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Quasi-isoelectric buffers for protein analysis in a fast alternative to conventional capillary zone electrophoresis ☆

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Abstract

Two different approaches are here reported for obtaining ultra-narrow p*I* cuts from 2-pH unit wide carrier ampholyte ranges, as commercially available, for use as quasi-isoelectric buffers in capillary electrophoresis separations of proteins. One of them uses multicompartment electrolyzers endowed with isoelectric membranes (Immobiline technology); the other employs the Rotofor equipment. Although the first approach results in more precise p*I* cuts, the latter technique is much faster, easier to handle and permits the immediate collection of 20 fractions in a single run. This results in ultra-narrow, ca. 0.1-pH unit intervals, uniformly spaced apart along the original wider gradient utilized for the fractionation. It is here shown that such quasi-isoelectric buffers, especially those in the pH 8–9 interval, have the unique property of coating the silica wall, thus preventing interaction of the proteins with the silica surface, that would otherwise totally disrupt the separation. On the contrary, such a shielding is not obtained in control, non isoelectric buffers (such as phosphate), that give very poor separations in uncoated capillaries. It is hypothesized that such a unique shielding effect is due to the oligo-amino backbone of the carrier ampholytes, typically composed (in the Vesterberg's synthetic approach) of 4–6 nitrogens spaced apart by ethylene moieties. Although such oligoprotic buffers should bear, in the isoelectric state, just one positive and one negative charge, they might be transiently ionized upon contact with the silanols, thus inducing a cooperative binding to the silica wall.

Keywords: Ultra-narrow-pH ampholytes cuts; Fractionation techniques; Isoelectric buffer; Uncoated-capillary CZE

1. Introduction

Zone electrophoresis in isoelectric buffers (IEB) has had a long gestation period, since the seminal papers by Mandecki and Hayden in 1988 (dealing with gel slab electrophoresis of DNA in histidine buffers) [1], by Bier et al. [2,3] (proposing preparative free-flow electrophoresis in a cycloserine buffer) and by Hjertèn et al. [4] (the latter dealing with capillary zone electrophoresis, CZE, of proteins in isoelectric Lys and other amphoteric buffers). There is an obvious advantage on the use of isoelectric buffers (IEB) in electrophoretic separations: due to their very low conductivity, they permit high voltage gradients along the separation axis, thus greatly shortening the analysis time, much as isoelectric focusing buffers [5] and immobilized pH gradients [6] do. However, CZE in alkaline buffers, such as Lys, as originally proposed by Hjertèn et al. [4], is also fraught with difficulties, due to the fact that proteins and peptides are strongly adsorbed by the silica wall, whose silanol groups are highly ionised at this operative pH (p*I* of Lys: 9.79). If peptide/protein separations are sought in uncoated capillaries, a possible solution is to work at a pH close to the neutralization point of the silica wall, determined as pH 2.3. Given an average p*K* value of the silanols of 6.3, at pH 2.3, essentially all silanols should be undissociated, thus should be unable to adsorb proteinaceous samples by an ion-exchange mechanism [7]. Over the years, we have tested and evaluated four such acidic, amphoteric buffers: cysteic acid (p*I* 1.85) [8], imino diacetic acid (IDA) p*I* 2.23 [9];

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aspartic acid (p*I* 2.77) [10] and glutamic acid (p*I* 3.22) [11]. Additional IEBs have been reported recently, based on diamino sulphate [12], quaternary ammonium dicarboxylic acid [13] and a bismorpholino derivative of a carboxylic acid [14]. Two theoretical papers have also dealt extensively with the fundamental properties of such IEBs and given guidelines for their selection [15,16]. Although some authors have expressed scepticism on the use of such IEBs [17,18], their concern seems to be limited mostly to IEBs close to neutrality, such as His, definitely not to IEBs with p*I* values well removed from neutrality, such as in the case of our acidic IEBs. In fact, the advantage of using IEBs vanishes at pH extremes, where the bulk water dissociation swamps the conductivity minima of IEBs. From this point of view, the best IEB appears to be Asp, since its p*I* value is located close enough to the silanol neutralization point, while being removed enough from too acidic pH values. For very low p*I* IEBs, such as cysteic acid and IDA, conductivity quenchers had in fact to be adopted. These acidic IEBs have performed very well not only for separation of peptides, but for complex protein mixtures as well (for a review, see [19]).

If, on the one hand, acidic IEBs allow to work with uncoated capillaries, alkaline IEBs do not solve the problem of protein adsorption onto the capillary wall. For this reason, the possible employment of ultra-narrow-pH-range carrier ampholyte fractions as alkaline IEBs is now under study. In fact, such fractions might represent good background electrolytes in CZE, since their particular chemical structure might interact with the capillary wall differently than "normal" IEBs. The chemical synthesis of such a battery of IEBs would be a cumbersome and costly project; a much more reasonable proposal would be to try to sub-fractionate available carrier ampholytes into extremely narrow pI intervals, thus providing an easy source of a large number of quasi-isoelectric buffers covering any desired p*I* value. This fractionation process is here reported by exploiting two different methods: preparative IEF in multicompartment electrolyzers with Immobiline membranes [20] or in the Rotofor unit [21]. Some unique (and unexpected) properties of such quasi-isoelectric buffers are also described.

