA, USA). Fused-silica capillaries (100 and 75 μm I.D., 375 μm O.D.) were obtained from Polymicro Technologies, Phoenix, AZ, USA. Hydroxyethylcellulose (HEC; number-average molecular mass, M_n 27 000) was purchased from Aldrich (Milwaukee, WI, USA).

2.2. Capillary electrophoresis

CZE was carried out with the Beckman (Palo Alto, CA, USA) P/ACE 2100 instrument equipped with automated Gold software. Thirty seven cm long and different I.D. (100 and 75 μm), uncoated capillaries were used. Several types of background electrolytes were tested: (a) the standard 80 m*M* phosphate buffer, pH 2.0, commonly adopted in peptide separations (in this case, the capillary was first pre-conditioned by washing with buffer containing 1.75% liquid linear polyacrylamide); (b) 50 m*M* isoelectric aspartic acid (pH=p*I*=2.77 at 25°C); (c) same as (b), but added with 0.5% HEC M_n 27 000; (d) same as (c) but added with 5% triflouroethanol. In all cases, the sample and standard

were loaded by pressure by applying 0.55 p.s.i. for 3 s (1 p.s.i.=6894.76 Pa). Separations were performed at 110 V/cm in buffer (a) and up to 800 V/cm in buffers b–d. Ultraviolet absorbance was monitored at 214 nm.

2.3. Isoelectric focusing (IEF)

In order to measure the precise p*I* of some of the major peptide fragments, an immobilized pH gradient (IPG) gel, spanning a pH 3–10 was made, according to recipes given in [21]. After focusing (overnight at 2500 V), a few sample tracks were excised and stained by the method of Righetti and Chillemi (which can precipitate and reveal ca. 75% of the total fragments) [22]. The remaining, unstained sample tracks were utilized for eluting some of the major fragments (as labelled in the CZE figures) which were then subjected to micro-sequencing and mass spectrometry for proper identification. For a quick comparison between CZE and IEF data, conventional IEF in presence of 2% Ampholine pH 3–10 and 20% glycerol was performed. In the latter case, focusing was continued for 2 h at 2000 V.

2.4. pH determinations

In order to assess potential pH changes with dilution of isoelectric Asp, the following potentiometric titration was performed. Doubly distilled, degassed water was prepared to contain 100 m*M* KCl. The solution was thermostated at 25°C and added with crystals of Asp acid, first up to 5 m*M*, then up to 10 m*M*; subsequently at 10 m*M* increments up to 50 m*M*. At each Asp addition, the pH was carefully assessed with a pHM64 Research pH meter, equipped with a GK2401C combination electrode from Radiometer (Copenhagen, Denmark).

2.5. β-casein digestion

Tryptic peptide maps of β-casein were obtained as follows: 10 mg/ml β-casein, dissolved in 50 m*M* CAPS buffer, pH 8.2, were added with trypsin to a trypsin/β-casein ratio of 2% (w/w). Hydrolysis continued for 4 h at 50°C. The reaction was stopped by adding acetic acid to pH 4.0.

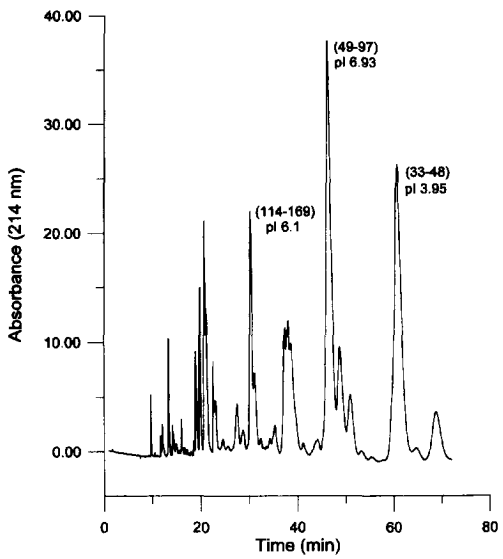

Fig. 1. CZE of tryptic digests of β-casein. Conditions: 37 cm×100 μm I.D. capillary, bathed in 80 m*M* phosphate buffer, pH 2.0. Sample application: by pressure, by applying 0.5 p.s.i. for 3 s. Separations were performed at 110 V/cm (current: 85 μA) and detection was at 214 nm. The three major peaks, labelled 1–3, are: (1) p*I* 6.1, fragment β-CN (114–169); (2), p*I* 6.93, fragment β-CN (49–97) and (3), p*I* 3.95, fragment β-CN (33–48). Note that the total running time is 80 min.

