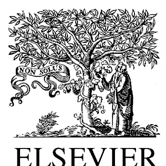

ELSEVIER

Journal of Chromatography A, 853 (1999) 71–82

JOURNAL OF CHROMATOGRAPHY A

General experimental aspects of the use of isoelectric buffers in capillary electrophoresis

Alessandra Bossi[a], Erna Olivieri[a], Laura Castelletti[a], Cecilia Gelfi[b], Mahmoud Hamdan[c], Pier Giorgio Righetti[a,*]

[a]*University of Verona, Department of Agricultural and Industrial Biotechnologies, Strada Le Grazie, Cà Vignal, 37134 Verona, Italy*

[b]*ITBA, CNR, L.I.T.A., Via Fratelli Cervi 93, Segrate 20090 Milan, Italy*

[c]*Glaxo Wellcome Medicines Research Center, Via Fleming 4, 37134 Verona, Italy*

Abstract

Four acidic, isoelectric buffers, for peptide and protein separations, have been recently described and adopted in capillary zone electrophoresis: cysteic acid [Cys-A, isoelectric point (p*I*) 1.85], iminodiacetic acid (IDA, p*I* 2.23), aspartic acid (Asp, p*I* 2.77) and glutamic acid (Glu, p*I* 3.22). These four buffers allow to explore an acidic portion of the titration curves of macroions, covering about 1.6 pH units (from pH 1.85 to ca. 3.45), thus permitting resolution of compounds having coincident titration curves at a given pH value. Given the rather acidic p*I* values of these buffers, their long-term stability has been investigated, by monitoring pH and conductivity changes upon increasing storage times. When dissolved in plain water, all four buffers appear to give constant pH and conductivity readings up to 15 days; after that, the conductivity keeps steadily increasing in a similar fashion. The same parameters, when the same buffers are dissolved in 6 *M* urea, appear to be stable for only one week, with the conductivity progressively augmenting after this period. A similar behaviour is exhibited by histidine (p*I* 7.70), a neutral, isoelectric buffer adopted for separation of DNA fragments. By mass spectrometry, Cys-A shows minute amounts (ca. 1%) of a degradation product after ageing for 3 weeks; in the same time period, Glu is extensively degraded (20%). No degradation species could be detected in IDA and Asp solutions. It is additionally shown that the acidic buffers are not quite stationary in the electric field, but can be transported at progressively higher rates (according to the p*I* value) from the cathodic to the anodic vessel. This is due to the fact that, at their respective p*I* values, a fraction of the amphotere has to be negatively charged in order to provide counterions to the excess of protons due to bulk water dissociation. Guidelines are given for the proper use and storage of such buffers. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Buffer composition; Isoelectric buffers; Amphoteric buffers; Peptides; Proteins

1. Introduction

Isoelectric buffers are not a novelty in separation science: their use stems from the theoretical concepts developed in the early sixties by Svensson-Rilbe, the inventor of isoelectric focusing (IEF) [1,2]. The implementation of IEF was brought about by Vesterberg's synthesis of the carrier ampholytes (CAs) [3], the soluble, amphoteric buffers needed to create and maintain, under an electric field, a pH gradient onto which amphoteric macroions would 'focus' (i.e., collect and condense into sharp zones at a particular point of the pH scale called the "isoelectric point", p*I*, or point of null net charge of such a

*Corresponding author. Tel.: +39-045-809-8901; fax: +39-045-809-8901.

E-mail address: righetti@imiucca.csi.unimi.it (P.G. Righetti)

0021-9673/99/$ – see front matter © 1999 Published by Elsevier Science B.V. All rights reserved.
PII: S0021-9673(99)00519-1

macroion). The CA buffers are a multitude of amphoteric compounds, generated by a random synthesis via addition of the double bond of acrylic acid to a mixture of linear and branched oligoamines, with p*I* values fairly well distributed along the pH scale and thus able to cover the grounds quite thoroughly along the pH 3–10 interval [4]. However, due to the fact that CAs represent a vast concoction of such buffers (perhaps comprising from 600 up to 5000 species) they cannot possibly be used for zone electrophoresis at a single pH value; if the electric circuit is closed in between a free acid as anolyte and a free base as catholyte they will automatically generate a pH gradient, thus their use is essentially limited to focusing techniques. However, a single amphoteric compound, provided it possesses a good buffering power at the p*I* value (condition well satisfied by a small value of Δp*K*, i.e. of the distance between the two protolytic groups across the p*I* of the amphotere) can be used as the sole component of the background electrolyte in zone electrophoresis. Perhaps one of the earliest reports on the use of such isoelectric buffers was that of Mandecky and Hayden [5], who adopted isoelectric histidine as the sole buffering species in gel slab electrophoresis of oligonucleotides. Another example can be traced in the preparative free-flow electrophoresis method of Bier et al. [6,7], where cycloserine was used as buffering ion. However, it was only in 1995 that Hjertèn et al. [8] proposed isoelectric buffers for capillary zone electrophoresis (CZE) analysis. This group demonstrated excellent fractionations of proteins and other small M_r analytes, in a time scale up to 100 s.

Soon isoelectric buffers were utilized also for separation of peptides. By and large, the experimental parameters adopted in developing peptide maps by CZE have been based on the use of acidic buffers (typically phosphate, formate, glycinate) at pH values ranging from 1.9 up to 2.8, in concentrations from 25 to 100 m*M* (for a review, see [9]). Acidic separation buffers for peptide mapping by CZE present some distinct advantages. First of all, at pH values well below the p*K* of the free silanols on the fused silica wall (assessed as p*K* 6.3, with a wall neutralization at pH 2.3) [10], their dissociation will be significantly suppressed and, in principle, the negative charge on the silica surface abolished. The electroosmotic flow (EOF) should thus be negligible, the peak capacity greatly increased and any interaction of the analyte with the wall suppressed. Additionally, the low pH value ensures that the vast majority of peptides will bear a net positive charge, thus allowing their migration at the cathodic end of the capillary, past the detector window. However, at low-enough pH values effectively minimizing peptide–wall interaction (i.e. at or below pH 2), the buffer and bulk water conductivities are so high that only low voltage gradients can be adopted (typically not higher than 200 V/cm), thus greatly lengthening the analysis times (in many reports, up to 60–70 min are required for fully developing complex peptide maps). During such long runs, peptide zones considerably broaden and peak resolution worsens. In view of these shortcomings, Nembri and Righetti [11] recently suggested the use of isoelectric aspartic acid as a background electrolyte, operating at pH= p*I*=2.77 (at 25°C). These authors could produce peptide maps of casein in only 10–12 min (as opposed to 80 min in standard phosphate buffer, pH 2.0) at voltage gradients as high as 800 V/cm, with much increased resolution. The method has been successfully used for generating peptide maps of α- and β-globin chains from tryptic digests of human adult hemoglobin [12], and has been shown to be highly competitive in respect to analogous separations by RP-HPLC. In search for additional background electrolytes, Bossi and Righetti [13] reported the use of iminodiacetic acid (IDA) as a unique isoelectric buffer, possessing a remarkably low p*I* (2.23 at 100 m*M* concentration) and an extreme solubility both in neat water and in a number of hydro–organic solvents. More recently, the potential use of cysteic acid in peptide separations has also been suggested [14]. Isoelectric buffers have been quite successful in a number of protein separations as well, ranging from glyadins (wheat storage proteins) [15], to zeins (maize storage proteins) [16] to human globin chains [17]. Recent reviews have also appeared on this topic [18,19] and a theoretical paper [20] has summarized the fundamental properties of such buffers.

