ELSEVIER

Journal of Chromatography B, 699 (1997) 149–171

JOURNAL OF CHROMATOGRAPHY B

Review

Peptide analysis by capillary (zone) electrophoresis

Irene Messana[a,b], Diana Valeria Rossetti[a], Loredana Cassiano[a], Francesco Misiti[a], Bruno Giardina[a,b], Massimo Castagnola[b,c,*]

[a] *Istituto di Chimica e Chimica Clinica, Facoltà di Medicina, Università Cattolica, Rome, Italy*
[b] *Centro di Studio per la Chimica dei Recettori e delle Molecole Biologicamente Attive, CNR, Rome, Italy*
[c] *Istituto di Chimica Biologica, Università di Cagliari, Cagliari, Italy*

Abstract

In this review various aspects concerning the application of capillary (zone) electrophoresis for peptide analysis will be critically examined. First, the basic instrumental requirements of CE apparatus and the strategies employed to enhance sensitivity in the analysis of underivatized sample are described. Multidimensional separative techniques of complex peptide mixtures that use CE as final step and the coupling of CE with mass spectrometry are subsequently discussed. A theoretical section describes the relationships existing between peptide mobility and the pH of the separation buffer. These relationships evidence that proton dissociation constants and Stokes radius at different protonation stages can be calculated by measuring the electrophoretic mobility at different pH values. Investigation of peptide mobility dependence on pH allows us to establish the optimum conditions, in terms of resolution, for peptide separation. Subsequently, a critical discussion about semiempirical models predicting peptide mobility as a function of chemico-physical peptide properties is presented. A section is devoted to the description of principles of peptide affinity capillary electrophoresis, underlining the similarity with peptide–proton interaction. CE separations performed in aquo–organic solvents are also critically discussed, showing the good performance obtained by using water–2,2,2-trifluoroethanol solutions. Finally, selected CE applications for the determination of peptide chemico-physical properties and conventional analysis, like peptide mapping, are reported.

Keywords: Reviews; Peptides

Contents

*Corresponding author. Address for correspondence: Centro di Studio per la Chimica dei Recettori e delle Molecole Biologicamente Attive, CNR, c/o Istituto di Chimica e Chimica Clinica, Facoltà di Medicina e Chirurgia, Università Cattolica, Largo F. Vito 1, 00168, Rome, Italy.

0378-4347/97/$17.00
PII S0378-4347(97)00198-9

1. Introduction

The chemical analyst continuously has to contend with the low sensitivity of his instrumentation and the magnitude of Avogadros number. For example, even when the analyst's apparatus reaches a satisfactory absolute sensitivity at the femtomole (10^{-15} moles) level, he must collect at least around 1 billion molecules ($>6 \cdot 10^{8}$) of any substance present in his specimen, in order to obtain a signal which can overcome the instrumental noise. Following collection, the analyst must be able to utilize some structural, physical or chemical distinction between the various substances in the sample, either to isolate identical molecules from all the others, or to obtain a specific signal for any kind of molecule present in the sample. In this respect, the analysis of biological specimens is certainly a most difficult task since the number of different similar substances present often can be counted in the thousands. Frequently, inability of the analytical technique to differentiate or isolate molecules with small structural differences leads to the false assumption of substance homogeneity. On the contrary, different macromolecular conformations or weak, but stable, interactions between the molecules of the sample, can induce structural distinctions, providing misleading multiple signals.

Nonetheless, the demand for more accurate qualitative and quantitative information on biological systems and the requirements of commercial biotechnological products which would fulfil criterion of quite absolute purity are continuously increasing. In this respect, capillary electrophoresis (CE) allows new and interesting analytical possibilities to develop and is generating a small revolution in chemical and biochemical analysis, similar to the advent of HPLC techniques. The very high absolute sensitivity of this technique induced some authors [1,2] to feel it would be easier to express the sensitivity in terms of number of molecules, rather then in terms of moles. In fact, through laser induced fluorescence they reached a sensitivity at the level of zeptomoles (10^{-21} moles), corresponding to several hundred molecules. CE selectivity, in the so called zonal conditions, (CZE, because the analytes migrate as separated, independent, non-contiguous zones), is also high and can be explained through well known theoretical equations, principally related to the charge-to-size ratio of the analyte. (Recently Knox [3] recommended the use of the term capillary electrophoresis (CE), since the word "zone" is now obsolete, therefore this term will be used whenever possible).

Furthermore, the very large number of CE options allows one to operate under completely different experimental conditions, for example, either using micellar conditions (capillary micellar electrochromatography, CMCE) or gel filled capillaries (capillary gel electrophoresis, CGE), or polymeric solutions (polymer-solution capillary electrophoresis, PSCE), otherwise employing either capillary isotacophoretic (CITP) or isoelectrofocusing (CIEF) conditions.

CE is quite useful in the field of peptide analysis, especially for small polar peptide separation, which is difficult to perform using other techniques. In addition, high sensitivity and selectivity might oblige the use of CE as the elective technique in quality

control of peptides produced by biotechnological procedures, in the near future. This review will focus upon several aspects of CE peptide separations, in the attempt to offer an extensive view of the present state-of-the-art and of the most important analytical information that can be obtained by this technique. In addition, several perspectives of peptide CE applications will be outlined. A limited number of references, selected among the very large number of significant papers published on this field, will be cited. We apologize for any oversight, but a comprehensive list of all the relevant articles published in the field of the CE application to peptide analysis is nowadays impossible.

2. Instrumentation

2.1. Basic apparatus and detectors

The basic apparatus for peptide CE separations does not require supplementary options. All common commercial instruments are suitable for typical separations. Appropriate instrumental arrangements will be described in cases of particular experimental strategies. Options such as multiple sampling and efficient temperature control are advisable. Elective detection is by spectrophotometric absorbance, which, of course, must operate in the low ultraviolet range (200–220 nm). Commercial fluorescence detectors are not usually useful for direct peptide detection. In fact, the intrinsic peptide fluorescence is principally due to tryptophan, with a minor contribution of tyrosine. Tryptophan excitation wavelength is in the 200–300 nm range, which is not covered by common commercial laser. This problem can be overcome by the use of appropriate fluorotags, as will be described in Section 2.5. Sensitivity is increased by two to three orders of magnitude compared to absorbance detection. On the other hand, the use of fluorescent reactants can generate problems deriving from unreacted reagents and difficulties could arise in reproducing quantitative analysis even with the use of internal standards [4]. Indirect detection [5] can be also used, by adding either fluorescent or high absorbing substances to separation buffers. In this case, analytes are detected as negative peaks. This option is very attractive, although a different detector design could be significant for instrumental improvement [6,7]. A sensible increase in sensitivity is also obtainable in this case, even though it is not easy to achieve an accurate quantitative analysis. Even though there is great deal of interest in the use of electrochemical detection [8] for CE analysis, commercial instruments do not usually offer it. Coupling with a mass spectrometer could be very useful [9] for a fast peptide identification (if money is not a problem). These options will be more extensively described in the sections devoted to the enhancement of peptide CE sensitivity and to CE hyphenation (Section 2.5 Section 2.7).

2.2. Capillary

While there are not any particular requirements for CE peptide separation essential apparatus, attention should be payed to capillary choice. In fact, it was well established that physico-chemical structure of the capillary wall plays a relevant role in the separation [10,11]. Electric field generates a net buffer flow (electroosmotic flow) by acting on diffusion part of electric double layer at the inner surface of the capillary. Electroosmotic flow is usually directed towards the cathode [12,13]. This characteristic is not negative "per se", since electroosmotic flow can be measured and modulated as a function of various parameters and hence can be used to modify analysis times (these topics were extensively reviewed [14–16]). Problems arise from the electrostatic interactions between peptides and inner capillary wall, that may generate sensible decrease of efficiency and reproducibility [17]. For these reasons many researchers recommended the use of coated capillary, as reported in excellent reviews [18–20]. Although some covalent coatings are stable even at moderately basic pH values, to date a universal coating has not been described due to instability at high basic pH values [21]. For these reasons, best separation performance in peptide separations were usually obtained at acidic pH [22,23].

2.3. Fraction collectors

CE requires very low analysis volumes and, even under electroosmosis, very low solution flow, thus, the building of apparatus for automated fraction collection is not easy. The problem can be solved by immediate peptide detection through coupling with

appropriate instruments; a mass spectrometer is the elective choice (Section 2.7). However, since peptide collection is essential for unambiguous identification, many efforts have been made [24] to solve this problem. Some of them were based on discontinuous collections. Electrophoresis is halted immediately before the peptide exit from the capillary and the peak is collected either into appropriate tanks under low pressure mobilization [25,26], or into a low-volume collection buffer under field-programmed voltage [27,28]. Otherwise collection may be continuous onto a wetted and moving membrane [29]. The various laboratory-made equipment demonstrated that multiple collection provided enough sample either to perform Edman sequencing or to investigate post-translational modification throughout mass spectrometry [30]. These collection methods required the presence of a strong electroosmotic flow in the capillary.

Among described choices, attention was addressed to an automated apparatus for continuous collection. A recent laboratory-made apparatus is based on a rotating moist membrane maintained at ground potential in electric contact with the capillary terminal [31]. Tracht et al. [32] used a silver-coated capillary in order to establish electric contact with the electrophoretic buffer, while the peptide is captured onto a binding membrane. Recently, Chiu et al. [33] have described another laboratory-made collector, based on electrical connection established either by using a coaxial capillary flow cell or through the treatment of the outer capillary surface with a gold-filled epoxy resin. The former option is most useful with low or absent electroosmosis, whereas the latter is preferred when dilution of the sample must be minimized. The authors described a more than 80% sample recovery and the fractions were successfully analyzed by electrospray mass spectrometry. These studies demonstrated that automated CE fraction collectors are in great progress and suggested a prompt development of commercially available equipment.