2. Materials and methods

2.1. Reagents

Ampholine™ pH 7–9 (0.4 g/mL), Immobiline™ with various p*K* values (3.6, 6.2, 8.5 and 9.3) and protein p*I* markers (High range p*I* kit, pH 3–10) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Fluka (Buchs, Switzerland) provided phosphoric acid, sodium hydroxide and Tris. Acrylamide and bis-acrylamide powders were from Bio-Rad (Hercules, CA).

2.2. Ampholine fractionation with multicompartment-electrolyzer (MCE)

Ampholine pH 7–9 were diluted with milli-Q water to a final concentration of 2% (w/v) and then fractionated in an IsoelectrIQ MCE device (Proteome Systems, NSW, Australia). The 2-pH unit Ampholine mixture was first fractionated into four 0.5-pH unit intervals by using a four-chamber MCE device, after that each fraction was fractionated again into two or three 0.2-pH unit narrow ranges.

2.2.1. Buffering membranes

The MCE fractionation chambers were separated by means of 1-mm thick polyacrylamide buffering membranes, which were prepared onto glass microfibre filters (Whatman GF/D). Each membrane was prepared by titrating a buffering Immobiline, with its p*K* value as close to the desired final pH as possible, with the acidic Immobiline p*K* 3.6. The concentration of the buffering Immobiline was calculated with the software Doctor pH, so as to obtain an approximate buffering power (β) of 10 for each membrane. The titrating Immobiline (p*K* 3.6) was gradually added until obtaining the desired pH value. Acrylamide/bis-acrylamide stock solution (3.3% C) was added to the polymerizing mixtures so as to obtain a final concentration of 10% T. Every membrane solution was finally titrated to pH 7.0 with acetic acid so as to allow the polymerization, which took place at 40 °C for about 1 h. Afterwards, the membranes were washed with water twice for 20 min and finally assembled in the MCE device.

2.2.2. Ampholine two-step fractionation

The first fractionation was carried out in a four separation chamber device, assembled with the following buffering membranes: pH 7.0, 7.5, 8.1, 8.5 and 9.1. The anode and cathode chambers were filled with 50 mM acetic acid and 200 mM Tris free base, respectively, while the four separation chambers were loaded with 24 mL of the 2% Ampholine pH 7–9 solution (6 mL in each chamber). Fractionation was carried out by applying 300 constant volts for 2–4 h, until current stopped decreasing and the pH value in each chamber was as close to the expected one (the average value of the interval) as possible. This fractionation step was performed twice, in order to collect eight fractions to be combined into four ones with a final volume of about 12 mL, sufficient for the loading of the two or three chambers used in the second run. With the second fractionation, the first four pH intervals were fractionated again by means of two- or three-chamber devices, cast as described in Table 1. Anolyte and catholyte and the applied voltage were as in the first fractionation, and the process was stopped when the expected pH value in each chamber was reached.

Table 1
Second Ampholine fractionation

First fractionation intervals	No. of chambers in the second fractionation	Buffering membranes used
7.0–7.5	2	7.1, 7.3, 7.5
7.5–8.1	3	7.5, 7.7, 7.9, 8.1
8.1–8.5	2	8.1, 8.3, 8.5
8.5–9.1	3	8.5, 8.7, 8.9, 9.1

For each of the first four fractions (first column), the number of the chambers used in the second fractionation (second column) and the buffering membranes employed (third column) are listed.

2.3. Ampholine fractionation with the Rotofor

Ampholine pH 7–9 were diluted with milli-Q water to a final concentration of 5% (w/v) and then fractionated in a Rotofor device (Bio-Rad, Hercules, CA), equipped with a mini focusing chamber (18 mL total volume) with 20 fractionation compartments. Anolyte and catholyte were, respectively, 0.1 M phosphoric acid and 0.1 M sodium hydroxide. These solutions were also used to activate and store the anion and cation exchange membranes employed at the anodic and cathodic sides of the focusing chamber. Ampholytes were allowed to focus for 2 h by applying a constant voltage of 700 V and setting the following limiting parameters: 15 mA maximum current and 12 W maximum power. During the focusing process, the system was cooled with an external refrigerated water bath set at 10 °C. After 2-h focusing, all of the 20 fractions were collected at once as soon as the focusing was stopped, so as to minimize the spontaneous diffusion among the fractions.