Although it would appear that CZE in isoelectric buffers will become a technique of growing importance in the years to come, there remains a main missing link in this field: a proper study on the

behaviour of these buffers upon prolonged use. We report here data on the long-term stability of isoelectric buffers, both alone and in presence of additives such as urea, and offer general guide-lines on their correct utilization.

2. Experimental

2.1. Reagents and protein samples

IDA, cysteic acid, aspartic acid, glutamic acid, the dipeptide Asp–Asp and histidine were obtained from Fluka (Buchs, Switzerland). Fused silica capillaries (75 μm I.D.×375 μm O.D.) were from Polymicro Technologies (Phoenix, AZ, USA) and were used as such, without inner coating. The Centricon 30 membranes were from Millipore (Bedford, MA, USA). Hydroxyethylcellulose (HEC, number-average molecular mass, M_n, of 27 000) was from PolySciences (Warrington, PA, USA). The zein extracts from the maize lines W64A+ (wild type), W64Ao2 (opaque 2 mutant) and W64Afl2 (floury 2 mutant) were a kind gift from Dr. A. Viotti, CNR, Milan, Italy.

2.2. Capillary electrophoresis

Capillary zone electrophoresis (CZE) was carried out with the Bio Rad Bio Focus 3000 instrument. Uncoated capillaries of 50 cm×75 μm I.D. were used. In order to check the long-term stability of each of these isoelectric buffers when used as catholyte and anolyte, CZE was run for hours to days without changing the electrode reservoirs. At the times indicated in the various graphs, aliquots of anolyte and catholyte were harvested and their pH and conductivity values carefully measured at room temperature. For zein analysis (in this case a 30 cm×50 μm I.D. capillary was used), the following electrolyte solutions were adopted: washing buffer (in between runs): 40 m*M* isoelectric aspartic acid (pH=p*I*=2.77 at 25°C), added with 0.5% HEC (M_n=27 000) and 8 *M* urea (apparent pH 3.9); running (and electrode) buffer: 0.5% HEC, 40 m*M* Asp, 6 *M* urea; overnight equilibration buffer for the capillary: 0.5% HEC and 25 m*M* Asp; sample buffer: 0.5% HEC, 5 m*M* Asp, 8 *M* urea, 2% β-mercaptoethanol.

2.3. pH determinations

In order to assess potential pH changes upon prolonged storage the solution pH was carefully assessed with a pHM64 Research pH Meter, equipped with a GK2401C combination electrode from Radiometer (Copenhagen, Denmark). All isoelectric buffers were dissolved at 50 m*M* concentration, either in plain water or in 6 *M* urea solutions.

2.4. Conductivity measurements

The conductivity of neat solutions of the various buffers, or in presence of 6 *M* urea, was measured at 25°C with an Orion conductivity meter fitted with a 1-cm cell.

2.5. Mass spectrometry (MS)

MS measurements were performed by using a VG platform from Micromass, UK, coupled to a liquid chromatography (LC) instrument, with a HP1100 binary pump, from Hewlett-Packard (Palo Alto, CA, USA). LC–MS conditions were: column, C_{18} ODS3 (15×0.4 cm) operated at a 1 ml/min flow rate; eluent: water–TFA (99.9:0.1) (isocratic). A flow splitter allowed 20 μl/min into a single quadrupole MS instrument operated in the positive electrospray ionization mode.

3. Results

3.1. Long-term stability of amphoteric, isoelectric buffers

Table 1 reports the physico-chemical properties of the four acidic, isoelectric buffers investigated in the present report, together with their chemical formulas. The first study undertaken has been an assessment of the shelf-life of isoelectric buffers when stored at room temperature, dissolved as pure species in plain water as a solvent. In this case, the final pH of such solutions will approach the p*I* value of each compound. This study was deemed necessary due to the fact that quite a number of them have strongly acidic p*I* values, thus they could potentially degrade at such local pH values. As indicators of potential degra-

Table 1
Properties of amphoteric, acidic, isoelectric buffers

Electrolyte	Chemical formulas	pK_1	pK_2	pK_3	pI	$pI-pK_{prox}$
Cysteic acid	$HO_3S-CH_2-CH(NH_2)-COOH$	1.55	2.15	9.3	1.85	0.3
Imino diacetic acid	$HOOC-CH_2-NH-CH_2-COOH$	1.73	2.73	——	2.23	0.5
Aspartic acid	$HOOC-CH_2-CH(NH_2)-COOH$	1.88	3.66	9.3	2.77	0.89
Glutamic acid	$HOOC-CH_2-CH_2-CH(NH_2)-COOH$	2.19	4.25	9.3	3.22	1.03

dation, pH and conductivity changes as a function of storage time were evaluated. Fig. 1 gives these variations for four compounds, in order of increasing p*I* values: cysteic acid (A), IDA (B) Asp (C) and Glu (D). In all cases, these changes have been monitored for a period of up to 3 weeks. There seems to be a general trend emerging: the pH and conductivity of all solutions remain stable for a period of ca. two weeks (except for Glu, where both parameters keep increasing steadily already after a few days), then there appear to be fluctuations in both parameters. The pH changes, in some cases, seem to be very minute (e.g., Fig. 1C, Asp) or to be non-monotonic, so they have been neglected. On the contrary, the conductivity increases steadily for all solutions analyzed, and this increment (in some cases quite marked) appears to be substantial after a period of 3 weeks.