2.4. Separation buffers

Buffers for peptide CE must supply a conductive medium through which the charged peptide migrate freely in the electric field. They must also have low UV absorbance. For these reasons traditional buffers used in slab gel electrophoresis are normally not suitable for CE. The following factors should be considered in the buffer choice: influence of cations and anions on solute migration, dissipation of Joule heating, buffering capability, solubility and stability, interaction with capillary wall and hence effects on electroosmosis [34–37]. Examples of most common used buffers for peptide CE are reported in Table 1.

In our experience a suitable option is represented by phosphate buffers 50–100 m*M* at acidic pH and by phosphate–borate buffers 10–40 m*M* at alkaline pH, the latter implemented by strong basic additive (i.e., 5 m*M* lysine) in order to reduce peptide–wall interactions. At neutral pH peptides usually have a small charge, consequently the analysis time in separations performed using coated capillary could be lengthy. Thus, to profit from electroosmotic flow, neutral and alkaline pH separations are usually performed with uncoated capillary. In this case, to reduce peptide–wall interactions, buffers based on zwitterionic substances, such as MES (2-[N-morpholino]ethanesulphonic acid) or HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]) are acceptable options.

2.5. Sensitivity enhancement

By direct absorbance detection CE absolute sensitivity is about two to three orders of magnitude higher than that of HPLC techniques. Nonetheless, in terms of concentration, CE sensitivity is slightly lower than HPLC (detection limit about $1 \cdot 10^{-5}$–1·

Table 1
Some selected buffers suitable for CE peptide separations

Buffer composition	m*M*	pH
Phosphate (Na^+, K^+)	40–100	2–4
Malonate (Na^+, K^+)	40–80	2.5–4
Citrate (Na^+)	20–80	2.5–4.5
Malate (Na^+)	40–80	3–4.5
Succinate (Na^+)	40–80	4–6
MES[a] (Na^+)	40–80	5.5–6.5
HEPES[a] (Na^+)	40–80	6–7.5
Tris–glycine (acetate)	25:50–50:200	6.5–8.5
Phosphate–borate (Na^+, K^+)	20:5–50:20	8.5–10.5
CAPS[a] (Na^+)	40–80	9.5–11

[a] MES (2-[N-Morpholino]ethanesulphonic acid); HEPES (N-[2-hydroxy-ethyl]piperazine-N′-[2-ethanesulphonic acid]); CAPS (3-[cyclohexyl-amino]-1-propanesulfonic acid).

10^{-6} M), since the CE injection volume is about three orders of magnitude smaller. Thus, the development of tools which may increase instrumental sensitivity is one of the major goals of CE peptide separations.

The most simple strategy consists of the preconcentration of the sample by employing discontinuous buffer systems. Using staking conditions with different buffer composition, Schwer and coworkers were initially able to increase the detection limit of about 30 times [38] and subsequently 50 times [39].

Alternatively, as mentioned in Section 2.1, the use of fluorescence detectors can greatly improve detection limits. Direct fluorescence detection of peptides is limited to their tryptophan and tyrosine content. In addition, these two amino acids are poor fluorophores and their excitation occurs in the 210–290 nm wavelength range. Commercial equipment is not fitted with lasers covering this range. However, several authors reported that the use of home-made detectors [40–42], equipped with Kr or Ar lasers, reached detection limits in the order of $1 \cdot 10^{-9}$ M. Recently, Timperman et al. [43] used a laboratory-made wavelength-resolved detection system to reach a detection limit of about $2 \cdot 10^{-10}$ M for tryptophan containing peptides. The absolute sensitivity in an injection volume of about $5 \cdot 10^{-9}$ l (5 nl) corresponded to about $1 \cdot 10^{-18}$ mole (equal to 1 amole; 60 000 molecules). Moreover, acquisition of emission spectrum allowed them to distinguish between tryptophan and tyrosine containing peptides.

Nonetheless, since many peptides lack either tryptophan or tyrosine, different strategies should be used in order to improve detection limits in their absence. Pre-column peptide derivatizations with fluoro-tags were described [44] including the use of fluorescamine, fluorescein isothiocyanate (FITC), 9-fluorenylmethyl chloroformate (FMOC) and *o*-phthaldialdehyde (OPA) [44]. A particular fluoro-tag, 3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde (CBQCA), was recently specifically designed for ultrasensitive detection of primary amines [45]. The excitation wavelength of these reactants was coherent with the wavelength of He–Cd laser (442 nm) and was successfully employed to reveal small peptides [46]. Orwar et al. [47], employing 2,3-naphtalenedicarboxy-aldehyde as fluoro-tag for glutathione, were able to perform single cell-analysis. Fluorescence detection appears to be the elective method for a great improvement of peptide CE separation. Lee et al. investigated these possibilities and discussed the laser-induced fluorescence detection of a single molecule in a capillary [48]. Using a near-infrared laser beam in a flowing stream confined in a capillary, they were able to detect a single molecule. A practical application in peptide CE of these insuperable detection limits must still surmount enormous experimental difficulties. Moreover, these sensitivity limits lead to speculation about the general significance of monomolecular analysis, while the usual analytical signals derive from means of molecular behaviour.

2.6. Hyphenation: multidimensional separations

Great interest was devoted to the coupling of CE with other separative techniques, HPLC in particular. RP-HPLC peptide separations are based on peptide polarity, whereas CE separations are based on charge-to-size ratio. Nonetheless, peptide with high charge should be also characterized by high polarity. Thus, when a comparison between RP-HPLC elution times and CE migration time was performed, slight correlations were observed [23,49]. Although the two methods cannot be considered strictly orthogonal, their different selectivity can offer a powerful tool for 2-dimensional (2-D) mapping of complex peptide mixtures.

Various authors performed automated coupling between HPLC and CE separations [50–53]. Due to its small injection volume, CE is always at the end of the coupling. An acceptable compromise between chromatographic flow-rate and electrophoretic analysis time must be made. In fact, the critical parameter is represented by the electrophoretic time. Its reduction, obtained by working on capillary length and on applied voltage, must be as low as possible in order to avoid undersampling of the chromatographic fractions and to shorten the total analysis times. Loss in CE resolution can be rewarded by the good performance of the coupling [53]. Recent improvements of Moore and Jorgenson [54] allowed them to couple fast RP-HPLC (characterized by relatively low performance) and fast optical-gated CE separations in order to obtain 2-D peptide mapping in

less than 10 min. Resolution lost in the fast HPLC gradient was regained in the second CE dimension. The total peak capacity of the whole system was found to be around 650. Since total peak capacity is the product of the peak capacity of each separative dimension, an extension to higher dimensions generates a very great improvement of total peak capacity even with low performance. Moore and Jorgenson continued in this vein [55] and coupled size-exclusion chromatography (SEC) to RP-HPLC and subsequently to optical-gated CE obtaining a 3-dimensional (3-D) analysis of peptides. The total analysis time was about 8 h and depended upon the slow flow-rate of the SEC separation, since the other two dimensions accounted for a total analysis time of about 7 min. Although the peak capacity of the SEC step was low, the total peak capacity was found to be around 3000. The broad use of 3-D separations cannot be considered routine, due to the difficulties in the representation.

2.7. Hyphenation: CE–MS coupling

One of the most relevant CE problem concerns the identification of unknown peptides, due to the very low sample loading and recovery difficulties. Mass spectrometry (MS) seems to be the detector most suitable to solve this problem. The CE–MS coupling is particularly attractive in consideration of the very low flow-rate of electroosmosis and the consequent reduction of mass flow buffer components at the interface. In this respect CE–MS has the advantage over HPLC–MS coupling and reflects about a 30-fold increase in peptide detection sensitivity [56] and simplification of the electrospray ionization interface [57]. Hyphenation between CE and MS has been extensively reviewed [9]. Coupling with electrospray ionization (ESI) [58–61] and fast atom bombardment (FAB) [62,63] has been largely described. In addition matrix assisted laser desorption ionization interface (MALDI) [64] and selected ion monitoring (SIM) [65,66] have been developed. The detection limits of these couplings have been evaluated in the order of the low femtomole and large applications for the analysis of peptide modification has been demonstrated [67,68].

CE–MS coupling can be considered the most powerful improvement to peptide CE analysis and various types of commercial equipment are presently available. Nonetheless, in order to completely exploit the potentiality of the coupling, further improvement of the interface [69,70] or strategies centered on the preconcentration step [71] are still necessary.

3. Theory of peptide CE

This section is devoted only to theoretical equations involving peptide separation. Thus we will not cover equations concerning electroosmosis, equations describing the connection of electrophoretic performance with instrumental parameters or equations treating general electrophoretic behaviours, unless they direct involve peptide separation.

Many factors can influence peptide electrophoretic mobility and proton activity is the most relevant, thus the next section covers this topic.

3.1. Peptide mobility as a function of pH

In CE conditions and in the presence of electroosmotic flow the peptide observed mobility results from vectorial addition of electroosmotic and electrophoretic mobilities:

$$\mu_{obs} = \mu_{ep} + \mu_{eo} \quad (1)$$

Using coated capillaries electroosmosis can be reduced to zero and under these conditions Eq. (1) can be simplified to:

$$\mu_{obs} \cong \mu_{ep} \quad (2)$$

where the electrophoretic mobility is controlled by the basic equation:

$$\mu_{ep} = \frac{qZ}{6\pi\eta r_s} \quad (3)$$

where q is the electron charge ($1.60 \cdot 10^{-19}$ coulomb), η the solution viscosity (water: $8.95 \cdot 10^{-4}$ N s/m^2 at 25°C), r_s the Stokes radius (i.e., the radius of the sphere equivalent to hydrated peptide) and Z corresponds to the number of elementary charges (dimensionless). Thus, electrophoretic mobility is simply related to the ratio Z/r_s. For weak electrolytes, such as peptides, the charge and the Stokes radius are affected by running buffer properties,

particularly by proton activity. In a solution at high proton activity, i.e., low pH values, the peptide is fully saturated and has a positive charge equal to the total number of the basic groups. As the pH value increases C-terminal carboxy groups and side chains of aspartic and glutamic acid, which have low affinity for the proton, will dissociate. These dissociations generate negative charges on the peptide, that could partly compensate the positive charges still present. Therefore, dissociation will involve groups having middle proton affinity, as N-terminal amino group and side chain of histidine, when pH values are almost neutral. This dissociation provides loss of some positive charges. Finally, further increase of pH values will induce the dissociation of high proton affinity groups like side chains of arginine and lysine and side chains of cysteine and tyrosine will dissociate causing the disappearance of positive charges and the generation of negative charges.