2.4. Capillary electrophoresis experiments

Capillary electrophoresis measurements were performed with a HP^{3D}CE capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany), provided with a diode array spectrophotometric detector. Data were handled with the HP Chemstation software and Microsoft Excel. The separation capillary was made of fused-silica (Composite Metal Services, Hallow, WR, UK), 33.5 cm long (25 cm effective length) and with an internal diameter (I.D.) of 50 μm. As background electrolyte (BGE) various Ampholine narrow-pH fractions were used, with pH values ranging from pH 7.8 to 8.3 and concentrations spanning from 1 to 2% (w/v) (Table 2). The running voltage was 25 kV, and the capillary cartridge temperature was set at 25 °C, while the sample tray was maintained at 20 °C by means of an external bath. The protein sample (High range p*I* kit, pH 3–10, Amersham), which was a mixture of 10 proteins with p*I* values in the pH range 5.20–10.25 (Table 3), was reconstituted in milli-Q water to a final protein concentration of 0.2 mg/mL. Sample injection into the capillary was hydrodynamic, by applying a pressure of 30 mbar for 3 s. The detection wavelength was set at 214 nm, while the reference was at 450 nm. New capillaries were always activated by means of successive 10-min flushes with 1 and 0.1 M NaOH, followed by a 10-min wash with water. Before every use, the capillary was equilibrated (1 min) with the BGE, which was also used to wash (30 s) the capillary between successive runs. Before changing the Ampholine fraction used as BGE, the system was always washed with 0.1 M H_3PO_4 for 1 min, followed by 1 min water and 1 min with the new BGE. Washes were performed at a pressure of 4.5 bar.

Table 2
Ampholine fractions with different pH values and concentrations tested as background electrolytes in capillary electrophoresis experiments

Ampholine fraction (pH value)	Concentration (%, w/v)
7.87	1.2, 1.4, 1.6, 2.0
8.02	1.6, 2.0
8.24	1.6, 2.0
8.29	1.2, 1.4

Table 3
High range p*I* marker kit (Amersham) protein composition

Id. No.	High range p*I* markers	p*I*
1	β-Lactoglobulin A	5.20
2	Bovine carbonic anhydrase B	5.85
3	Human carbonic anhydrase B	6.55
4	Horse myoglobin-acidic band	6.85
5	Horse myoglobin-basic band	7.35
6	Lentil lectin-acidic band	8.15
7	Lentil lectin-middle band	8.45
8	Lentil lectin-basic band	8.65
9	Trypsinogen	9.30
10	Cytochrome C	10.25

p*I* marker proteins are listed (second column) along with their p*I* values (third column) and the identity numbers (first column) used to label peaks in Fig. 7.

3. Results

Fig. 1 outlines the MCE-based approach used for obtaining narrow p*I* cuts from a 2-pH unit, basic carrier ampholyte mixture. Since only five chambers were available for fractionation (the two extreme ones being used as electrode reservoirs), we tried three approaches for obtaining 0.2 pH fractions (in the example p*I* 7.9–8.1, 8.1–8.3 and 8.3–8.5 intervals in the first approach, and p*I* 8.5–8.7, 8.7–8.9 and 8.9–9.1 in the second approach). The first two approaches were dubbed "non-homogeneous" in that much wider pH cuts would be obtained in one or two of the terminal chambers. In turn, such wider p*I* cuts were sub-fractionated in a cascade fashion, so as to generate additional narrower p*I* cuts, again spanning a 0.2 pH interval (such interval being deemed to be the narrower one obtainable under our experimental conditions). In the third approach, the 2-pH unit commercial Ampholine range was sub-fractionated first in four p*I* cuts encompassing about 0.5-pH units (thus called homogeneous intervals), each being further sub-fractionated, again in a cascade fashion, into 0.2 p*I* cuts. An example of the results of the first approach is given in Fig. 2 . It is seen that, in such an experimental set-up, the pH profiles of the various fractions tend to diverge from the expected ones (left panel), approaching a linear distribution instead of the non-homogeneous one predicted by the separation-chamber set-up. However, when the pH 8.5–9.1 interval collected in the extreme basic chamber is sub-fractionated into three narrower homogeneous p*I* cuts (each 0.2-pH unit wide), the expected and effective pH profiles tend to coincide (right panel) and to present, this time, a quasi-linear profile. Moreover, when the third approach is tried (Fig. 3), both the first fractionation and the cascade sub-fractionation into 0.2 p*I* cuts, performed in a way so as to have all of the intervals in each fractionation of the same width, appear to follow much more closely the theoretically expected p*I* values. What these results suggest is that, notwithstanding the particular buffering membranes adopted in each fractionation, Ampholine species might focus along the separation device creating a quite linear pH gradient, which seems to be only slightly disturbed by