Fig. 2 displays the monitoring of the same parameters for the set of four compounds dissolved in 6 *M* urea. Here too it is difficult to draw any conclusions from the shape of the pH curves: they either fluctuate or give minimal absolute changes with time, except for Asp and Glu which show a steady increase in pH, for a total of 0.2 and 0.4 pH units, respectively, over a period of 3 weeks. On the contrary, conductivity changes are quite marked and appear to increase constantly for all buffers studied. In addition, the onset of such conductivity increments occurs quite early as compared to the same solutions in the absence of urea: typically already after a week.

Fig. 3 monitors the behaviour of the corresponding basic amphoteric buffer, notably His. His, at present, has been used mostly for DNA separations [21,22]. In the absence of urea (Fig. 3A), not much can be stated in terms of pH variation, which basically fluctuates around its initial value. On the contrary, the conductivity keeps increasing already after the

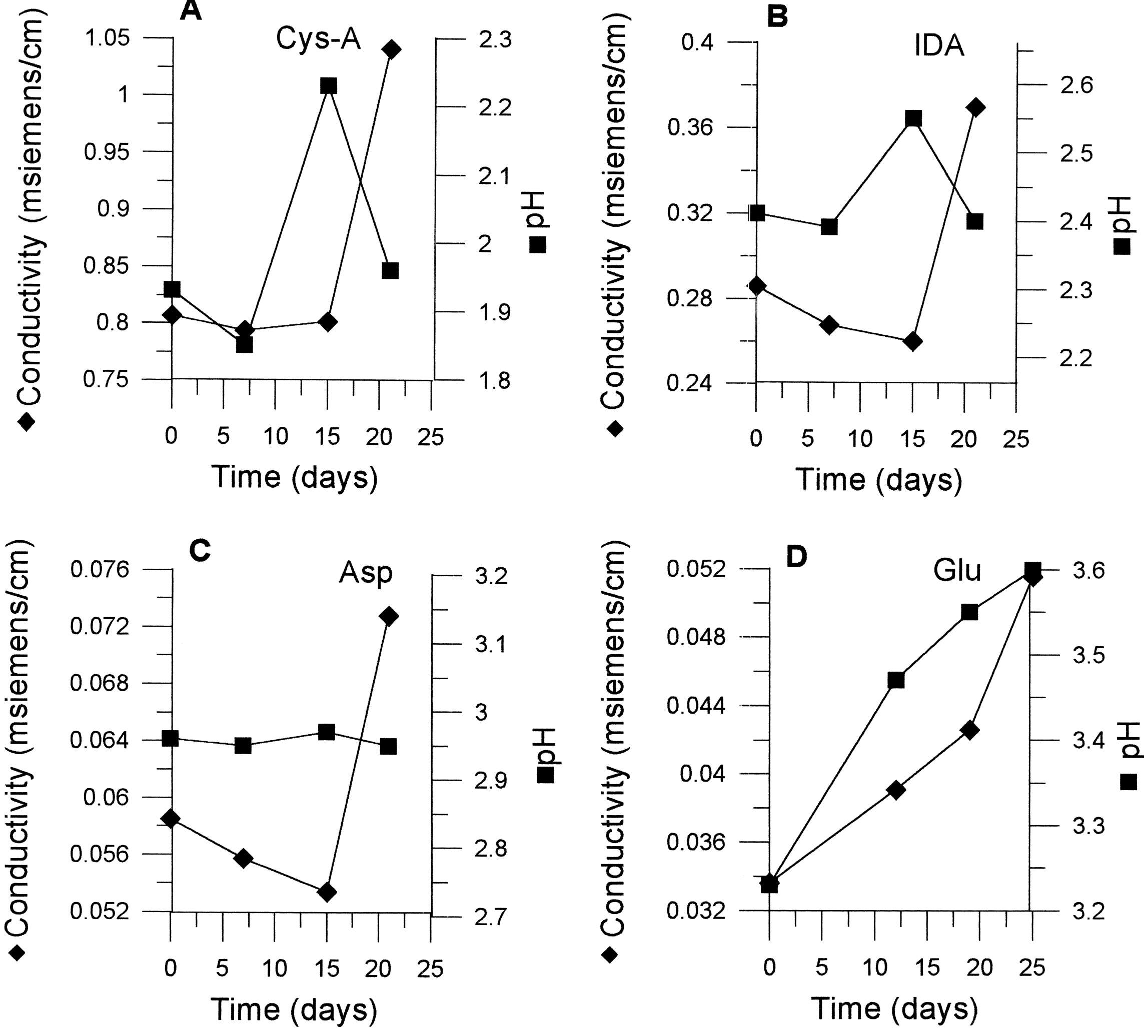

Fig. 1. Ageing of four isoelectric buffers as monitored by pH and conductivity changes with time. The pH and conductivity of solutions of cysteic acid (A), iminodiacetic acid (B), aspartic acid (C) and glutamic acid (D), dissolved at 50 m*M* concentration in plain water have been monitored for a period of up to 21 days. Note the general trend of all solutions to increase the conductivity after ca. 15 days.

first few days from the preparation of the solution. When the same buffer is dissolved in urea (Fig. 3B) two phenomena are immediately apparent: the pH increases by 0.2 pH units at the end of 3 weeks and the conductivity steadily augments as well, twice as fast as compared with His in plain water.