3. Results

Fig. 1 shows the CZE separation of the tryptic digest of β-casein in the standard 80 m*M* phosphate buffer, pH 2.0. Between 20 to 25 major and minor peaks are distinguishable in the electropherogram. Among them, three major peaks could be precisely mapped by subjecting them to IPGs, followed by band excision, microsequencing and mass spectrometry. They are: peak 1, p*I* 6.1, fragment β-CN (114–169); peak 2, p*I* 6.93, fragment β-CN (49–97) and peak 3, p*I* 3.95, fragment β-CN (33–48). This separation, however, suffers from some inconveniences: first of all, the analysis time is too long, since ca. 80 min are required for complete map development. Secondly, and probably due to the long running time, not all peaks are resolved to base-line and some zones appear to be envelopes of more than one band, since shoulders are clearly visible in some of them. Additionally, the peak widths are unacceptably large: e.g., the p*I* 3.95 fragment, one of the last eluting peaks, has a width at 4σ of 2.5 min. Due to

the relatively large bore capillaries utilized (100 μm), and needed for increased sensitivity, it was not possible to perform the CZE run at voltage gradients higher than 110 V/cm, as predicted by the thermal theory of Bello and Righetti [24,25], since even at this relatively modest field strength the current is already close to 90 μA.

In order to shorten the analysis time, we have tried isoelectric buffers as the sole buffering species. The most acidic amphoteric compound available is aspartic acid (Asp, pH=p*I*=2.77 at 25°C). Fig. 2 shows the results obtained in this buffer system, by applying a four-times higher voltage gradient (400 V/cm). Although the running time is reduced to about 12 min, the results are quite disappointing: except for the peaks eluting in the very first few minutes, all the others are broad and asymmetric, suggesting a strong interaction of the larger peptides with the capillary wall (at pH 2.77 some silanols will be ionized and will certainly interact with positively charged peptides). One could use coated capillaries, but this would add an extra experimental burden.

It is well known that a number of polymers, when added to the background electrolyte, can dynamically

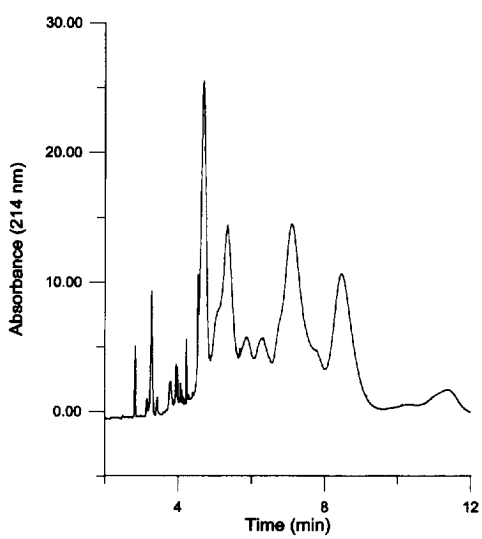

Fig. 2. CZE of tryptic digests of β-casein in a 37 cm×100 μm I.D. capillary, bathed in 50 m*M* isoelectric aspartic acid (pH approximating the p*I* value of 2.77). All other conditions as in Fig. 1, except that the separation was performed at 400 V/cm (current: 25 μA). Note the reduction of transit times to only 12 min, but the very poor separation and peak tailing, suggesting adsorption of several peptides to some ionized silanols.