Therefore, if a generic peptide ($X^{(charge)}$) having j basic groups and i acidic groups is considered, its dissociation can be described as follows:

$$X^{j} \underset{K_1}{\rightarrow} X^{j-1} \underset{K_2}{\rightarrow} .... \underset{K_{j-i}}{\rightarrow} X^{-i} \tag{4}$$

The above equation presupposes the equivalence of all species having the same charge.

Since proton exchange is much faster than the amount of time required for analysis, electrophoretic mobility observed at a given pH may be regarded as a means of the mobility of any k different charged form, each form participating with its percentage x_k.

$$\mu_{obs} = \mu_j x_j + \mu_{j-1} x_{j-1} + ... + \mu_{-i} x_{-i} = \sum_{k=j}^{k=-i} \mu_k x_k \tag{5}$$

and, hence

$$\mu_{obs} = \frac{q}{6\pi\eta} \sum_{k=j}^{k=-i} \frac{k}{r_{s_k}} \cdot x_k \tag{6}$$

The molecular percentage of any form, namely the molar fraction, can be expressed as a function of proton activity and of dissociation constants, as follows:

$$x_{j-n} = \frac{[X^{j-n}]}{[X^j] + [X^{j-1}] + [X^{j-2}] + .. + [X^{-i}]} = \frac{\beta_n/[H^+]^n}{P} \quad (n = 0, 1, 2, ..., j+i) \tag{7}$$

where P is the binding polynomial

$$P = \beta_0 + \beta_1/[H^+] + \beta_2/[H^+]^2 + ... + \beta_{j-i}/[H^+]^{j-i} \tag{8}$$

and

$$\beta_0 = 1;\ \beta_1 = K_1;\ \beta_2 = K_1K_2;.....;\ \beta_{j+i} = K_1K_2K_3.....K_{j+i} \tag{9}$$

Eqs. (6)–(9) account for difference of the Stokes radius of the various charged forms. This equation has too many variables in order to be of practical use if a peptide has many dissociable groups. In addition, as aforementioned, it does not consider that the total charge of any form could derive from different charge distributions. As an example, mobility, in a peptide with C-terminal and N-terminal free groups and lysine and aspartic acid as charged side chains, at low pH values, results from a high percentage of fully saturated peptides with two positive charges, one localized on lysine and the other one on the N-terminus. A pH increase provides the dissociation either of the C-terminal or of the side chain of aspartic acid, leading to large percentages of two distinct forms with one net positive charge (deriving from the sum of two positive and one negative charges), which are considered equivalent in Eqs. (4)–(9). Equations considering microscopic dissociation constants could account for their percentages [72,73]. Since CE separation is usually not able to differentiate the various forms, these equations have no practical use.

If the peptide is small and the pH modifications are restricted to a limited range in order to provide the dissociation of single groups, such as the carboxy C-terminus in acidic pH range, the theoretical treatment described in Eqs. (6)–(9) can provide a measure of C-terminal dissociation constants and of Stokes radius at different protonation stages [74].

For example, Eqs. (6)–(9) may be simplified as follows, for peptides having more than one basic

group and C-terminus as the only acidic group, at acidic pH range:

$$\mu_{obs} = \frac{q}{6\pi\eta}\left[\frac{j}{r(j)}\cdot\frac{1}{1+K/[H^+]} + \frac{j-1}{r_{(j-1)}} \cdot \frac{K/[H^+]}{1+K/[H^+]}\right] \quad (10)$$

where K is the dissociation constant of C-terminus carboxylic group.

Eq. (10), upon simple algebraic modification, changes into:

$$\mu_{obs} = \frac{q}{6\pi\eta}\left[\frac{j}{r(j)}\cdot\frac{[H^+]}{[H^+]+K} + \frac{j-1}{r_{(j-1)}}\cdot\frac{K}{[H^+]+K}\right] \quad (11)$$

and, in order to transform it into a function of pH:

$$\mu_{obs} = \frac{q}{6\pi\eta}\left[\frac{j}{r(j)}\cdot\frac{10^{-pH}}{10^{-pH}+10^{-pK}} + \frac{j-1}{r_{(j-1)}} \cdot \frac{10^{-pK}}{10^{-pH}+10^{-pK}}\right] \quad (12)$$

Eq. (12) for $pH \ll pK$ reduces to:

$$\mu_{obs} = \frac{q}{6\pi\eta}\cdot\frac{j}{r_{(j)}} \quad (13)$$

and for $pH >> pK$ to:

$$\mu_{obs} = \frac{q}{6\pi\eta}\cdot\frac{j-1}{r_{(j-1)}} \quad (14)$$

which allow us to measure the Stokes radius of the different protonated forms.

The following three possibilities may occur, each with different consequences on mobility, as shown in Fig. 1. When $r_{(j)}$ is equal to $r_{(j-1)}$ Eq. (12) can be transformed into:

$$\mu_{obs} = \frac{q}{6\pi\eta r_s}\cdot\left[\frac{j\cdot 10^{-pH}}{10^{-pH}+10^{-pK}} + \frac{(j-1)\cdot 10^{-pK}}{10^{-pH}+10^{-pK}}\right] \quad (15)$$

It obviously does not account for Stokes radius changes. In this case, i.e., at a constant radius, curve shape is invariant. This is evidenced by the derivative of this equation as a function of pH:

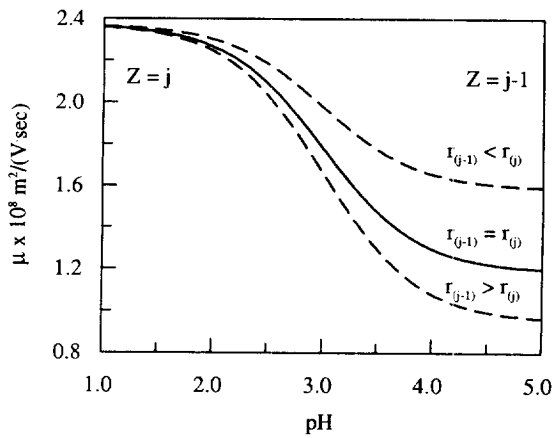

Fig. 1. pH dependence of mobility values of a generic peptide with a Stokes radius of 8.0 Å and a C-terminus pK value of 3.00, according to Eq. (12). Continuous line corresponds to a peptide $Z=+2$ charged that does not change its Stokes radius by proton loss, whereas dashed lines correspond to a 2.0 Å decrease (top line) and a 2.0 Å increase (bottom line) of the Stokes radius, respectively.

$$\left(\frac{\partial\mu}{\partial pH}\right)_r = -\frac{q}{6\pi\eta}\cdot\frac{1}{r_s}\cdot\ln_e(10)\cdot\frac{10^{(pH+pK)}}{(10^{pH}+10^{pK})^2} \quad (16)$$

This function assumes the maximum absolute value at $pH=pK$ (when saturation reaches 50%):

$$\left|\left(\frac{\partial\mu}{\partial pH}\right)_r\right|_{(pH=pK)} = \frac{q}{6\pi\eta}\cdot\frac{\ln_e(10)}{4r_s} = 9.48\cdot 10^{-18}\cdot\frac{0.576}{r_s} \quad (17)$$

In contrast, when $r_{(j)}$ is different from $r_{(j-1)}$ the curve is stretched or compressed (Fig. 1), since the mobility of X^{j-1} form is either lower or higher than expected, respectively. In these cases, partial derivative of Eq. (12) as a function of pH is:

$$\left(\frac{\partial\mu}{\partial pH}\right)_r = -\frac{q}{6\pi\eta}\cdot\left(\frac{j}{r_j} - \frac{j-1}{r_{(j-1)}}\right)\cdot\ln_e(10)\cdot\frac{10^{(pH+pK)}}{(10^{pH}+10^{pK})^2} \quad (18)$$

The absolute value of the above equation at pH

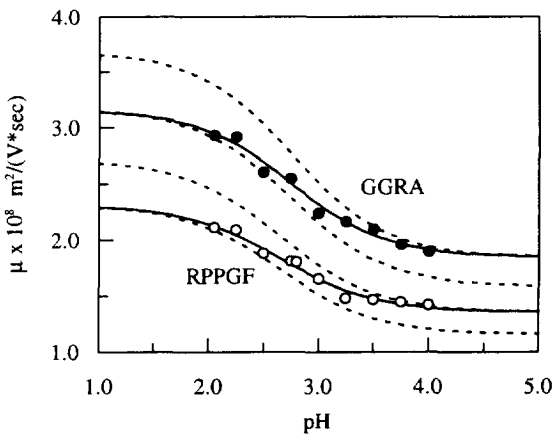

Fig. 2. Mobility values of GGRA and RPPGF (bradykinin fr. 1–5) peptides as a function of pH. Continuous line was obtained by fitting experimental data according to Eq. (12). By this procedure the values of C-terminus pK and Stokes radius at different protonation stages reported in Table 2 were determined. Dashed lines represent theoretical mobility values obtained without considering Stokes radius modifications. CE conditions: Na–phosphate buffers 80 mM, 25°C, 25 kV, coated capillary. Data obtained from Ref. [74].

equal to pK is dependent upon Stokes radius modifications:

$$\left|\left(\frac{\partial\mu}{\partial \mathrm{pH}}\right)_r\right|_{(\mathrm{pH}=\mathrm{p}K)} = \frac{q}{6\pi\eta}\cdot\frac{\ln_e(10)}{4}\cdot\left(\frac{j}{r_j}-\frac{j-1}{r_{(j-1)}}\right)$$

$$= 9.48\cdot 10^{-18}\cdot 0.576\cdot\left(\frac{j}{r_j}-\frac{j-1}{r_{(j-1)}}\right) \qquad (19)$$

Paradoxically, if proton loss provides a very strong Stokes radius decrease, mobility modification as a function of pH cannot be observed at all.