3.2. Net migration of isoelectric buffers in the electric field

Another practical point of interest to users of CZE is how often one should replenish the anolyte and catholyte solutions, so as to avoid electrode polarization and marked changes of buffer pH due to buffering ion and counter ion depletion. This point, in principle, should not be critical when using isoelectric buffers, since, due to the fact that they should not bear a net charge, their depletion at either electrode should be negligible. Fig. 4 explores potential pH and conductivity variations as a function of time of operation of the CZE instrument under a constant electric field of 20 000 V. The data

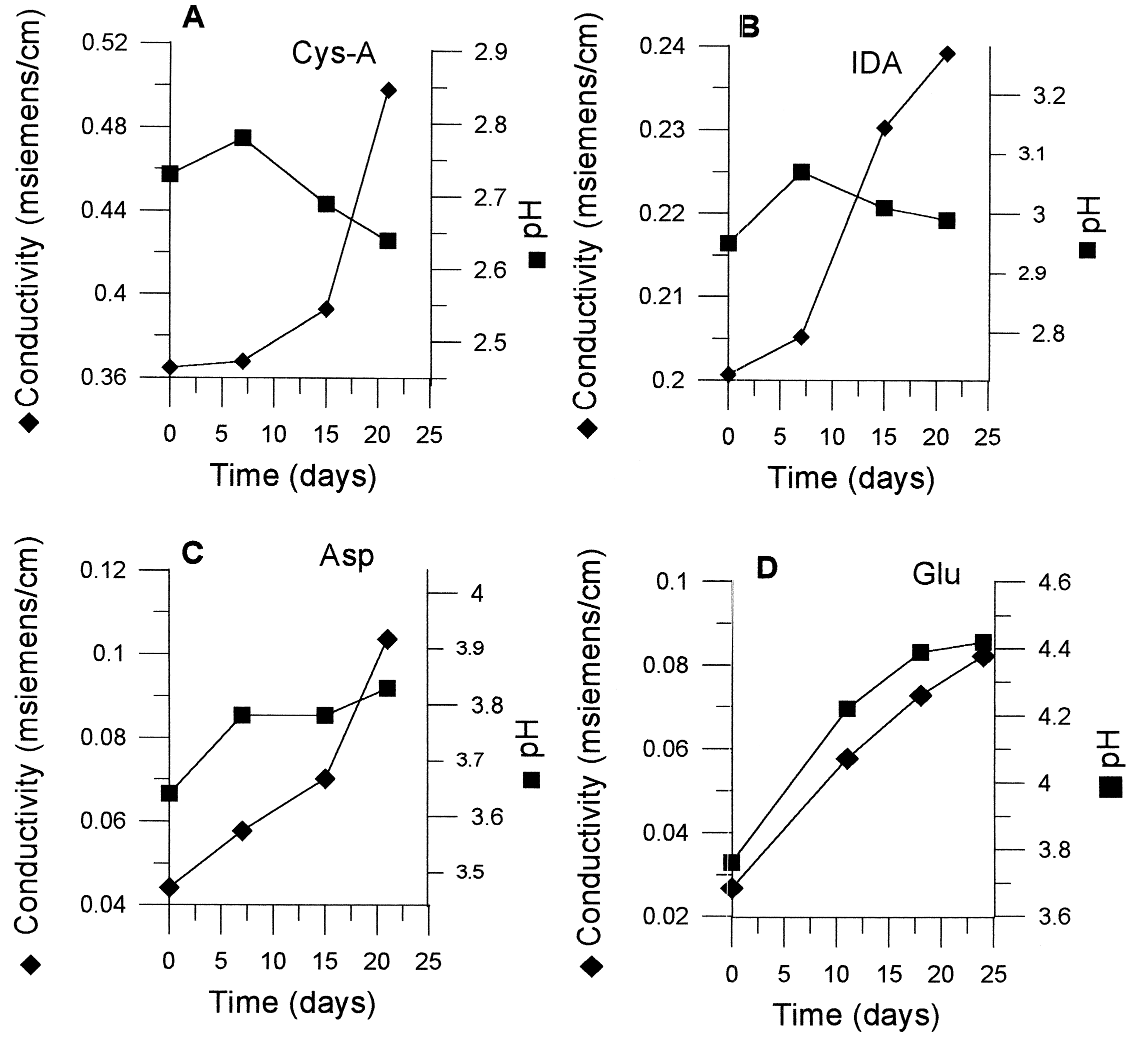

Fig. 2. Ageing of four isoelectric buffers as monitored by pH and conductivity changes with time. The pH and conductivity of solutions of cysteic acid (A), iminodiacetic acid (B), aspartic acid (C) and glutamic acid (D), dissolved at 50 m*M* concentration in 6 *M* urea have been monitored for a period of up to 21 days. Note the general trend of all solutions to increase the conductivity already after one week.

are expressed as difference in pH and conductivity between the two electrode vessels. It should be noted that, upon continuous operation, the pH keeps increasing at the cathode, whereas the conductivity keeps augmenting at the anode. This means that there must be a net transport of Asp from the cathodic to the anodic vessels. If that is the case, the pH should keep increasing at the cathode and decreasing at the anode [13]. Decrements of pH in the anodic vessel should bring about increments of conductivity. This is to be expected. In fact, at progressively more acidic pH values, a fraction of the amphotere should be non-isoelectric, in order to neutralize the excess positive charges brought about by bulk water ionization. E.g., in a solution of Asp at pH 3.0, there must be 1 m*M* proton concentration which must be neutralized by a 1 milli-molar fraction of Asp being non-isoelectric, but negatively charged. This excess, non-isoelectric molar fraction will constantly migrate towards the anode, creating an unbalance in concentration of Asp in the two electrodic vessels. This phenomenon can be neglected during

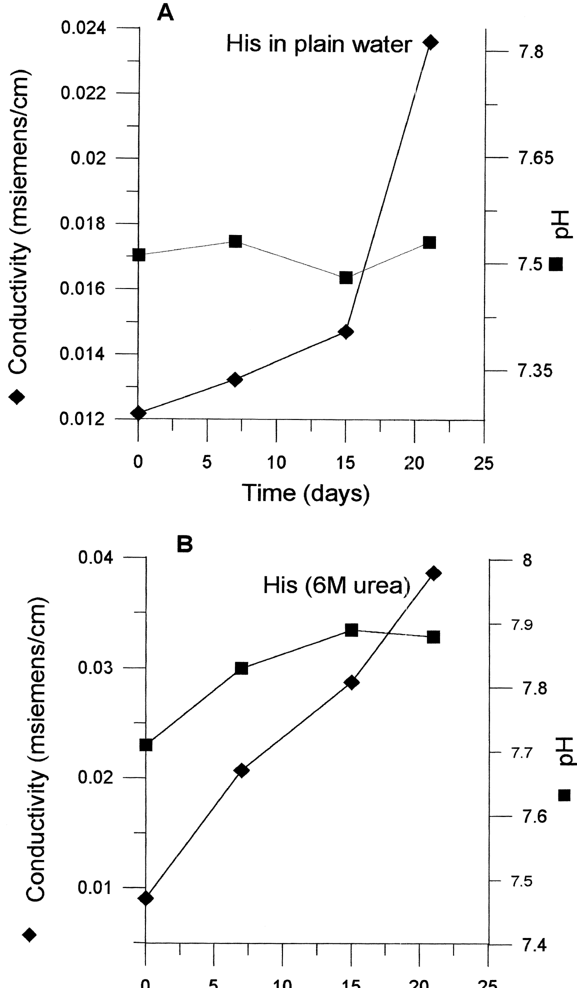

Fig. 3. Ageing of isoelectric His buffer as monitored by pH and conductivity changes with time. The pH and conductivity of solutions of His, dissolved at 50 mM concentration either in water (A) or in 6 M urea (B) have been monitored for a period of up to 21 days. Note the general trend of all solutions to increase the conductivity already after a few days. In water, however, the pH does not seem to change, whereas it keeps increasing in 6 M urea solutions up to pH 7.9.