coat the capillary, thus quenching any potential analyte-wall interaction (for a review, see [26]). We have thus adopted the same 50 m*M* Asp buffer, but added with 0.5% HEC of 27 000 M_n. As shown in Fig. 3A, already at 200 V/cm the analysis time is halved and the peaks appear now much sharper and considerably less skewed. At progressively higher voltage gradients (from 400 up to 800 V/cm) the transit times are progressively decreased, down to only 5 min total running time at 800 V/cm (Fig. 3D). It can be easily appreciated that quite a few peak doublets, which were only apex-resolved at low (200 V/cm) voltages, are almost base-line resolved at 600 V/cm. This is largely due to the much reduced peak width concomitant with the much shortened analysis time: e.g., in Fig. 3D the band width of the p*I* 3.95 peak at 4σ is only 20 s, as opposed to 2.5 min in Fig. 1. However, it would appear that the safest running voltage is 600 V/cm, rather than 800 V/cm, since in this last case (compare Fig. 3C and D) some peak degradation is visible, probably due to the very high current developed at 800 V/cm (90 μA). In principle, 800 V/cm in isoelectric Asp should carry less current than the one monitored, but the addition of HEC increases the basic current intrinsic to the background Asp electrolyte, suggesting that either the HEC powder contains some adsorbed salt, or that the HEC polymer itself is not neutral but carries some charged groups, thus contributing to some extent to the final current of the system (as an example, at 400 V/cm in 50 m*M* Asp the current is 25.7 μA and with the addition of HEC it increases by 28% to 33 μA). Due to these reasons, although we have tested higher levels of HEC (0.75% and 1%) we much prefer a level of 0.5% HEC since we obtain maximum benefit for the separation without the added burden of an excessive current. Moreover, higher levels of HEC (e.g., 1%) substantially increment the viscosity of the solution, thus considerably lengthening the analysis time.

In an attempt at further ameliorating the system, we have tried to manipulate selectivity by addition of organic modifiers. Recently, both our group [27] and Castagnola et al. [28] have reported the use of trifluoroethanol as an additive for modulating peptide separations. In our case, 20% trifluoroethanol was adopted for solubilizing peptides in preparative IPGs