3.2. Examples of measure of peptide dissociation constants and Stokes radius at various protonation stages

Fig. 2 shows the curves obtained by best-fit procedures according to Eq. (12) of the mobilities of some model peptides, such as GGRA and bradykinin fragment 1–5 (RPPGF). Dashed lines in the figure represent curves calculated according to Eq. (15), which assumes that proton loss does not modify Stokes radius. The disagreement between these curves and experimental data clearly indicates that Eq. (15) is unable to describe the peptide behaviour. Table 2 shows the radii and the values of the C-terminus dissociation constant, obtained by best-fitting procedures performed according to Eq. (12) [74]. Peptide behaviour shown in Fig. 2 derives from a Stokes radius decrease, probably linked to the formation of intra-molecular ion pairs, although this is not a general rule [74].

Interesting results are also obtained for peptides which have only dissociable C- and N-termini. In this case, since the second term is zero, the Eq. (12) reduces to:

$$\mu_{\mathrm{obs}} = \frac{q}{6\pi\eta}\cdot\frac{1}{r_{(+1)}}\cdot\frac{10^{-\mathrm{pH}}}{10^{-\mathrm{pH}}+10^{-\mathrm{p}K}} \qquad (20)$$

The above equation at a pH $\ll$ pK becomes:

$$\mu_{\mathrm{obs}} = \frac{q}{6\pi\eta}\cdot\frac{1}{r_{(+1)}} \qquad (21)$$

Fig. 3 shows the results obtained applying this equation to Met-enkephalin (YGGFM; ME), Leu-enkephalin (YGGFL; LE) and their common 1–4 tetrapeptide YGGF. Whereas at pH values lower than 2.7 YGGF displays higher mobility, in agreement with its smaller dimension, at higher pH values the

Table 2
pK and Stokes radius values at different protonation stages[a] obtained by best-fitting of experimental data of Fig. 2 according to Eq. (12)

Peptide sequence	pK	Stokes radius (Å)	
	(C-terminus)	$Z=+2$	$Z=+1$
GGRA	2.75±0.04	6.0±0.2	5.2±0.2
RPPGF (bradykinin fragment 1–5)	2.64±0.03	8.2±0.2	7.0±0.2

[a] Data from Ref. [74].

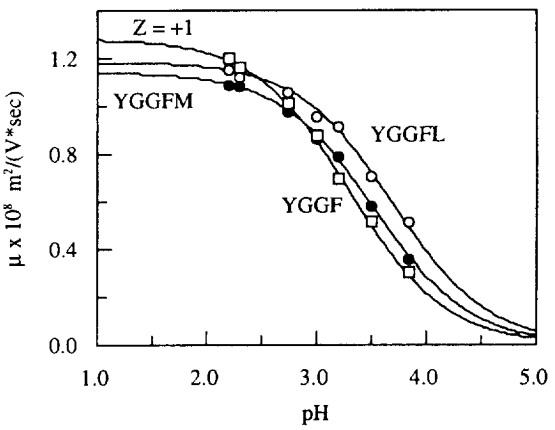

Fig. 3. Mobility values of Met-, Leu-enkephalin and YGGF peptide as a function of pH. Lines were obtained by fitting experimental data according to Eq. (20). This procedure provided the C-terminus pK and Stokes radius values reported in Table 3. CE conditions as in Fig. 2. Data obtained from Ref. [74].

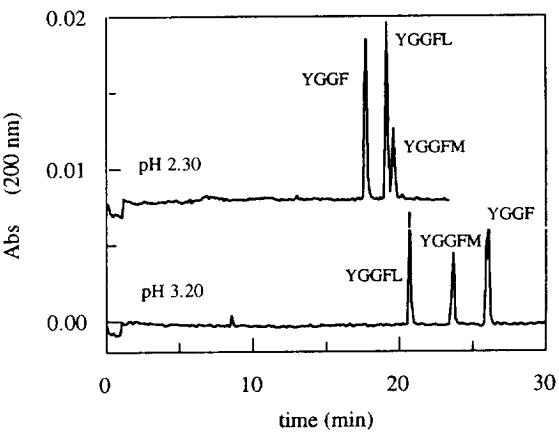

Fig. 4. Electropherogram of Met-, Leu-enkephalin and YGGF peptides at two different pH values showing the inversion of migration order. CE conditions as in Fig. 2. Data obtained from Ref. [74].

larger ME and LE move faster (Fig. 4). This apparently singular event may be explained taking into account pK values of ME, LE and YGGF, obtained by the best fit of Eq. (20), that evidences the higher acidity of the YGGF C-terminus (Table 3). pK values measured throughout CE are in perfect agreement with those obtained by microtitration [75].

The general Eqs. (6)–(9) can also offer information when side chain dissociable groups (i.e., glutamic acid) are present in the peptide [74]. On the basis of the results reported here and in literature [75–77], it can be affirmed that CE has transformed electrophoresis into a relevant tool for the determination of chemico-physical analyte properties, giving quantitative information concerning the peptide dissociation constants and conformational transitions related to proton loss.

3.3. Resolution

On the basis of the data presented in Section 3.2, two distinct peptide mobility possibilities may be observed: the first, when proton saturated forms are predominant, characterized by a mobility control exerted by Stokes radius, and the second, in a pH range corresponding to half peptide saturation, where mobility is mainly under dissociation constant control.

Thus, if two peptides have similar charge properties but different Stokes radius, separation should be achieved at pH values low enough to ensure high saturation, in order to exploit Stokes radius differences. Whereas, if different C-terminus dissociation constants are expected, the best resolution would be observed at a pH value near pK. This can be better quantified according to Jorgenson and Lukacs [78] and our previous studies [79]. In fact, the CE

Table 3
pK and Stokes radius values obtained by best-fitting of the mobility values of Figs. 3 and 6 according to Eq. (20)[a]

Peptide sequence	pK (C-terminus)		Stokes radius (Å)
	by CE	by microtitration [75]	$Z=+1$
YGGF	3.30±0.03	–	7.4±0.2
YGGFM (Met-enkephalin)	3.52±0.02	3.45±0.01	8.2±0.1
YGGFL (Leu-enkephalin)	3.69±0.02	3.69±0.01	8.0±0.1
GGNA	3.60±0.02	3.65±0.01	6.3±0.1
GGQA	3.59±0.02	3.61±0.01	6.4±0.1

[a] Data from Ref. [74].

resolution can be calculated by the following equation [79]:

$$R_s = \frac{1}{4} \cdot \frac{1}{\sqrt{r_{s1} + r_{s2}}} \cdot \frac{r_{s1}Z_2 - r_{s2}Z_1}{\sqrt{r_{s1}Z_2 + r_{s2}Z_1}} \cdot \sqrt{\frac{2qV}{kT}} \quad (22)$$

where k is the Boltzmanns constant ($1.38 \cdot 10^{-23}$ J/K), V the applied voltage (volt) and T the absolute temperature (K). Eq. (22), in the case of two peptides having equal charge and different radius, may be reduced to:

$$R_s = \frac{1}{4} \cdot \frac{\Delta r_s}{r_{s1} + r_{s2}} \cdot \sqrt{\frac{2qV}{kT}} \quad (23)$$

and, in the case of two peptides with equal radius and different charge may be reduced to:

$$R_s = \frac{1}{4} \cdot \frac{\Delta Z}{\sqrt{Z_1 + Z_2}} \cdot \sqrt{\frac{qV}{kT}} \quad (24)$$

Eqs. (23) and (24), under ideal separation conditions, provide the results reported in Fig. 5. Either a difference in the Stokes radius of 0.3 Å or a difference in pK values of 0.04 would be necessary in order to obtain a resolution value of 10. Thus, high resolution values are observed as a function of small differences either of charge or of radius. Unfortunately, it is impossible to employ both on a contemporary basis. It is in fact worthwhile to note that: (a) when the choice leans towards radius driven separation, pH value should be as low as possible in order to work at high saturation (a good pH value is around 2.2); (b) when the choice leans towards charge driven separation, pH value should be slightly higher than mean pK values of the two peptides (a good pH value should be around 3.0–3.5); (c) a pH value around 2.5–3.0 should be avoided, since in this range the inversion in the control can provide a strong reduction of the resolution, as observed in the separation of enkephalin related peptides (Fig. 3).

When peptides are very similar, differences in Stokes radius could be too small to ensure a good separation. Nonetheless, since resolution increases as the Stokes radius decreases (Eq. (23)), minimal structural differences for very small peptides could be enough to obtain a good separation [74]. This is the case for GGNA and GGQA peptides (Fig. 6), which differ only in a CH_2 group. Their pK difference (Table 3) is too small to obtain an acceptable separation at a pH value near the pK. However, the peptides are well resolved at low pH, where the separation is under Stokes radius control.

Eqs. (22)–(24) show that temperature and voltage also play a role in the resolution: low temperature and high voltage increase resolution. On the other hand, since an increase of voltage is linked to a temperature increase, the best experimental conditions should be established for each analytical prob-

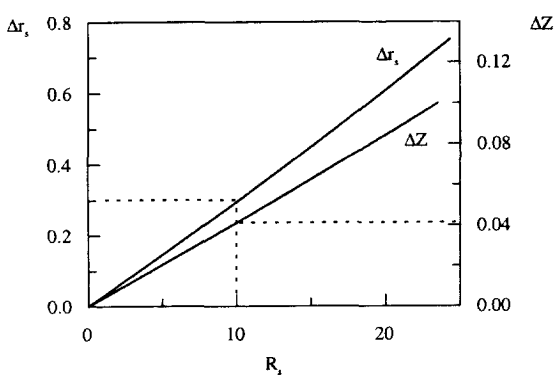

Fig. 5. Simulation of resolution values obtainable in CE separations of two peptides (10.0 Å and $Z = +1$ as starting values) as a function of Stokes radius difference (Δr_s) and charge difference (ΔZ). The simulation was performed according to Eqs. (23) and (24).