the first 10 hours of operation, but after that it becomes quite appreciable. We have tried to measure this phenomenon in a direct way, by injecting anolyte and catholyte solutions after given periods of continuous electrophoresis. However, since Asp is a UV-transparent buffer, we have used the dipeptide Asp–Asp, which has similar properties (pI 3.04,

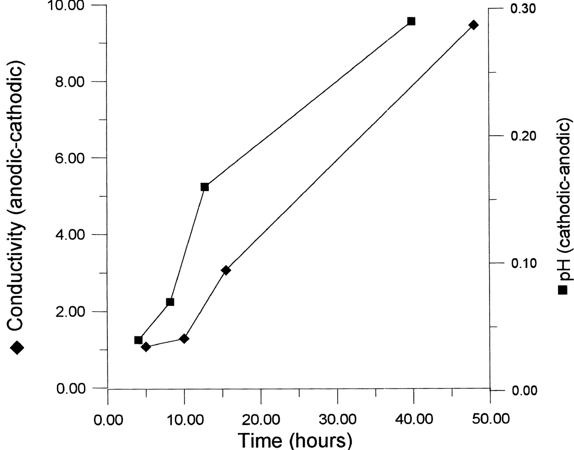

Fig. 4. Net migration of isoelectric Asp in the electric field. The same solution of 40 mM Asp was loaded in the capillary and used as catholyte and anolyte (0.85 ml vessels). At the given time intervals, the pH and conductivity were monitored in the anodic and cathodic vessels. The conductivity data refer to the difference between the anodic and cathodic, whereas the pH values represent the difference between the cathodic and anodic vessels. Conditions: constant electric field of 20 000 V over 50 cm capillary length.

pI–pK_{prox}=0.34). As shown in Fig. 5, it can be appreciated that, after 10 h of electrophoresis, the concentration of the dipeptide has diminished at the cathode and increased at the anode by >20%, due to electrophoretic transport of the non-isoelectric, negatively-charged fraction (at 25 h, the anode contains 37% more Asp–Asp as compared to the cathode).

3.3. On the potential degradation of isoelectric buffers

We have next investigated what happens to the four acidic buffers upon storage in solution, since they might potentially degrade, due to their rather acidic pI values. MS analysis failed to reveal any degradation products in IDA and Asp solutions aged up to 21 days at room temperature. The situation was somewhat different in the case of Cys-A: as shown in Fig. 6, a minute amount of a possible degradation product (ca. 1%) can be detected in the total ion current (TIC) profile of the 3-week old Cys-A (lower left panel), which is absent in the TIC profile of fresh Cys-A (upper left panel). The mass spectrum (upper

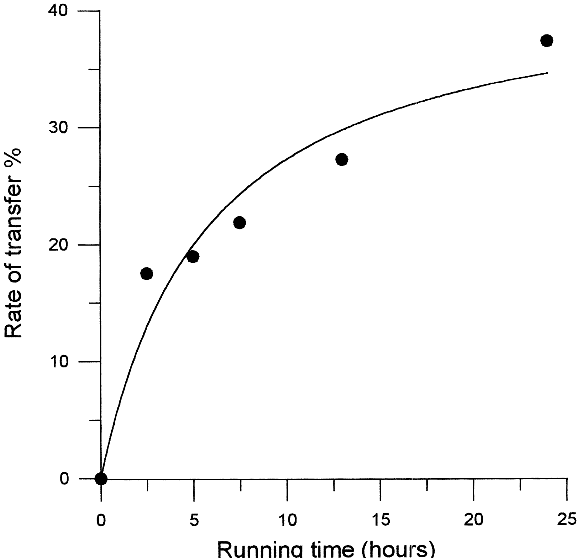

Fig. 5. Measurement of net migration of the isoelectric dipeptide Asp–Asp. Aliquots of anodic and cathodic reservoir solutions were harvested at the times indicated and subjected to electrophoresis in Tris–borate buffer, pH 9.6. After peak integration, the difference in dipeptide molarity between the two vessels was expressed as rate of transfer from one vessel to the other. Conditions: constant electric field of 5000 V over 30 cm capillary length.

right panel) associated with the fresh solution contains the signals of mass to charge (*m*/*z*) ratios of: 170 $[\text{Cys-A}+\text{H}]^+$; 339 $[2\text{Cys-A} +\text{H}]^+$ and 124 $[\text{Cys-A}-\text{CO}_2+\text{H}]^+$. On the other hand, the minor compound in aged Cys-A (retention time, t_R, of 1.98 min) gives a single peak of *m*/*z* 61 as well as a weak dimer at *m*/*z* 122, caused by a known aggregation effect within the electrospray source, which we interpret as acetic acid, produced by degradation of Cys-A. Much more pronounced, instead, is the degradation occurring to Glu (see Fig. 7). The extra peak appearing in aged Glu (lower left panel, t_R 2.02 min) appears to be dominated by the *m*/z 61 species, as in the case of Cys-A (lower right panel). Fresh Glu (upper left panel) gives the protonated species at *m*/*z* 148 $[\text{Glu}+\text{H}]^+$; 295 (traces of dimer); 102 $[\text{Glu} -\text{CO}_2 +\text{H}]^+$ and 84 $[\text{Glu} -\text{CO}_2 -\text{NH}_2]^+$. The extent of degradation is also quite severe: ca. 20% in a period of 3 weeks.