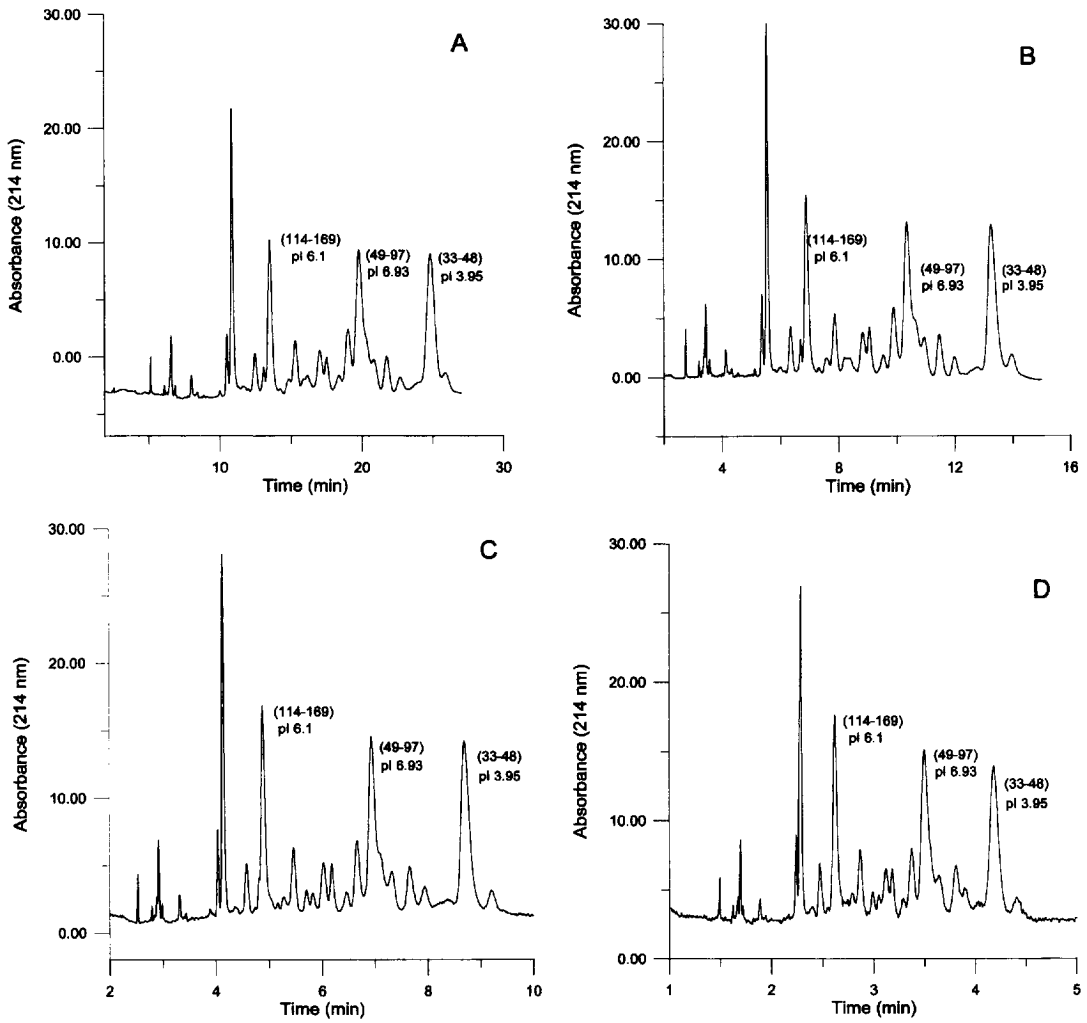

Fig. 3. CZE of tryptic digests of β-casein in a 37 cm×100 μm I.D. capillary, bathed in 50 m*M* isoelectric aspartic acid (pH approximating the p*I* value of 2.77) added with 0.5% HEC (M_n 27 000). A, run at 200 V/cm (current: 15.6 μA); B, 400 V/cm (current: 33 μA); C, 600 V/cm (current: 57 μA); D, 800 V/cm (current: 90 μA). All other conditions as in Fig. 1. Note the marked decrease in running times and the absence of peptide adsorption to the capillary wall.

[27]. In [28], up to 37% trifluoroethanol was added as co-solvent for improving the separation of large apolar peptides. Unfortunately, amounts >5% of trifluoroethanol precipitate isoelectric Asp. Nevertheless, a buffer comprising 50 m*M* Asp, 0.5% HEC and 5% trifluoroethanol gave an excellent performance in developing peptide maps (Fig. 4A and 4B). It is seen that now most peaks are base-line resolved and, in Fig. 4B, an extra minor peak, eluting and trapped at the base of the p*I* 3.95 peptide, is now well resolved to its right. Of course, if one were willing to sacrifice sensitivity, and use narrower bore capillaries, e.g., 75 μm I.D. as in most other applications, the present system is fully compatible with voltage gradients of up to 1400 V/cm (data not shown).

4. Discussion

In the present report, we have optimized a novel method for peptide separation (based on isoelectric

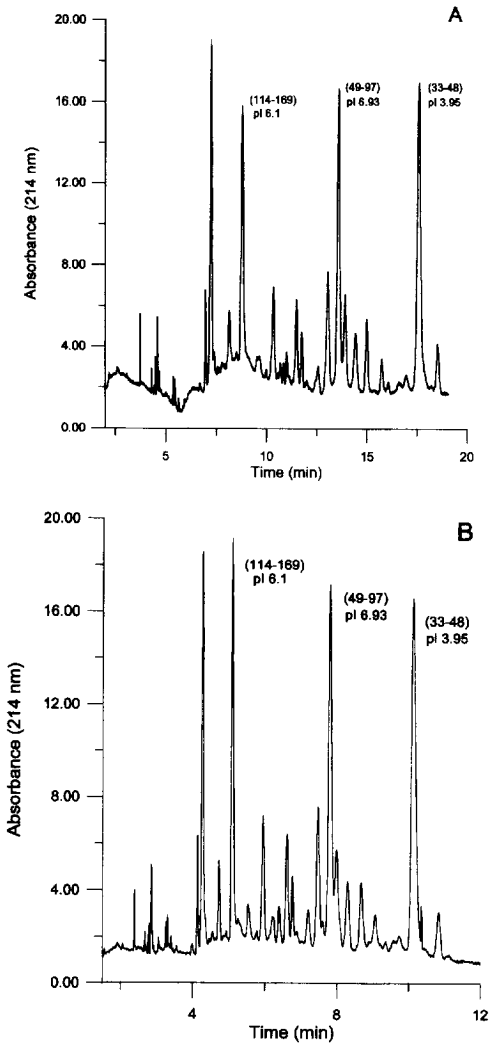

Fig. 4. CZE of tryptic digests of β-casein in a 37 cm×100 μm I.D. capillary, bathed in 50 m*M* isoelectric aspartic acid (pH approximating the p*I* value of 2.77) added with 0.5% HEC (M_n: 27 000) and with 5% trifluoroethanol. A, 400 V/cm (current: 33 μA); B, 600 V/cm (current: 58 μA). All other conditions as in Fig. 1.