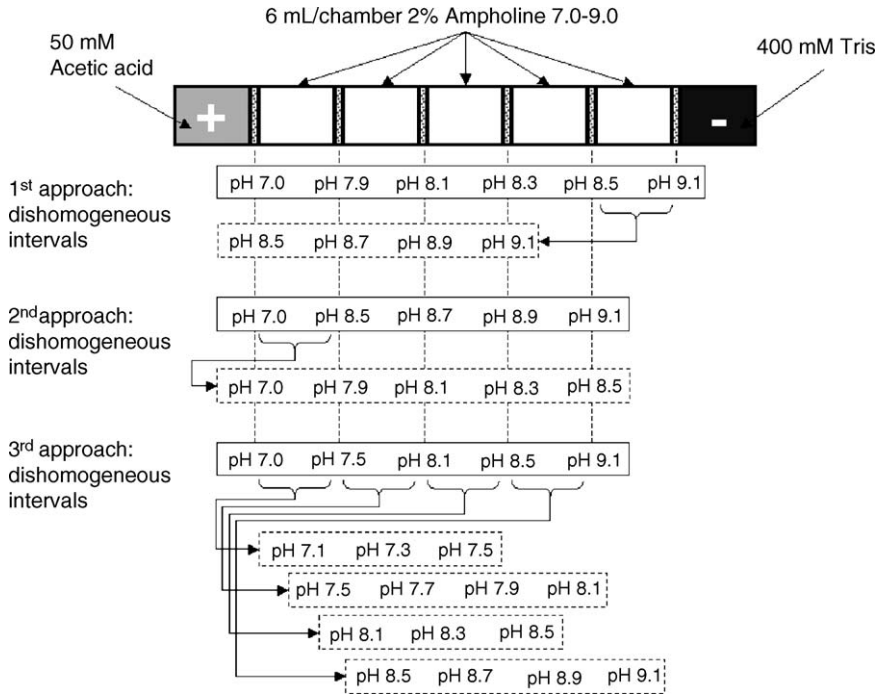

Fig. 1. Scheme of the experimental protocols for Ampholine fractionation with a multicompartment electrolyzer.

the membranes. In fact, in all of our experiments, the actual pH profile was always quite linear, coinciding with the theoretical one only when homogeneous fractionation intervals were adopted, so as to support the natural distribution of the focused Ampholines. Nevertheless, it should be remembered that the Vesterberg-type carrier ampholytes do not have a homogeneous buffering power along the pH interval, the acidic components (especially those focusing in the pH 4–6 interval) having substantially higher buffering capacity as compared to their basic counterparts [22,23].

We have next tried the fractionation approach with the Rotofor apparatus, as devised by Bier's group. Fig. 4 shows the experimental set-up and the various steps involved in this protocol. Due to the fact that this instrument is sub-divided into 20 chambers, 20 fractions can be obtained directly within a single electrophoretic run. When utilizing the same pH 7–9 Ampholine interval, it is clear that, in principle, one can obtain directly 20, 0.1-pH unit intervals, the narrowest possible span under these experimental conditions. Such fractions would then automatically offer a quasi-isoelectric buffer system, comprising fractions evenly spaced along the pH 7–9 interval. That this hypothetical situation can be verified in practice is demonstrated in Fig. 5A, which shows that, indeed, except for the two extremes (possibly contaminated by some products diffusing from the electrodic reservoirs, or by some by-products of the synthetic approach), the experimental pH profile closely follows the the-

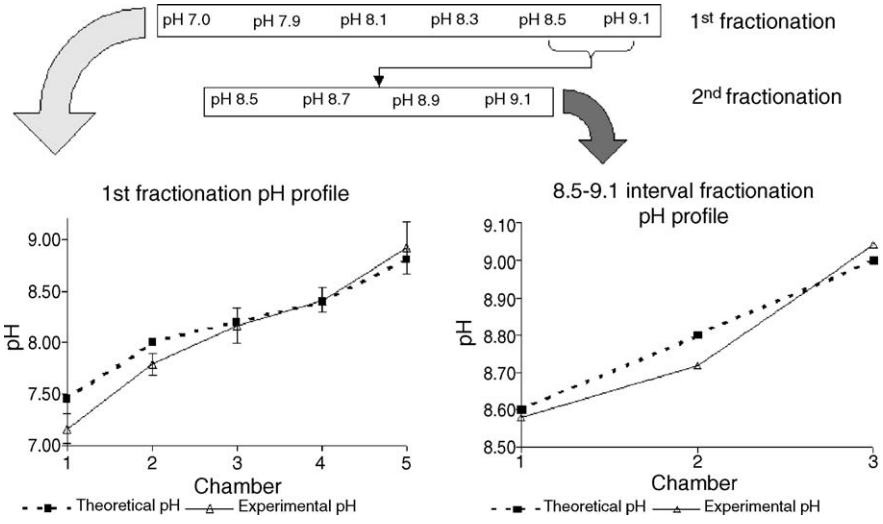

Fig. 2. pH profiles obtained in the case of the "first approach" of Fig. 1 (non-homogeneous intervals). Left panel: pH intervals as obtained with the top set of membranes (first row); right panel: pH intervals obtained by the cascade sub-fractionation of the pH 8.5–9.1 interval.