3.4. Examples of protein separations

Although we have reported in a number of papers

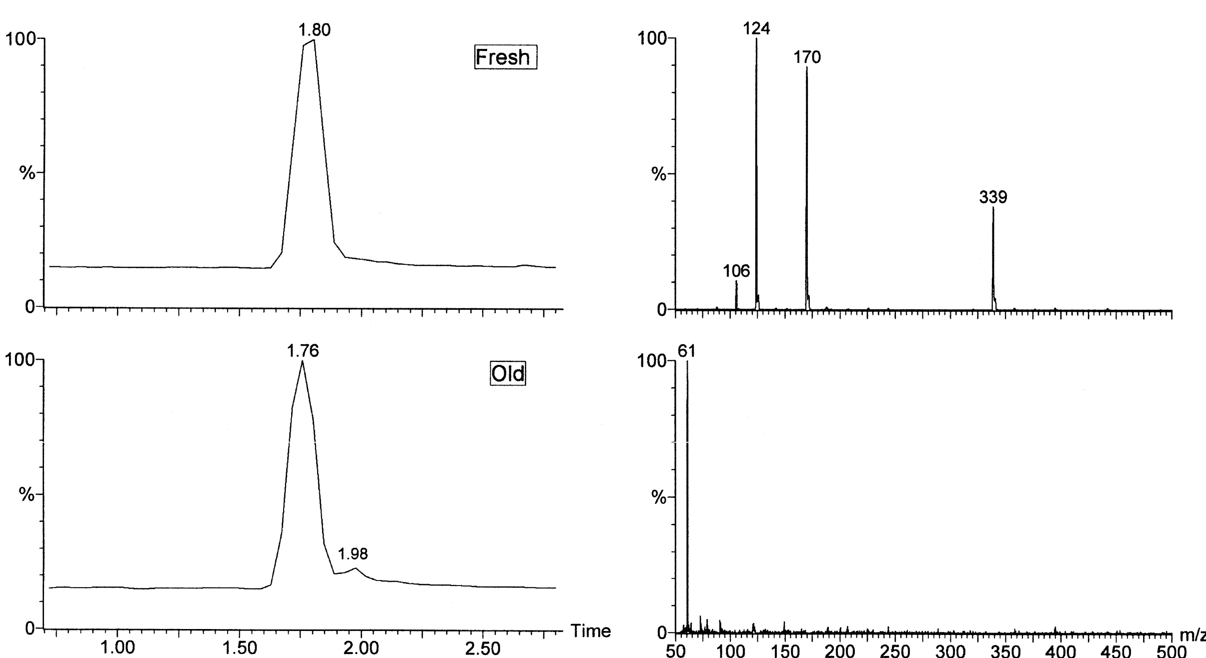

Fig. 6. Left panels: total ion current (TIC) chromatograms of fresh (upper) and aged (lower) Cys-A. Right panels: the electrospray mass spectra associated with the TIC peaks of $t_R = 1.8$ min (fresh) and $t_R = 1.98$ min (aged) Cys-A.

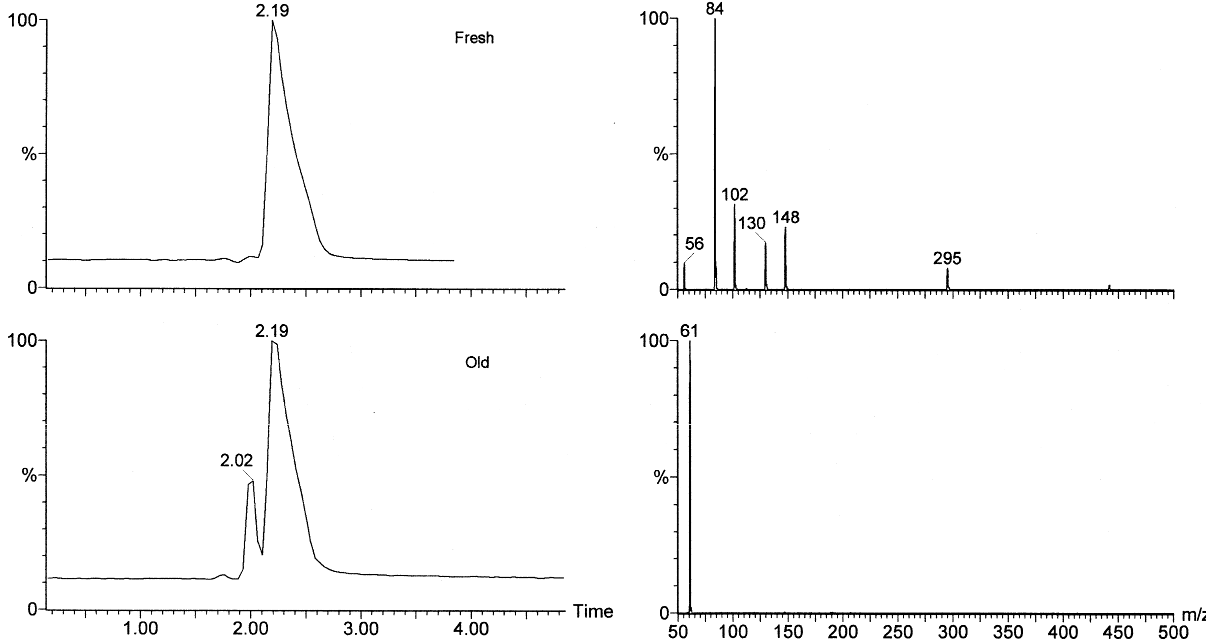

Fig. 7. Left panels: TIC chromatograms of fresh (upper) and aged (lower) Glu. Right panels: positive electrospray mass spectra associated with the TIC peaks at $t_R = 2.19$ min (fresh) and $t_R = 2.02$ min (aged) Glu.

separations of peptides, proteins and nucleic acids [11–19], we will offer here an example on the separation power of CZE in isoelectric buffers. Fig. 8 shows the electrophoretic pattern of zeins from three related lines, the W64A+ and its floury 2 (fl2) and opaque 2 (o2) variants. Peak assignment and numbering was done after collecting and comparing all the zein patterns developed by analysing 21 different maize lines. A total of 31 zein peaks, variously present in the different lines, were considered as relevant for maize line identification. Peak assignment and numbering was made possible by multivariate statistical analysis. Equal numbers are considered to represent the same zein species in all different lines tested. W64Afl2 was found to be one of the lines comprising the largest number of peaks (>20) in all the samples tested, in agreement also with earlier data by IEF [24]. Most lines seem to express fewer zein species, typically a dozen or less. It is seen, from these zein spectra, that these 3 maize lines are easily distinguishable.