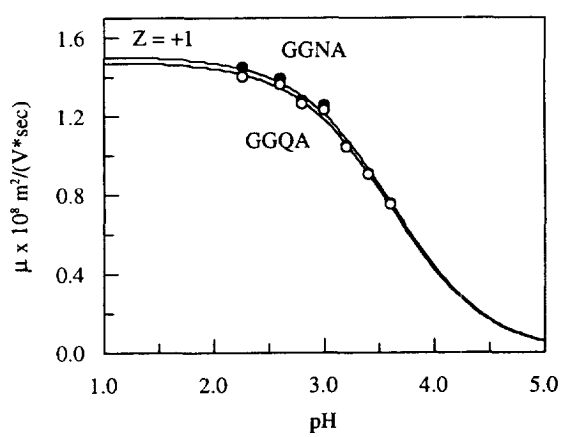

Fig. 6. Mobility values of GGNA and GGQA peptides as a function of pH. Lines were obtained by fitting experimental data according to Eq. (20). This procedure provided the values of C-terminus pK and Stokes radius reported in Table 3. CE conditions as in Fig. 2. Data obtained from Ref. [74].

lem and ionic strength and capillary dimension should also be considered [80].

3.4. Ionic strength

Changes in ionic strength may account for differences in observed peptide mobilities [35,81,82]. Ionic strength has influence on the buffer conductibility, and hence on different Joule effects. It can modify the electric double layer of the capillary wall and hence may alter electroosmosis and interactions between peptide and capillary. Finally, even with coated capillaries, it can account for modification of peptide effective charge and activity. Small changes in buffer composition generate only small ionic strength variations, that have negligible effects on peptide mobilities [45]. Otherwise, if the buffer composition is sensibly changed, ionic strength effects must be considered [35,81,82]. Chae and Lenhoff [83] established relationships which accounted for the effect of ionic strength in computation of protein electrophoretic mobility in free solution. The computation, based on tensorial extension of Debye–Hückle–Henry theory, use mathematical formulations too complex to be routinely applied.

3.5. Semiempirical models predicting peptide mobility

Some authors proposed the use of semiempirical theoretical models in order to make an acceptable prediction of peptide mobility. This type of prediction could be useful, both for the identification of particular peptides in a large library and for predicting the running order in the separation of complex peptide mixtures, such as in endoprotease mapping. Recently, Basak and Ladisch have reviewed the different models reported in literature [82]. The first model was presented by Grossmann et al. (GCL model) [84]. They correlated peptide mobility to the charge and to the number of peptide amino acids (nn_a) throughout the following semiempirical equation:

$$\mu = A \cdot \frac{\ln(Z+1)}{nn_a^B} + C \tag{25}$$

Thereafter, Rickard et al. (RSN model) [85] proposed that the mobility was directly proportional to the charge and inversely proportional to the peptide molecular mass (M_r), as follows:

$$\mu \propto \frac{Z}{M_r^A} + B \tag{26}$$

Subsequently, several models derived from these two were proposed [82,86–88]. Some authors [89,90] tested the two models and found that both are satisfactory in order to obtain a reliable prediction of peptide electrophoretic mobility. Nonetheless, the peptide mobility behaviour described in the previous sections leads us to a further discussion on the matter. In fact, proposed models are centered on theoretical predictions of peptide charge and Stokes radius, according to equations somewhat related to Eq. (3). Model performance depends upon choices made in order to reach a good relationship between the knowledge of the primary sequence and the two above mentioned peptide properties.

Computation of peptide charge is usually performed considering any dissociable group separately [91]. Thus, for a generical i acidic group:

$$AH \Leftrightarrow A^- + H^+ \quad K_i = \frac{[A^-] \cdot [H^+]}{[AH]}$$

The fraction of negative charge is given by:

$$N_i = \frac{[A^-]}{[AH] + [A^-]} = \frac{K_i/[H^+]}{1 + K_i/[H^+]} = \frac{10^{(pH - pK_i)}}{1 + 10^{(pH - pK_i)}} \tag{28}$$

and assuming that the dissociation of each group is independent of the others, total negative charge (N_t) of a peptide can be expressed:

$$N_t = \sum_i \frac{10^{(pH - pK_i)}}{1 + 10^{(pH - pK_i)}} \tag{29}$$

Instead, for a j basic group, we have:

$$BH^+ \Leftrightarrow B + H^+ \quad K_j = \frac{[B] \cdot [H^+]}{[BH^+]}$$

The fraction of positive charge is then:

$$P_j = \frac{[BH^+]}{[B]+[BH^+]} = \frac{1}{1+K_j/[H^+]} = \frac{1}{1+10^{(pH-pK_j)}} \quad (30)$$

and total positive charge of a peptide with j basic groups is given by:

$$P_t = \sum_j \frac{1}{1+10^{(pH-pK_j)}} \quad (31)$$

Thus Z, at any pH value, can be obtained by the sum:

$$Z = P_t - N_t \quad (32)$$

This expression of Z corresponds to a proton saturation function for a peptide with $i+j$ independent binding sites, and leads to an equation which corresponds exactly to Eq. (15) describing a multi-sites peptide, without considering changes in Stokes radius.

Since proton activity has a great deal of influence on peptide mobility, some considerations on pH separation must be made. When the separation is performed at pH values ranging between 3.0 and 3.5, where the mobility is pK driven, Eqs. (28)–(32) reduce to:

$$\mu = \frac{q}{6\pi\eta}\cdot\frac{1}{r_s}\cdot\left(j - \sum_y \frac{10^{(pH-pK_y)}}{1+10^{(pH-pK_y)}}\right) \quad (33)$$

where y is the number of acidic groups, with the exception of cysteines and tyrosines. In fact, mobility change depends only upon dissociation of the C-terminus and of the side chains of aspartic and glutamic acid. In these conditions, a good knowledge of the dissociation constants of these groups is necessary to carry out an acceptable mobility prediction. The pK values utilized by the GCL model were those of free amino acids, while the CSN model utilized a pK set related to mean values reported for peptides (Table 4). Even though the CSN model use better set of values in comparison to the GCL model, the mobilities observed in terms of enkephalin related peptides (Fig. 3), indicated that these pK values provide only approximate predictions, since the C-terminus dissociation constant depends upon peptide length and sequence.

When the separation is performed at pH values

Table 4
Acidic dissociation constants of amino acid ionizable groups

Amino acid	Free amino acid			Mean values in peptides (Ref. [85])		
	C-terminus	N-terminus	Side chain	C-terminus	N-terminus	Side chain
Ala (A)	2.34	9.87	–	3.20	8.20	–
Arg (R)	1.91	9.02	12.48	3.20	8.20	12.50
Asn (N)	2.06	8.82	–	2.75	7.30	–
Asp (D)	2.02	9.85	3.82	2.75	8.60	3.50
Cys (C)	1.93	10.40	8.26	2.75	7.30	10.30
Gln (Q)	2.17	9.13	–	3.20	7.70	–
Glu (E)	2.15	9.57	4.18	3.20	8.20	4.50
Gly (G)	2.35	9.78	–	3.20	8.20	–
His (H)	1.79	9.18	6.08	3.20	8.20	6.20
Ile (I)	2.34	9.72	–	3.20	8.20	–
Leu (L)	2.35	9.67	–	3.20	8.20	–
Lys (K)	2.17	9.06	10.66	3.20	7.70	10.30
Met (M)	2.28	9.24	–	3.20	9.20	–
Phe (F)	2.37	9.21	–	3.20	7.70	–
Pro (P)	1.98	10.62	–	3.20	9.00	–
Ser (S)	2.20	9.18	–	3.20	7.30	–
Thr (T)	2.09	9.10	–	3.20	8.20	–
Trp (W)	2.40	9.42	–	3.20	8.20	–
Tyr (Y)	2.20	9.11	–	3.20	7.70	–
Val (V)	2.30	9.68	–	3.20	8.20	–

low enough to ensure high proton saturation, the mobility is approximately related to the number of basic groups according to the equation

$$\mu = \frac{q}{6\pi\eta} \cdot \frac{j}{r_s} \qquad (34)$$

Thus it would only be necessary to make a good prediction of the Stokes radius, since j is known from the sequence. Therefore, the best of the two choices described in the RSN and GCL models, is the one regarding the peptide molecular mass, which provides more accurate results than the number of peptide amino acids, because it accounts for differences deriving from the side chains.

We have performed a correlation on various peptides based upon the RSN model (Table 5, Fig. 7) at two different pH values, the first one allowing C-terminus high saturation (pH 2.25) and the second one corresponding approximately to C-terminus half saturation (pH 3.00). The best fit procedures gave different values of the power of molecular mass (0.62 at pH 2.25 and 0.54 at pH 3.00, respectively) at the two pH values. This difference between the exponents is in agreement with a prevalent Stokes radius decrease as a function of peptide proton loss (see also Fig. 2). The correlation performed at pH

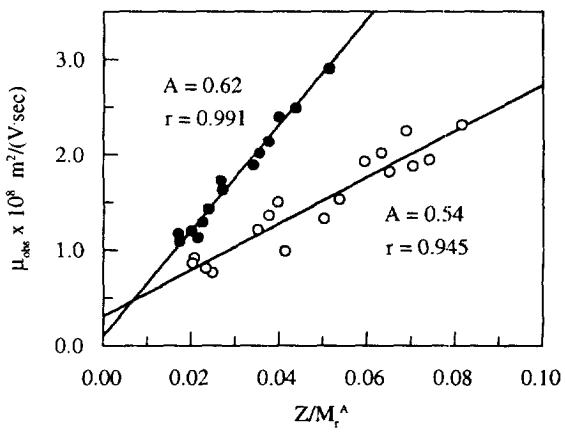

Fig. 7. Correlation between measured mobilities at two different pH values (pH 2.25: closed symbols; pH 3.00: open symbols) and the Z/M_r^A ratio, according to RSN model [85], for the peptide set reported in Table 5. The best power of M_r (A) obtained by non-linear best-fitting procedures is different at the two pH values (0.62 and 0.54, respectively). This behaviour corresponds to general Stokes radius decrease due to the proton loss.