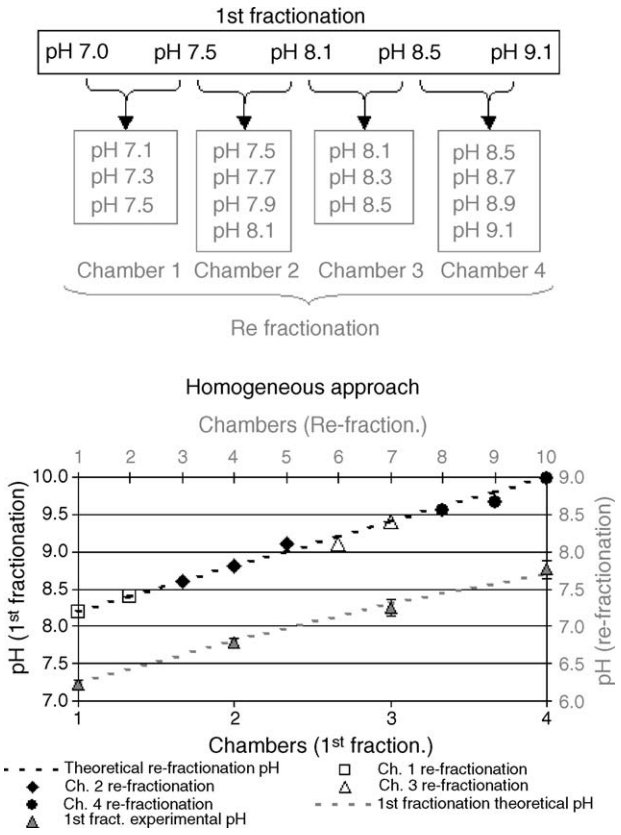

Fig. 3. pH profiles obtained in the case of the "third approach" of Fig. 1 (homogeneous intervals). Table at the top: the upper row represents the fractions obtained in the wider pH interval (lower pH profile in the graph to the bottom); the four columns below represent the 0.2-pH unit wide ranges obtained by subfractionating the wider intervals in a cascade fashion, as plotted in the upper pH profile of the graph.

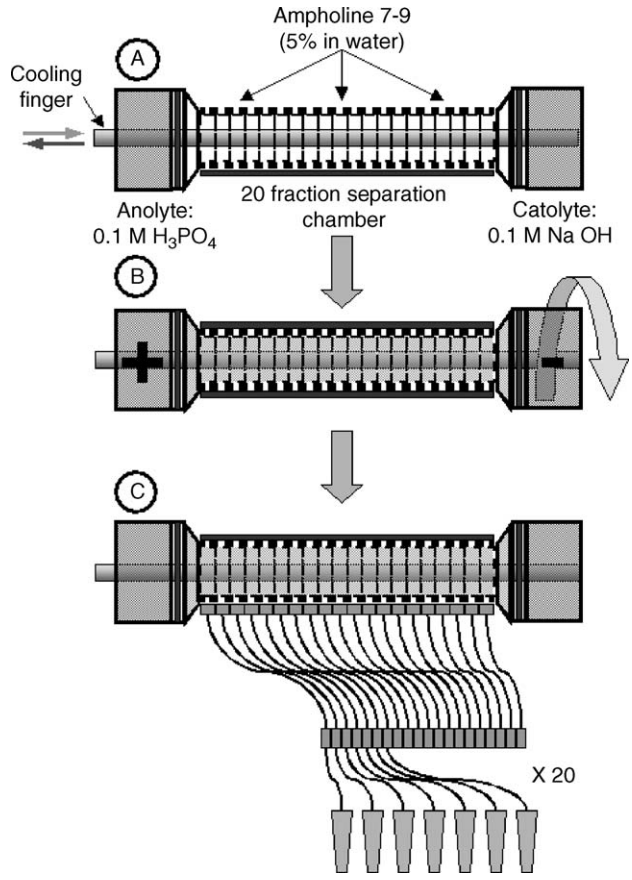

Fig. 4. Experimental set-up for Ampholine fractionation in the Rotofor. (A) The separation device is loaded with 18 mL 5% Ampholine water solution; (B) start of the focusing, with the entire device slowly rotating around the separation axis; (C) simultaneous collection of the 20 focused Ampholine fractions.

oretical one (the fact that the experimental pH profile is always somewhat lower than the theoretical one could be due to adsorption of atmospheric CO_2, as typical for alkaline fractions). By examining the graph, one can appreciate that quite a few of the fractions are spaced apart by the ΔpI increment of 0.1-pH unit. Fig. 5B gives the pH and conductivity profiles of 21 fractions obtained by mixing the 80 fractions from four different runs. It can be seen that the conductivity is really minimal (as expected from focused Ampholine fractions) and quite even, except for a few fractions at both extremes of the pH interval, a fact reinforcing the notion that these fractions must be contaminated by highly conducting species.