buffers, added with linear polymers and with organic modifiers) by testing such method on a real-life sample, i.e., a tryptic digest of β-casein. By this approach, we could guarantee that the method should have wide applicability to any sample of biological interest, since our peptide mixture comprises not only the typical small fragments of M_r 500–600, but also some quite large fragments liable to generate problems of adsorption to the silica wall, e.g., (refer to Fig. 1), peak 1 (p*I* 6.1) has an M_r value of 6063; peak 2 (p*I* 6.93) exhibits a mass of 5319 and peak 3 (p*I* 3.95) has an M_r value of 2064.

The use of isoelectric buffers has not been explored much in the electrophoretic literature outside their normal use, i.e., for isoelectric focusing [20]. Thus, in the field of zone electrophoresis of nucleic acids, we were able to find only a report by Mandecki and Hayden [29] and, more recently, by Gelfi et al. [30] in the analysis of oligonucleotides. In the field of proteins, and other small M_r compounds, Hjertén et al. [31] have explored a number of different amphoteric compounds and given proper guide-lines for their use. These authors report separations at voltage gradients as high as 2000 V/cm. Their results with protein separations have been modelled also by Blanco et al. [32] who obtained simulated protein separations in close agreement with the experimental ones in [31]. In the light of the data in [30–32], and the present data, it is clear that such isoelectric buffers have substantial advantages, which should be exploited also in CZE. In fact, while it is common knowledge that CZE allows high field strengths, due to rapid heat dissipation through the narrow-bore channel, nevertheless there is a limit to the applied voltage, and rarely separations are seen exploiting voltage gradients higher than 200–300 V/cm. Here we have proven that as much as 800 V/cm can be applied, not only without loss of resolution, in fact with an increment of resolving power and peak sharpness. This is no doubt due to the extremely low conductivity of such isoelectric buffers (typically less than 1/10 the conventional ones) allowing much higher voltage gradients for the same amount of joule heat production. However, a note of caution should be expressed against the indiscriminate use of such amphoteric buffers. Many users might be induced to think that Good's buffers might play such a role in CZE. Nothing is farther from truth. Good's buffers are good only for what they are supposed to do, i.e., when titrated around the p*K* value of the tertiary amino group, where they exhibit the much needed buffering power (β power). When used as the sole buffering component, i.e., as isoelectric buffers, they are disastrous since, at their p*I* value (which for most of them falls in the pH 4–5

range) [33], their β power is practically nihil, due to the very large value of pI−pK (3 to 4 pH units!). One should remember the golden rules of Svensson-Rilbe, as laid out more than 30 years ago (as reviewed in [20]), on the concept of carrier ampholyte: among the vast number of amphoteric species, only a few of them are carriers, i.e., display good conductivity and buffering capacity at their pI value. The hallmark of a carrier ampholyte is the value of the difference pI−pK. We give in Table 1 a list of five common amino acids possessing ionizable groups in their side chain, arranged in order of decreasing β power (as calculated with the program of Giaffreda et al. [34]), i.e. of increasing pI−pK values. It is seen that, for equimolar concentrations (here fixed at 50 mM), the best species, in terms of β power at the pI value, is Lys, closely followed by Asp. His and Arg have a rather minute buffering power at their pI values and the worst possible buffer is definitely Arg. This brings up an interesting corollary: what is the minimum buffering power one might want to guard to ensure a good CZE run? Based on our experience with isoelectric His (where a minimum of 100 mM buffer was required) and with Asp (with which, when lowering the molarity to only 20 mM, in an attempt to increase the trifluoroethanol concentration in the liquid phase, poor results were obtained) we tentatively set this minimum β power at ≥10 mequiv. l^{-1} pH^{-1}. It must be emphasized, however, that Hjertèn et al. [31] fix this limit at much lower values, since they report good separations even in only 5 mM Lys (which would have a β power of only 2.6 mequiv. l^{-1} pH^{-1}). However, we much prefer stronger β powers for the following reasons: (a) when working at rather acidic (as in the present case with peptides) or rather basic conditions, water too begins to buffer substantially; (b) when working with widely differing analyte mixtures (or with very acidic analytes, such as in the case of nucleic acids) one has to ensure that all components in the system are properly buffered, thus the need for increased buffering power.