Table 5
Values of molecular mass, charge and observed mobility[a] at different pH values of various peptides

Peptide	M_r	Z^b (pH 2.25)	$\mu \cdot 10^8$ (pH 2.25)	Z^b (pH 3.00)	$\mu \cdot 10^8$ (pH 3.00)
GGRA	359	1.90	2.92	1.61	2.24
AAAY	395	0.90	1.13	0.61	0.76
YGGF	443	0.90	1.20	0.61	0.80
YGGFL (Leu-enkephalin)	556	0.90	1.09	0.61	0.91
RPPGF (bradykinin fr. 1–5)	573	1.90	2.09	1.61	1.53
YGGFM (Met-enkephalin)	574	0.90	1.17	0.61	0.86
ETYSK	627	1.89	1.88	1.58	1.33
HFRW (α-MSH[c] fr. 6–9)	645	2.90	2.90	2.61	2.31
KETYSK	755	2.89	2.64	2.58	1.94
EHFRWG (ACTH[c] fr. 5–10)	831	2.89	2.49	2.58	1.87
SAVTALWGK (Hb T-β_2)	932	1.90	1.73	1.61	0.98
MEHFRWG (ACTH[c] fr. 4–10)	962	2.89	2.39	2.58	1.81
LLVVYPWTQR (Hb T-β_4)	1112	1.90	1.42	1.61	1.36
VVAGVANALAHK (Hb T-β_{14})	1149	2.90	2.01	2.61	1.92
EFTPPVQAAYQK (Hb T-β_{13})	1379	1.89	1.29	1.58	1.21
VLGAFSDGLAHLDNLK (Hb T-β_9)	1670	2.79	1.63	2.13	1.50
TYFPHFDLSHGSAQVK (Hb T-α_6)	1834	3.85	2.13	3.37	2.01

These values were fitted according to a function $\mu \propto Z/M_r^A$ (Fig. 7).
[a] $m^2/V/s$; coated capillary; Na–phosphate buffer 80 mM; 25°C; 25 kV.
[b] Values calculated by Eqs. (28)–(32) using the mean pK values [85] of Table 4.
[c] α-MSH: α-melanocyte stimulating hormone; ACTH: adrenocorticotropic hormone.

2.25 is noticeably better in terms of the correlation coefficient (0.991 as opposed to 0.945) and intercept.

In summary, it must be recognized that the prediction performance is greatly dependent upon the pH of the electrophoretic buffer. Above and beyond the error introduced by the estimation of the Stokes radius, the prediction would be even worse when a poor estimation of the dissociation constants is added. Since the dissociation constant of histidine displays great variability, the worst prediction should be achieved at neutral or slightly acidic pH values.

Since these semiempirical models usually can be utilized with buffers having similar compositions, considerations about ionic strength effects are not strictly necessary [79]. Obviously, if the objective is the comparison between results obtained in sensibly different conditions, ionic strength effects must be considered [76,79].

4. Peptide affinity capillary electrophoresis (ACE)

One of the most interesting options of peptide CE, usually defined as "affinity electrophoresis", is its ability to quantify specific interaction with whatever ligand [92]. Specific interaction must simply result in modification of the charge-to-size ratio and therefore of the electrophoretic mobility [93]. The mobility modifications of the specific peptide(s) at a saturating ligand concentration allow it (them) to be detected in a vast library [94,95]. In addition, mobility dependence from ligand concentration allows the measurement of the association constants and the study of the kinetics of association–dissociation process [96]. In this respect, affinity CE (ACE), due to its sensitivity, to the absence of spurious interaction with an electrophoretic support as well as its ability to alter experimental parameters, offers several advantages in comparison to conventional affinity gel electrophoresis. Since any ligand may be considered, separations based on pH related mobility modifications could be defined by the term "proton affinity electrophoresis", even though this term has been debated [74]. In fact, the basic treatment of the proton binding process must be considered equivalent and must abide by rules common to any other ligand [97,98], even though the following properties are distinguishing: (i) proton binding sites are usually known; (ii) proton binding is usually defined throughout dissociation constants, while other ligand bindings are generally expressed by association constants; (iii) the measure and buffering of proton activity is a basic job of any analytical laboratory.

4.1. Some considerations on ACE theory

The binding process of a ligand x to a peptide P having a single binding site can be described by the association equilibrium:

$$P + x \Leftrightarrow Px$$

and the association constant, K_{ass}, of this reaction should be expressed in terms of the species activities. Assuming that the ratio between P and Px activity is equivalent to the corresponding concentration ratio, K_{ass} is expressed by:

$$K_{ass} = \frac{[Px]}{[P]x}$$

where x represents the activity of the free ligand at equilibrium. The use of saturation function (Y) is one of the best ways to quantify the binding process. This function corresponds to:

$$Y = \frac{[Px]}{[P] + [Px]} = \frac{Kx}{1 + Kx} \tag{35}$$

and its value at established x activity can be obtained from an electrophoretic run assuming that the observed mobility μ_{obs} is given by:

$$\mu_{obs} = Y \cdot \mu_b + (1 - Y) \cdot \mu_f \tag{36}$$

where μ_b and μ_f are the mobilities of the completely bound and of the free peptide measured at saturating and zero ligand activity, respectively. If the binding process is simplified by assuming that the peptide conformation is not modified by ligand interaction, Eqs. (35) and (36) may be transformed into:

$$Y = \frac{\mu_{obs} - \mu_f}{\mu_b - \mu_f} = \frac{Kx}{1 + Kx} = \frac{10^{(\log K + \log x)}}{1 + 10^{(\log K + \log x)}} \tag{37}$$

This basic equation can be utilized in various forms [72,73,99]. Models of ligand-binding description were recently revised by Rundlett and Armstrong [100] and by Winzor [101]. These studies

implicitly suggest the use of uniform symbols. Several transformations are called double-reciprocal plot, x-reciprocal or Scatchard plot. It is well known that these transformations are useful in order to linearize experimental data and obtain association constant values characterized by as few errors as possible. In this way data is fitted through the best line obtained by least square procedures. The present availability of non-linear multi-parametric best-fitting procedures offers the chance to perform a measure of chemico-physical properties according to any reasonable non-linear equation. Thus, several other plots are profitable and allow a better understanding of ligand interaction. One of them is the famous Hill plot [102], largely used for studying the cooperativity of hemoglobins. However, the best option is the so-called binding titration curve, since the error on saturation function is uniform over the complete range of variables [99] (Fig. 8). Titration curve is obtained plotting Y as a function of the logarithm of ligand activity according to Eq. (37), and the logarithm of ligand activity is proportional to the chemical potential of the ligand. The value of $-\log(x)$ at 50% saturation corresponds to $\log(K_{ass})$. In fact:

$$\frac{1}{2} = \frac{10^{(\log K + \log x_{50})}}{1 + 10^{(\log K + \log x_{50})}} \tag{38}$$

hence $10^{(\log K + \log x_{50})} = 1$ and $\log(K) = -\log(x)_{50}$

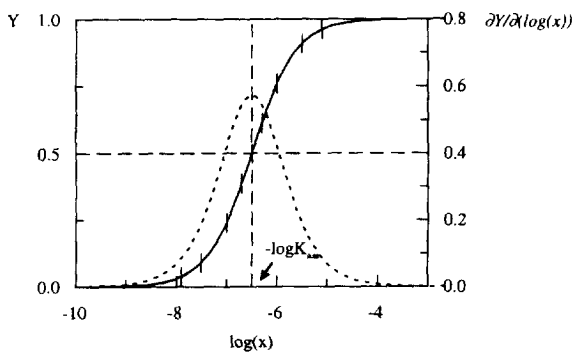

Fig. 8. Simulation of the saturation function (Y) of a generic peptide with a pK_{ass} value of 6.5 as a function of log of ligand activity, obtained according to Eq. (37). The bars correspond to the error in Y determination [99]. Dashed line is the derivative of Y function (according to Eq. (39)) and corresponds to peptide buffering capacity.

The first derivative of Eq. (37), $[\partial Y/\partial(\log x)]$, which expresses the buffering capacity of the peptide compared to the ligand potential [103], is characterized by an invariant shape for peptides having a single binding site and is only shifted along abscissa axis as a function of the $\log K$ values:

$$\frac{\partial Y}{\partial(\log x)} = \ln_e(10) \cdot \frac{10^{(\log K + \log x)}}{\left(1 + 10^{(\log K + \log x)}\right)^2} \tag{39}$$

The maximum value is always 0.576 (corresponding to $\ln_e(10)/4$ and its position on the abscissa axis allows for a second measure of $\log K$.

This approach on monosite ligand binding is once again strictly equivalent to Eq. (15) used for one-site proton dissociation and does not take into account a possible change of Stokes radius due to ligand binding. In view of this fact and similarly to what was established for the proton, Eq. (36) can be transformed into:

$$\mu_{obs} = \frac{q}{6\pi\eta}\left(\frac{Z_b}{r_b}Y + \frac{Z_f}{r_f}(1 - Y)\right) \tag{40}$$

and by substitution:

$$\mu_{obs} = \frac{q}{6\pi\eta}\left(\frac{Z_b}{r_b} \cdot \frac{Kx}{1 + Kx} + \frac{Z_f}{r_f} \cdot \frac{1}{1 + Kx}\right) \tag{41}$$

Thus, titration curve can be expressed by the following equation:

$$\mu_{obs} = \frac{q}{6\pi\eta}\left(\frac{Z_b}{r_b} \cdot \frac{10^{(\log K + \log x)}}{1 + 10^{(\log K + \log x)}} + \frac{Z_f}{r_f} \cdot \frac{1}{1 + 10^{(\log K + \log x)}}\right) \tag{42}$$

Deviations of experimental data from the curve shape described by Eq. (37) can be explained taking into account Stokes radius modification due to ligand interaction and may be interpreted according to Eq. (42). However, since the sites of ligand interactions are not known a priori, deviations from Eq. (37) for charged ligands could also be indicative of multiple site interactions and/or of cooperative binding. On the other hand, if the ligand is not charged, mobility modification related to its concentration should be obviously attributed principally to conformational transitions.