The various fractions obtained via Rotofor fractionation were tested in CZE for separating a mixture of 10 p*I* markers, in comparison with controls run in non-isoelectric buffers, notably phosphate [although the choice of this last buffer might not have been optimal, since it induces electromigration dispersion; on the other hand, Tris did not improve dramatically the separation (not shown)] [24,25]. A summary of these results is shown in Figs. 6 and 7. It should be noted, first of all, that the phosphate buffer performs poorly: out of the 10 peaks expected, only a few are resolved and quite poorly. The shape of the peaks suggests strong adsorption of several protein species onto the silica wall. This hypothesis is confirmed by the fact that, at progressively higher buffer molarities (from 20 up to 100 mM) some of the peaks sharpen, suggesting inhibition of binding to the wall, as customary under higher ionic strength conditions [26]. On the contrary, good separations are obtained in the quasi-isoelectric p*I* cuts, especially the one with a nominal p*I* value of 8.29 (Fig. 7): at the higher Ampholine concentration run (1.4%), one can appreciate the elution of all 10 p*I* markers, most of them exhibiting sharp and symmetric zones, suggesting absence of interaction with the wall. This is quite unique, considering that, in both cases, the CZE runs have been performed in uncoated capillaries. The significance of these data will be discussed below.

4. Discussion

Fractionation of carrier ampholytes into narrower p*I* cuts has been tried, in the past, by a number of approaches (for a review, see ref. [5], pp. 56–57). More recently, novel approaches have been described by Bossi and Righetti [27] and by Peltre's group [28,29], the former via the multicompartment electrolyzer (MCE) approach, the latter via a preparative IEF in a thick granulated gel (Sephadex G 75 superfine), as described by Radola [30]. The present report offers some fresh insight into this process. First of all, it shows clearly that, notwithstanding the high precision in p*I* cuts obtainable via the MCE instrument, this approach

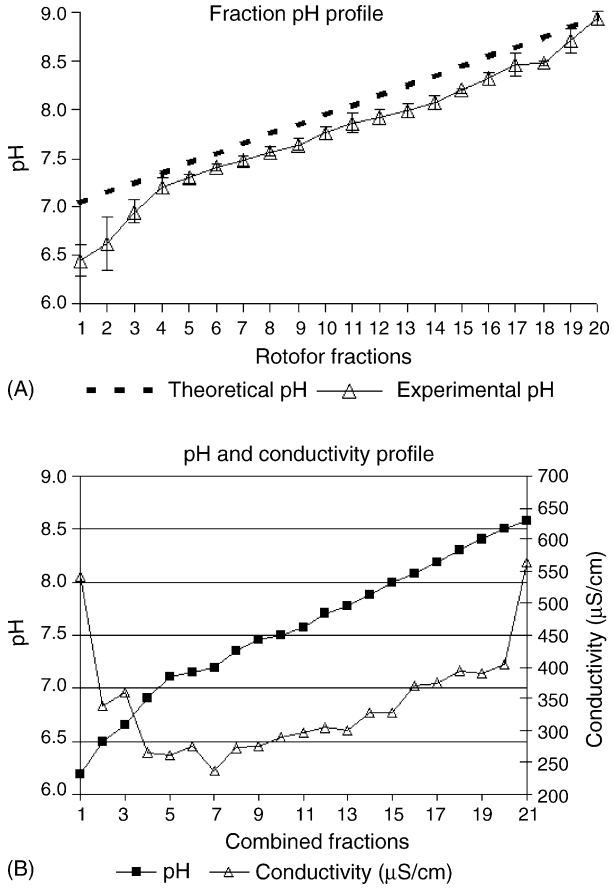

Fig. 5. Panel A: pH profiles obtained by fractionating a pH 7–9 interval into 20 fractions. Experimental conditions:
- 18 mL Ampholine 5% in water (pH 7.95);
- 0.1 M H_3PO_4 (anode), 0.1 M NaOH (cathode);
- 700 V, 12 W MAX, 15 mA MAX;
- Coolant at 10 °C;
- Focusing time: 2 h.

Panel B: pH and conductivity profiles of 80 fractions (20 × 4 runs) combined into 21 new fractions according to their pH values.