The situation is in fact much more complex than that. According to Rilbe [35] the pH of a pure solution of an ampholyte is a complex function, which depends on the hydrogen ion activity, on the concentrations of the undissociated (or zwitterionic) ampholyte, in equilibrium with its cationic and anionic forms, on two dissociation constants close to the pI value and on the activity factor for monovalent ions. The final equation linking all these parameters to the total concentration of the ampholyte is difficult to interpret since it is of the fourth degree on the hydrogen ion activity. Nevertheless, the following general rules can be extrapolated: (a) at low concentrations of acidic ampholytes, pH>pI; (b) at low concentrations of basic ampholytes, pH<pI; (c) as a corollary of the above, when the concentration of the ampholyte in solution is increased, the pH approaches the pI. In order to check the validity of these assumptions, we have performed the experiment illustrated in Fig. 5: it is seen that, indeed, at very low concentrations (5 mM) a solution of isoelectric Asp is quite removed from the pI (the pH of the solution is 3.36). As the concentration is increased, the pH progressively decreases and, at 50 mM, it tends to the theoretical pI (measured pH value: 2.80; theoretical pI: 2.77). In view of all these considerations, we feel that our suggestion of using relatively high concentrations of zwitterionic buffers (of the order of 50 mM for Asp, but as high as 100 mM for His) is not unjustified.

Table 1
Buffering power of acidic and basic amino acids as a function of pI–pK at constant molarity

Compound	Molarity (M)	pI[a]	pI–pK	Buffering power[b]
Lysine	50	9.74	0.79	27.4
Aspartic acid	50	2.77	0.89	26.4
Glutamic acid	50	3.22	1.03	20.5
Histidine	50	7.47	1.50	6.1
Arginine	50	10.76	1.72	5.5

[a]At 25°C in pure water.
[b]In mequiv. l^{-1} pH^{-1}.

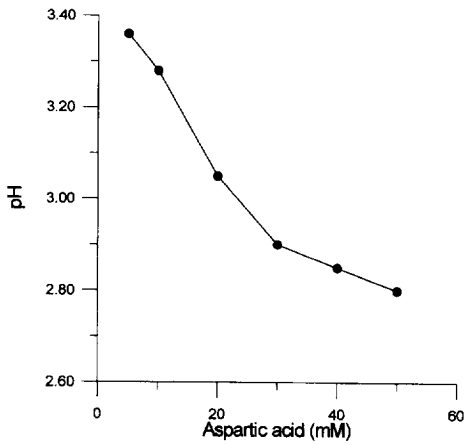

Fig. 5. pH of isoelectric Asp solutions as a function of Asp molarity. Doubly distilled, degassed water was added with 100 m*M* KCl, thermostated at 25°C and then added with crystals of Asp, first at 5 m*M* increments (5 and 10 m*M*) then at 10 m*M* increments up to 50 m*M*. pH was assessed after each addition with a pHM64 research pH Meter from Radiometer.

5. Conclusions

We feel that CZE in zwitterionic buffers has much to offer for general separation purposes, including analysis of macromolecules such as proteins and nucleic acids. CZE users are encouraged to always calculate the β power of their running buffers, which, together with its conductivity, seems to be one of the most important parameters in ensuring proper running conditions.

6. Abbreviations

Arg	Arginine
Asp	Aspartic acid
CAPS	3-(Cyclohexylamino)-1-propanesulfonic acid
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio)-1-propanesulfonate
CTAB	Cetyltrimethylammonium bromide
DTAB	Dodecyltrimethylammonium bromide
HEC	Hydroxy ethyl cellulose
His	Histidine
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
Lys	Lysine
MECK	Micellar electrokinetic chromatography
p*I*	Isoelectric point
SDS	Sodium dodecylsulfate
TEMED	N,N,N′,N′ tetramethylethylene diamine
TPCK	L-1-tosylamide-2-phenylethyl chloromethyl ketone

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