Conclusively, Eqs. (40)–(42) introduce new pa-

rameters in the interpretation of experimental data relating to binding. While this fact represents an experimental enrichment it also provides further uncertainty in the interpretation of the molecular events linked to mobility modification. As an example, in the interpretation of the data of Heegard and Robey, it is difficult to establish if the observed mobility changes upon binding of Ca^{2+} and phosphorylcholine to human C-reactive protein derived either from conformational transitions or from the presence of multiple interaction sites [104].

During ACE experiments devoted to the determination of peptide chemico-physical properties particular attention must be paid to the following conditions:

(a) The use of coated capillary and suitable buffers, in order to avoid undesired peptide interactions with the inner capillary wall. However, ligand interactions should be studied close to physiological conditions, where many peptides, near the isoelectric point, show low mobilities and strong interactions with the wall. These problems are partially resolved by the use of appropriate zwitterionic buffers, that do not interfere with ligand association (Section 2.4). The measure of electroosmotic flow is mandatory in order to obtain effective peptide mobilities, when uncoated capillaries are used. In this respect, peptide electrophoretic mobilities can be corrected by the mobility of reference substance added to the sample [105].

(b) Binding curves must be obtained in isotherm conditions [106]. Hence, an appropriate control of temperature during the electrophoretic run must be fulfilled.

(c) Activity of the free ligand at equilibrium must be known, except that the fraction of ligand bound to peptide is negligible compared to the total ligand concentration. Free ligand activity is not always known in terms of the binding process characterized by high K_{ass}, where runs are performed at very low ligand concentration. Dynamic properties of electrophoresis have the advantage over simple measurements at equilibrium because peptide solution is continuously refreshed during separation. In some cases, similarly to what happened for pH control, buffered ligand solutions can be used.

(d) The modification of the ligand concentration causes a contemporaneous ionic strength modification. Thus, mobility variations due to this modification may be observed.

(e) The width of the electrophoretic peak depends upon the ligand exchange rate. If the exchange rate is fast compared to the run time, the electrophoretic (spatial [23,79]) peak width will be similar at any saturation degree. A progressive lowering of the exchange rate will provide gradually larger Gaussians for those peaks obtained at a partial saturation degree. If the exchange rate is sensibly lower than the run time, two different bound and free peptide peaks should be observed providing information concerning kinetic and stoichiometry of the binding, as observed in the interaction of several drugs to proteins [107,108].

4.2. ACE applications

Interest in ACE application in the field of peptide and protein analysis is growing. Apart from the possibility of detecting particular components in vast peptide libraries [94,95], several studies were devoted to the use of ACE for the investigation of drug–peptide interactions [109,110]. ACE was also used for the determination of binding constant in antibody–antigen interaction [111]. This strategy could be the basis for future CE alternative methods to the widespread immuno-blotting analysis. Furthermore, ACE was used in the study of the interaction of sugars and lectins [112], as well as of a ligand to its specific receptors [113]. This interaction may be at the basis of a biosensor separation system [113]. The ligand was also covalently bound to a replaceable soluble polymer matrix [114].

Since ACE includes all the separations based on specific interactions of any ligand with peptides, many applications of CE may be included into the "affinity" classification. Specific interactions can improve the performance in peptide mixture separation. Mosher [115] studied the effect of metal ion-supplemented buffers on the resolution of peptide CE separations. In this regard, the study of Kornfelt et al. [116] on the improvement of CE peptide mapping observed using phytic acid is meaningful. Authors argued that the interaction of phytic acid was related to the peptide charge-to-size ratio and that only the mobility of the peptides positively charged at the pH of separation was

influenced. Thus, phytic acid interaction seems not to be highly specific, but can be used to deeply modify mapping selectivity. Also the chiral separation of peptides by optically active crown-ether can be included under the "affinity" definition. In fact, the peptide originates inclusion complexes with the crown ether which are characterized by different stability constants of diastereomeric complexes, thereby changing electrophoretic mobilities of enantiomers [117]. For these reasons, chiral separations must be optimized as a function of crown ether concentration according to rules common to any other ligand. Following similar criterion, the CE frontal analysis of enantioselective binding of drugs to protein described by Ohara et al. [118] can be included in the ACE class.

5. Separation in aquo–organic solvents

Due to great CE flexibility in the composition of electrophoretic solutions, Walbroehl and Jorgenson proposed the use of organic solvents on CZE analysis [119,120]. This option was not greatly studied on slab gel electrophoresis, since organic solvent can provide gel shrinkage. The addition of an organic solvent to CE separation buffers can offer various advantages. Sahota and Khaledi [121] established that formamide exhibits the best characteristics with respect to other solvents, including methanol and acetonitrile. The deep alteration of solution chemico-physical properties causes many effects on electrophoretic separation [122,123]. For example, electroosmosis was greatly reduced in uncoated capillaries [124]. Furthermore, the general increase in viscosity reduces and stabilizes running current, facilitates heat exchanges consequently improving the number of theoretical plates. Moreover, organic solvents deeply modify both charge and Stokes radius of peptides, adding a chance in manipulation of separation selectivity.

Among various organic solvents, we have focused our attention on 2,2,2-trifluoroethanol (TFE) [125] due to its characteristic of increasing solubility of apolar peptides and inducing stable secondary conformations in peptides which are otherwise unstructured in aqueous solution [126,127]. Thus, TFE contributes to transitions from random to defined and limited structures, with a consequent decrease in peak dispersion and improvement of CE separation. Finally, TFE reduces the hydrolysis processes providing coating stabilization [125].

The use of aquo–organic mixtures is highly advisable when peptide CE separations are characterized by insufficient performance. The separation of GGNA and GGDA peptides in TFE–water and in water is reported in Fig. 9 (data from Ref. [125]). The increase of resolution is impressive and is connected to enhancements in Stokes radius and dissociation constant differences.

The determination of chemico-physical properties of small peptides in aquo–organic solvents is possible by fitting experimental mobilities according to Eq. (12). In order to compare the results with those obtained in aqueous solutions and to avoid differences deriving from distinct viscosity values, the utilization of the product of mobility for viscosity (intrinsic mobility) is useful. The results obtainable in TFE–water on HFRW peptide (α-MSH: α-melanocyte stimulating hormone fr. 6–9) at 30% v/v (corresponding to a 0.0531 molar fraction) are reported in Fig. 10. The apparent pK decrease of about 0.5 units in TFE (Table 6) is in agreement with the decrease of TFE dielectric constant [125]. Moreover, while in water the proton loss provides a slight Stokes radius decrease, in TFE–water the proton loss provides a strong Stokes radius increase (from 8.9 to

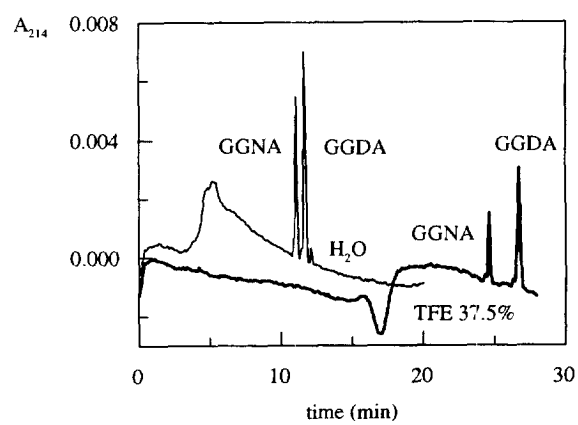

Fig. 9. Electropherogram of GGNA and GGDA peptides in water and TFE–water 37.5% (v/v) mixture at the apparent pH value of 2.8. Other CE conditions as in Fig. 2. Data obtained from Ref. [125].

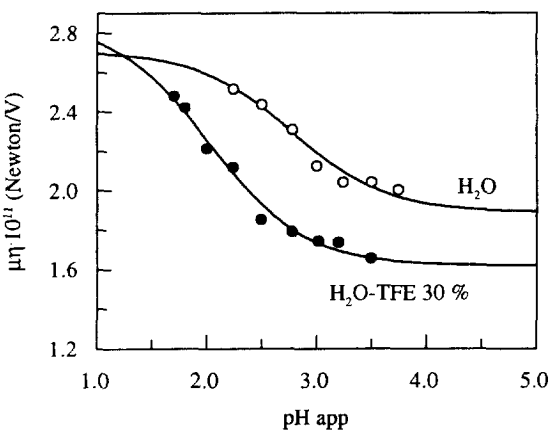

Fig. 10. Product of mobility values and viscosity (intrinsic mobility) of HFRW peptide (α-melanocyte stimulating hormone fr. 6–9) as a function of pH in water and TFE–water 30% (v/v) mixture. Lines were obtained by best-fitting experimental data according to Eq. (12). This procedure provided the values of apparent C-terminus pK and Stokes radius at different protonation stages reported in Table 6. Other CE conditions as in Fig. 2.

10.5 Å). This fact might be interpreted by increased TFE–peptide interaction when the peptide generates an intramolecular ion pair due to C-terminus dissociation. Since TFE is considered a solvent that forces peptide towards conformations similar to those assumed in the membrane environment [128], this information could be relevant to estimate structural variations linked to membrane–peptide interactions.

6. Selected applications

Two main fields of application are discussed: (i) use of CE as a powerful instrument for the determination of chemico-physical peptide properties; (ii) use of CE for conventional analytical aims.