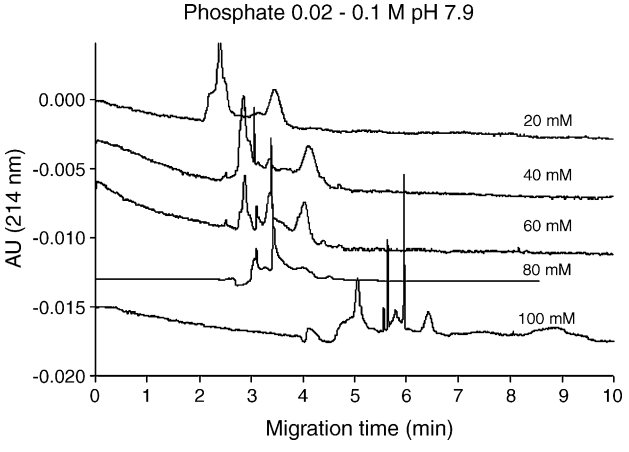

Fig. 6. CZE runs in control, non-amphoteric buffers of 10 p*I* marker proteins. The figure shows various runs in phosphate buffer pH 7.9 at increasing molarities from 20 up to 100 mM.

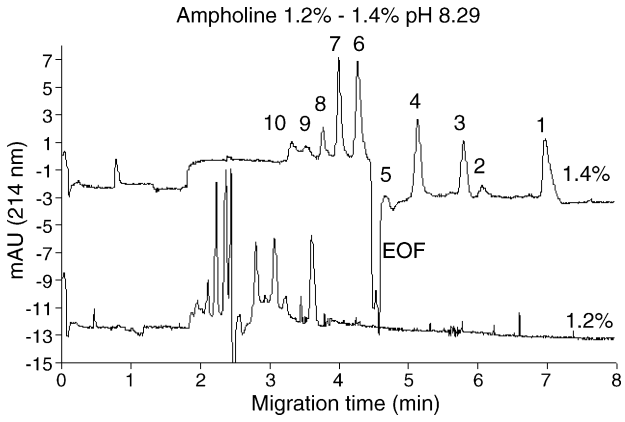

Fig. 7. Representative CZE runs of the same 10 p*I* marker proteins in a ultra-narrow Ampholine cut having a nominal p*I* of 8.29. Notice, in this last case, the proper separation of the 10 protein zones (labelled with numbers from 1 to 10, corresponding to the proteins listed in Table 3), with high resolution at an Ampholine concentration of 1.4%.

is too cumbersome, lengthy and difficult, since in order to obtain narrower and narrower p*I* cuts, one has to resort to a cascade set-up. Also, the preparation of the isoelectric membranes requires skills and the process is intrinsically lengthy due to the fact that such membranes slow down migration, since some sieving effects are operating also on Ampholine buffers, as their molecular mass is substantially higher than that of conventional buffers (in the range of 600–900 Uma) [31]. The Rotofor approach has some distinct advantages, notably the much reduced focusing times (barely 2 h) and the fact of enabling direct collection of 20 fractions, each with a nominal p*I* span of 0.1-pH units. For all practical purposes, these very narrow p*I* cuts are as narrow as could possibly be experimentally obtained and represent very valuable, quasi-isoelectric buffers.

Some unique properties of such IEBs are also here highlighted for the first time. It was stated in the introduction that the approach by Hjertén et al. [4] (consisting on the use of alkaline isoelectric buffers, mostly Lys and Arg) was unpractical, since at this high operative pH values strong adsorption of proteins to the silica wall would ensue. Thus, for a proper use, coating of the inner wall of the tube had to be performed, typically requiring covalent affixing of neutral, hydrophilic polymers that would shield the wall from contact with the proteins. In fact, in uncoated walls strong adsorption is indeed visible, even at moderately pH values (see Fig. 6). It is extraordinary that the narrow p*I* Ampholine cuts substantially reduce such an adsorption to the silica wall (see Fig. 7). This was unexpected. A possible mechanism: Ampholines are synthesized from an oligo-protic backbone, consisting of oligo-ethylene imines some 4–6 nitrogens long [22]. In general, at the p*I* value, any Ampholine species should have possibly only one positive and one negative charges, balancing each other and rendering null the net surface charge. We hypothesize, though, that, upon approaching the silica wall, where up to 50% of the silanols are ionized, even the uncharged nitrogens could be induced to carry a positive charge, thus neutralizing the negative charge of the wall. The binding to adjacent silanols on the wall by adjacent nitrogens on the oligomeric