4. Discussion

4.1. On the theory of electrophoretic transport in CZE

As already predicted by Jorgenson and Lukacs in 1981 [23], if longitudinal diffusion were the only significant source of band broadening, the number of theoretical plates (N) would be given by:

$$N = \mu V/2D$$

where μ is the analyte mobility, D its diffusion coefficient and V the voltage gradient applied. It is thus seen that high μ and high V values are the most direct way to high-resolution CZE. However, indiscriminately high voltage gradients cannot be applied for eliciting a fast separation, since resolution could worsen, instead of ameliorating. This is due to the fact that the plate height (H), in terms of thermal peak broadening, can be expressed as:

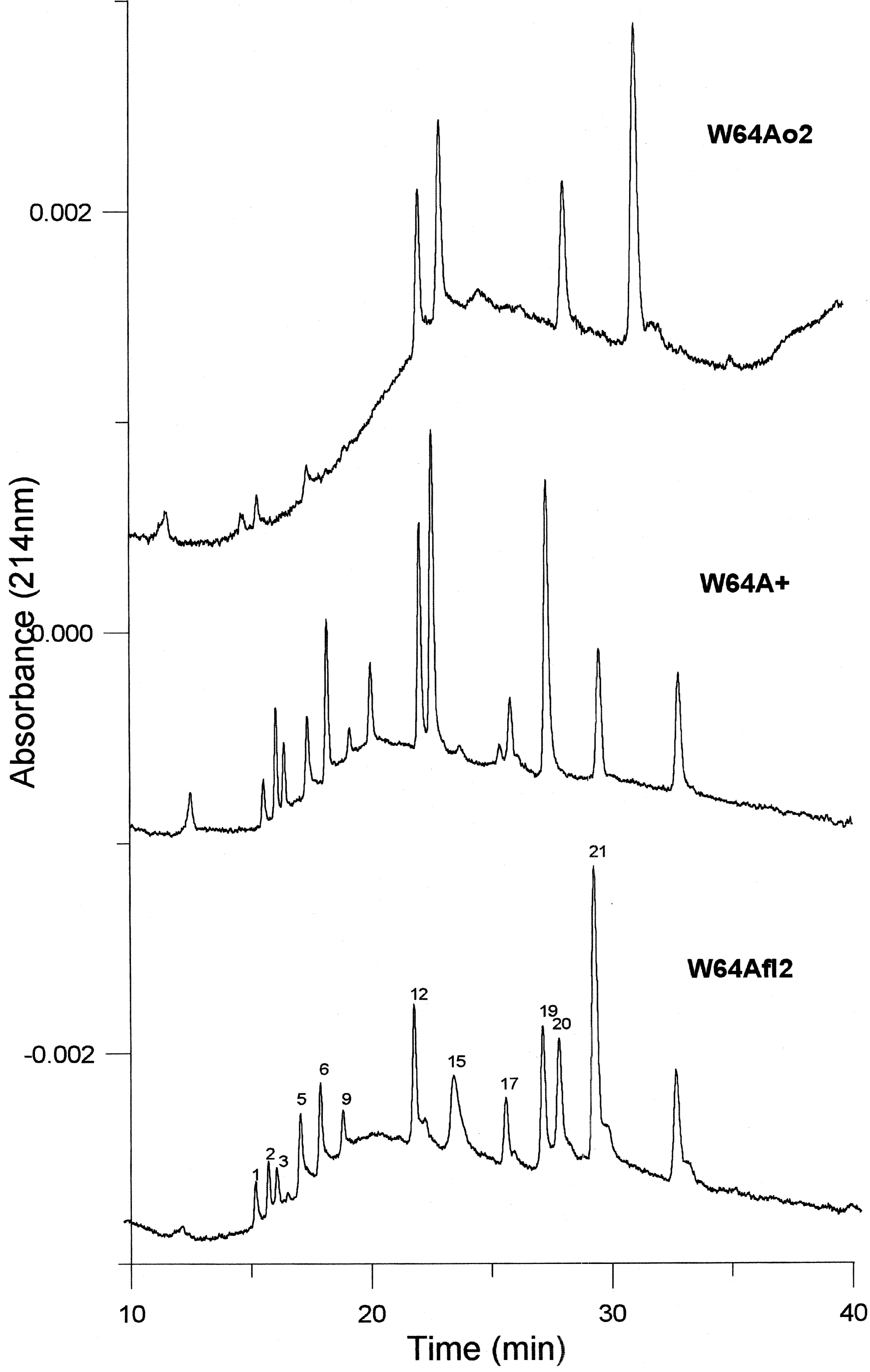

Fig. 8. Representative CZE runs of zeins, obtained by 70% ethanol, 2% β-mercaptoethanol extraction of ground endosperm, from three different lines of maize. Run performed in 40 m*M* Asp buffer, 6 *M* urea and 0.5% HEC (apparent pH: 3.7). Conditions: 30 cm×50 μm I.D. capillary, run at 800 V/cm and 30°C. Detection at 214 nm. From top to bottom: W64Ao2; W64A+; W64Afl2. Peak numbering as obtained by multivariate statistical analysis.

$$H = \frac{f^2 \lambda^2 E^5 R^6 \mu}{1536 D \kappa^2}$$

where f is the temperature factor of the mobility, λ the electric conductivity of the solution, κ its thermal conductivity, R the inner capillary radius and E the field strength. Thus, a simple and direct way for minimising H is to use buffers of low conductivity. It is concluded that maximization of N and minimization of H can be most effectively achieved with the use of amphoteric, isoelectric buffers. This is in fact the route taken by Hjertén et al. [8], in a fundamental paper which describes at length the theoretical and practical aspects of CZE in buffers of very low conductivity. However even when adopting such buffers, when analysing peptides and proteins, two different strategies can be adopted. This is apparent when exploring the typical titration curves (expressed as net charge vs. pH) of such macroions (as an example, see these profiles for globin chains in Figs. 4 and 5 of [17]). Maximization of net charge, for polypeptide chains, is typically achieved only at the extremes of the pH scale, e.g. in the pH 2–3 range (maximum positive charge) and in the pH 11–12 interval (maximum negative charge). Of these two possible strategies, Hjertén's group has adopted separations of proteins at alkaline pH values, by using isoelectric buffers with p*I* values ranging from pH 8.6 up to pH 9.7. On the contrary, our group has focused on the development of strongly acidic, isoelectric buffers, spanning the pH 1.85 to 3.2 interval. One could argue that both approaches are equally satisfactory, but we believe that our method offers distinct advantages over the one proposed in [8]. In fact, in order to avoid protein adsorption and to suppress electroendosmosis (EOF), Hjertén's group has to resort to silica tubes coated with linear polyacrylamide. On the contrary, due to the acidic pH values adopted by us, uncoated capillaries can be utilized, and are in fact recommended. Not only there is no protein adsorption at our low pH values, but also the EOF is negligible and this results in highly reproducible migration times of the analytes. In addition, as seen from the shape of the protein titration curves [17], in order to achieve maximum charge, if operating at alkaline pH values, one should adopt much higher pH values than those proposed in [8], typically above pH 11. At such very high pH values, coated capillaries will rapidly lose their coating; if used uncoated, the silica will deteriorate and start dissolving. In addition, very alkaline pH value require manipulations under anaerobic conditions, since atmospheric carbon dioxide can be rapidly adsorbed, with concomitant pH changes (and conductivity increments due to formation of carbonate ions). As an additional detrimental effect, proteins dissolved in 7 or 8 *M* urea will risk carbamylation, an ever present hazard in urea solutions, due to formation of cyanate at alkaline pH values. All these risks are simply not present in our methodology which utilizes acidic, isoelectric buffers.