buffer would produce a cooperative effect, thus ensuring tenacious adhesion to the wall. The final result would be a shielding of the negative charges of the silica wall, effectively impeding binding of the protein analytes. This mechanism does not exclude another concomitant effect: the possibility that these oligo-protic buffers would also interact with the protein analytes, thus modulating their mobility and further preventing their binding to the silica. If this is the case, it might turn out that indeed oligo-protic buffers would be much preferable to monoprotic ones, as currently adopted in most electrophoretic separations in vogue in present times. It would additionally appear that the fractionation of 2-pH unit Ampholine ranges into quasi-isoelectric, 0.1-pH unit spans to be adopted as IEB background electrolytes in CZE separations, could be a much better approach than trying to synthesize ad hoc amphoteric buffers containing solely one positive and one negative group. This synthetic approach might be quite demanding and might not confer to such buffers the unique properties that these oligo-protic buffers seem to possess. Moreover, the sub-fractionation of commercially available 2-pH unit carrier ampholytes offers the possibility of testing many fractions, eventually discarding those containing individual "bad" ampholytes (e.g., interacting with some proteins in the sample) and retaining the "good" ones. Additionally, the fact that these ultra-narrow p*I* cuts are not represented by a single chemical species, but surely by a mixture of them, could be beneficial as well. In fact, it might be such a limited diversity that ensures these unique silica shielding effects. One might try to guess how many species could be present in such 0.1-pH unit spans. The Vesterberg synthetic approach should ensure, over a 2-pH unit interval, from a minimum of 100 up to 200 different chemical entities [22]; thus, over a 0.1-pH unit span, one should expect some 5–10 different buffering compounds, possible only one or two representing the majority. It could be this micro-diversity that ensures, additionally, these unexpected shielding effects on the silica wall.

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References

[1] W. Mandecki, M. Hayden, DNA 7 (1988) 57.
[2] M. Bier, T. Long, J. Chromatogr. 604 (1992) 73.
[3] M. Bier, J. Ostrem, R.B. Marquez, Electrophoresis 14 (1993) 1011.
[4] S. Hjertèn, L. Valtcheva, K. Elenbring, J.L. Liao, Electrophoresis 16 (1995) 584.
[5] P.G. Righetti, Isoelectric Focusing: Theory, Methodology and Applications, Elsevier, Amsterdam, 1983.
[6] P.G. Righetti, Immobilized pH Gradients: Theory and Methodology, Elsevier, Amsterdam, 1990.
[7] M.S. Bello, L. Capelli, P.G. Righetti, J. Chromatogr. A 684 (1994) 311.
[8] A. Bossi, P.G. Righetti, J. Chromatogr. A 840 (1999) 117.
[9] A. Bossi, P.G. Righetti, Electrophoresis 18 (1997) 2012.
[10] F. Nembri, P.G. Righetti, J. Chromatogr. A 772 (1997) 203.
[11] A. Bossi, E. Olivieri, L. Castelletti, C. Gelfi, M. Hamdan, P.G. Righetti, J. Chromatogr. A 853 (1999) 71.
[12] S. Lalwani, G. Vigh, Electrophoresis 26 (2005) 3.
[13] S. Lalwani, E. Tutu, G. Vigh, Electrophoresis 26 (2005) 2047.
[14] T. Rodemann, C. Johns, W.S. Yang, P.R. Haddad, M. Macka, Anal. Chem. 77 (2005) 120.
[15] A.V. Stoyanov, P.G. Righetti, J. Chromatogr. A 790 (1997) 169.
[16] A.V. Stoyanov, P.G. Righetti, Electrophoresis 19 (1998) 1674.
[17] M. Horka, K. Slais, Electrophoresis 21 (2000) 2814.
[18] J.L. Beckers, Electrophoresis 24 (2003) 548.
[19] P.G. Righetti, C. Gelfi, A. Bossi, E. Olivieri, L. Castelletti, B. Verzola, P.G. Righetti, Electrophoresis 21 (2000) 4046.
[20] P.G. Righetti, E. Wenisch, M. Faupel, J. Chromatogr. 475 (1989) 293.
[21] N.B. Egen, M. Bliss, M. Mayersohn, S.M. Owens, L. Arnold, M. Bier, Anal. Biochem. 172 (1988) 488.
[22] O. Vesterberg, Acta Chem. Scand. 23 (1969) 2653.
[23] P.G. Righetti, M. Pagani, E. Gianazza, J. Chromatogr. 109 (1975) 341.
[24] P. Gebauer, P. Pantuckova, P. Bocek, J. Chromatogr. A 894 (2000) 89.
[25] P. Gebauer, P. Bocek, Electrophoresis 21 (2000) 2809.
[26] M. Chiari, M. Nesi, P.G. Righetti, in: P.G. Righetti (Ed.), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, Boca Raton, 1995, p. 1.
[27] A. Bossi, P.G. Righetti, Electrophoresis 16 (1995) 1930.
[28] J.M. Busnel, M.C. Hennion, G. Peltre, J. Chromatogr. B 818 (2005) 99.
[29] J.M. Busnel, F. Kilàr, V. Kasicka, M.C. Hennion, G. Peltre, J. Chromatogr. A 1087 (2005) 183.
[30] B.J. Radola, Ann. N.Y. Acad. Sci. 209 (1973) 127.
[31] A. Bianchi Bosisio, R.S. Snyder, P.G. Righetti, J. Chromatogr. 209 (1981) 265.