4.2. Guidelines on the proper use of isoelectric buffers

Up to the present, however, no systematic study had been performed on the long-term stability of such buffers. Given the present findings, we recommend the following:

1. The four acidic buffers (with the exception of Glu, which should not be left ageing for more than a few days), when dissolved in plain water as a solvent, should not be used for more than 2 weeks and discarded after this period;
2. The same four buffers, when dissolved in 6 *M* urea, should be used for only one week and discarded after this period (again with the exception of Glu, which in this case should be made fresh every day or every other day);
3. Isoelectric His, both alone and in 6 *M* urea, should be used for a maximum of one week.
4. Acidic buffers contain a fraction of non-isoelectric species, proportional to the p*I* value. The more acidic they are, the higher is this fraction. Thus, when used as anolytes and catholytes for extended periods of time, they should be changed with a frequency dictated by their respective p*I* values. E.g., for Asp (p*I* 2.77), replenishment of electrodic vessels can be performed after a rather extended period of time (up to 8 h of operation at 20 000 V), but for Cys-A we recommend changing the content of the electrodic compartments after each run (all these data refer to rather small electrodic vessels, of 0.85 ml volume).

5. Conclusions

A note of caution should be cast on our data on the degradation of isoelectric buffers when dissolved at pH = p*I*. We do not know if the degradation observed is of chemical or biological origin (e.g., via bacterial contamination of the solutions). Although the buffers are prepared in a quasi-sterile way (liquids and container sterilized) they are handled in a non-sterile way, as typical in the daily laboratory routine. We have thus placed the accent on what would occur to a typical user in a laboratory of Separation Science, where sterile handling of liquids is rarely implemented, if at all. It must be emphasized that solutions of amino acids, especially the naturally occurring ones, are a good pabulum for bacterial growth, although the strongly acidic environments in which these amphoteres are dissolved should discourage growth of most micro-organisms. Nevertheless we do not believe that such contamination could apply in our case, first of all because no macroscopic signals could be detected (e.g., turbidity of the solutions) and secondly because the degradation seems to be selective and limited, to a large extent, only to Glu.

Acknowledgements

P.G.R. is supported by grants from Agenzia Spaziale Italiana (No. ARS-98-179) and from MURST (Coordinated Project 40%, Folding e Unfolding di Proteine).

References

[1] H. Svensson, Acta Chem. Scand. 15 (1961) 321–345.
[2] H. Svensson, Acta Chem. Scand. 16 (1962) 456–466.
[3] O. Vesterberg, Acta Chem. Scand. 23 (1969) 2653–2666.
[4] P.G. Righetti, Isoelectric Focusing: Theory, Methodology and Applications, Elsevier, Amsterdam, 1983.
[5] W. Mandecki, M. Hayden, DNA 7 (1988) 57–62.
[6] M. Bier, T. Long, J. Chromatogr. 604 (1992) 73–83.
[7] M. Bier, J. Ostrem, R.B. Marquez, Electrophoresis 14 (1993) 1011–1018.
[8] S. Hjertén, L. Valtcheva, K. Elenbring, J.L. Liao, Electrophoresis 16 (1995) 584–594.
[9] M. Castagnola, I. Messana, D.V. Rossetti, in: P.G. Righetti (Ed.), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, Boca Raton, FL, 1996, pp. 241–275.
[10] M.S. Bello, L. Capelli, P.G. Righetti, J. Chromatogr. A 684 (1994) 311–320.
[11] F. Nembri, P.G. Righetti, J. Chromatogr. A 772 (1997) 203–211.
[12] L. Capelli, A. Stoyanov, H. Wajcman, P.G. Righetti, J. Chromatogr. A 791 (1997) 313–322.
[13] A. Bossi, P.G. Righetti, Electrophoresis 18 (1997) 2012–2018.
[14] A. Bossi, P.G. Righetti, J. Chromatogr. A 840 (1999) 117–129.
[15] L. Capelli, F. Forlani, F. Perini, N. Guerrieri, P. Cerletti, P.G. Righetti, Electrophoresis 19 (1998) 311–318.
[16] P.G. Righetti, E. Olivieri, A. Viotti, Electrophoresis 19 (1998) 1738–1741.
[17] P.G. Righetti, A. Saccomani, C. Gelfi, A. Stoyanov, Electrophoresis 19 (1998) 1733–1737.
[18] P.G. Righetti, C. Gelfi, M. Perego, A.V. Stoyanov, A. Bossi, Electrophoresis 18 (1997) 2145–2153.
[19] P.G. Righetti, A. Bossi, C. Gelfi, J. Cap. Electrophoresis 4 (1997) 47–59.
[20] A.V. Stoyanov, P.G. Righetti, J. Chromatogr. A 790 (1997) 169–176.
[21] C. Gelfi, M. Perego, P.G. Righetti, Electrophoresis 17 (1996) 1470–1475.
[22] C. Gelfi, D. Mauri, M. Perduca, N.C. Stellwagen, P.G. Righetti, Electrophoresis 19 (1998) 1704–1710.
[23] J.W. Jorgenson, K.D. Lukacs, Anal. Chem. 53 (1981) 1298–1301.
[24] C.M. Wilson, Plant Physiol. 82 (1986) 196–202.