Table 6
Values[a] of apparent dissociation constant and Stokes radius at different protonation stages of HFRW peptide (α-melanocyte stimulating hormone fr. 6–9) in water and in water–TFE mixture (30%, v/v)

Conditions	pK_{app}	Stokes radius (Å)	
		$Z=+3$	$Z=+2$
Water	2.77±0.03	9.4±0.2	9.0±0.2
Water–TFE (30%, v/v)	2.25±0.03	8.9±0.2	10.5±0.1

[a] The values were obtained by best-fitting of the mobility data of Fig. 10 according to Eq. (12).

6.1. Determination of chemico-physical peptide properties

Several studies were devoted to the determination of the effective peptide (protein) charge. In such an example Gao et al. [129] described the generation of either covalent or non-covalent "charge ladders" that permitted a measure of the effective protein charge without the knowledge of its composition, structure or sequence. The method implies that the charge ladder does not greatly modify the hydrodynamic protein properties. Otherwise, CE can be utilized to determine conformational peptide changes. In this respect CE seems to be particularly attractive in terms of the separation and the characterization of *cis* and *trans* peptide conformers at room [130] and subzero [131] temperatures. Different *cis–trans* conformational isomers are often present in proline containing peptides. CE analysis established the kinetic of the conformational transition [130]. On the basis of similar considerations CE is also attractive in terms of the monitoring of peptide folding–unfolding processes, induced either by temperature changes [132] or by denaturant [133].

6.2. Conventional analytical separations

Studies in the field of conventional use of CE for peptide analysis are continuously increasing. Selected examples concern the detection of particular amino acids within a peptide. Cobb and Novotny [134] have indicated that a derivatization reaction with benzoin can provide a sensitive detection of arginine, whereas peptides containing tyrosine can be selectively detected after formylation and subsequent derivatization with 4-methoxy-1,2-phenylenediamine. Thiols and therefore cystein containing peptides can be detected by electrochemical detection using a palladium field-decoupler and chemically modified electrodes [135].

Great interest is also devoted to the detection of post-translational modification of peptides. Obviously, compared to HPLC, CE is particularly suitable when the modification sensibly changes the peptide charge. Therefore, although until now few applica-

tions were described, CE should be very attractive for modifications of peptide N-terminus, lysine and arginine (ADP-rybosylation, acetylation). Gao et al. [136] showed that CE is particularly suitable for the determination of the acetylation of amino groups in insulin. Furthermore, by electrophoretic runs at different pH values, the determination of the acidity constants of the three basic groups is possible. Phosphorylation, for the intrinsic phosphate charge, deeply affects the total peptide charge; thus, phosphorylated peptides can be detected in complex mixtures [137]. Dawson et al. [138] have shown that CE can be used for a fast and sensible assay for protein kinases and phosphatases. In this respect, Fadden and Haystead [139] have developed a method for selective labeling of phosphoserine by 6-iodoacetamidofluorescein and characterization of phosphorylated peptides at the attomole level by CE and laser-induced fluorescence.

Chiesa et al. [140] have recently reviewed the different CE based strategies for the analysis of glycopeptides. Borate-based buffers have been widely utilized in order to increase the resolution throughout the formation of a complex with vicinal hydroxyl groups at moderately alkaline pH solutions. The complex generates an additional negative charge and permits a net separation between glyco- and apopeptides in mapping strategies [141–143]. In addition, CE can be largely utilized for the identification of oligosaccharides released from glycopeptide. The release can be induced by chemical [144–146] or enzymatic [147,148] hydrolysis. In this respect, following different strategies of derivatization [140], CE seems to be the up-and-coming elective method for carbohydrate analysis.

CE has been largely utilized for peptide mapping schemes [134]. Obviously, it is particularly attractive for a fast identification of protein variants. Ferranti et al. [149] showed that the CE–MS coupling permits a fast identification of several hemoglobin (Hb) variants. In our laboratories the determination of Hb J Oxford (α15 [A13] Gly→Asp) was possible, only on the basis of the CE mobility modification of Tα_5 peptide [150]. The peculiar geometry of CE apparatus permits to develop methods devoted to on-line mapping. The initial part of the capillary is enzyme-modified and it is used as a micro-reactor [151]. The final part is used for CE separation. Trypsin, pepsin as well as carboxypeptidase Y has been immobilized and tested for their hydrolytic properties on some proteins. Guzman [152] showed that, in addition to on-line digestion, a contemporary on-line derivatization with fluorescein-isothiocyanate (FITC) is possible. On this line, CE can be used for fast and sensitive analysis of protease and transpeptidase activity [153–155].

7. Perspectives and conclusions

Several future prospects regarding CE applications for peptide analysis may be hypothesized. Short term projects could include the study of conformational modifications as a function of various substances, the help in the search of appropriate peptides in combinatorial peptide libraries generated by robotic synthesis [156], the detection of known and unknown post-translational peptide modifications, the characterization of peptide microheterogeneity [157] and ACE studies. Long term prospects can obviously be only partially postulated and include the detection of a few molecules in a singe cell and the arrangement of miniaturized completely automated CE apparatus on a planar silicon chip. Some efforts in order to produce these instruments have already been made [158]. Great challenges in sample handling and derivatization, as well as sample detection, must be met [159]. However, the development of automated manufacturing procedures on planar silicon chip characterized by an analysis time of milliseconds promises real-time analytical applications in clinical diagnosis, biotechnology and environmental control.

CE can be thus considered a relatively new instrument in the hand of the peptide analysts and will allow them to fight against the magnitude of Avogadros number with a good chance of success. The CE sensitivity, indeed, can generate some analytical trouble. In fact, a common problem in the introduction of innovative separation techniques involves the fact that few substances satisfy criteria of absolute purity, when different chemical or biochemical preparations are submitted to CE analysis. Therefore, above and beyond providing important biochemical information, CE peptide separation will act as a stimulant for greater improvement in complementary techniques, will aid in the characteriza-

tion of minimal impurity quantities, help to further discriminate between sample microheterogeneity and conformational isomers and aid in the development of new separative and preparative techniques for the production of as homogeneous as possible peptide samples.

References

[1] D.Y. Chen, K. Adelhelm, X. Cheng, N.J. Dovichi, Analyst 119 (1994) 349.
[2] A.T. Timperman, K. Khatib, J.V. Sweedler, Anal. Chem. 67 (1995) 139.
[3] J.H. Knox, J. Chromatogr. A 680 (1994) 3.
[4] K.C. Chan, G.M. Janini, G.M. Muschik, H.I. Issaq, J. Liquid Chromatogr. 16 (1993) 1877.
[5] B.L. Hogan, E.S. Yeung, J. Chromatogr. Sci. 28 (1990) 15.
[6] E.S. Yeung, W.G. Kuhr, Anal Chem. 63 (1991) 275.
[7] G.J.M. Bruin, A.C. van Asten, X. Xu, H. Poppe, J. Chromatogr. 608 (1992) 97.
[8] M.C. Chen, H.J. Huang, Anal. Chem. 67 (1995) 4010.
[9] K.B. Tomer, C.E. Parker and L.J. Deterding, in P.G. Righetti (Editor), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, New York, 1996, Ch. 5, p. 123.
[10] G. Schomburg, Trends Anal Chem. 10 (1991) 163.
[11] K. Turner, LC·GC 9 (1991) 350.
[12] S. Hjertén, Chromatogr. Rev. 9 (1967) 122.
[13] R. Virtanen, Acta Polytech. Scand. 123 (1974) 1.
[14] J.W. Jorgenson, K.D. Lukacs, J. High Resolut. Chromatogr., Chromatogr. Comm. 4 (1981) 230.
[15] T. Tsuda, K. Nomura, G. Nakagawa, J. Chromatogr. 264 (1983) 385.
[16] R.A. Wallingford, A.G. Ewing, Adv. Chromatogr. 29 (1989) 1.
[17] S. Hjertén, M. Kiessling-Johansson, J. Chromatogr. 550 (1991) 811.
[18] M. Chiari, M. Nesi and P.G. Righetti, in P.G. Righetti (Editor), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, New York, 1996, Ch. 1, p. 1.
[19] A.M. Dougherty, C.I. Woolley, D.L. Williams, D.F. Swaile, R.O. Cole, M.J. Sepaniak, J. Liq. Chromatogr. 14 (1991) 907.
[20] K.A. Cobb, V. Dolnik, M. Novotny, Anal. Chem. 62 (1990) 2478.
[21] M. Nakatani, A. Shibukawa, T. Nakagawa, Electrophoresis 16 (1995) 1451.
[22] R.M. McCormick, Anal. Chem. 60 (1988) 2322.
[23] M. Castagnola, L. Cassiano, R. Rabino, D.V. Rossetti, F. Andreasi-Bassi, J. Chromatogr. 572 (1991) 51.
[24] C. Fujimoto and K. Jinno, in N. Guzman (Editor), Capillary Electrophoresis Technology, Marcel Dekker, New York, 1993, p. 509.
[25] P. Camilleri, G.N. Oklafo, C. Southan, Anal. Biochem. 196 (1991) 178.
[26] E.A. Lerner, R.J. Nelson, LC·GC 12 (1994) 36.
[27] A.S. Cohen, D.R. Najarian, A. Paulus, A. Guttman, J.A. Smith, B.L. Karger, Proc. Natl. Acad. Sci. USA 85 (1988) 9660.
[28] M. Albin, S.M. Chen, A. Louie, C. Pairaud, J. Colburn, J. Wiktorowicz, Anal. Biochem. 206 (1992) 382.
[29] X. Huang, R.N. Zare, J. Chromatogr. 516 (1990) 185.
[30] H.J. Boss, M.F. Rohde, R.S. Rush, Anal. Biochem. 230 (1995) 123.
[31] K.O. Eriksson, A. Palm, S. Hjertén, Anal. Biochem. 201 (1992) 211.
[32] S. Tracht, V. Toma, J.V. Sweedler, Anal. Chem. 66 (1994) 2382.
[33] R.W. Chiu, K.L. Walker, J.J. Hagen, C.A. Monnig, C.L. Wilkins, Anal. Chem. 67 (1995) 4190.
[34] S.E. Moring, in P.G. Righetti (Editor), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, New York, 1996, Ch. 2, p. 37.
[35] A. Cifuentes, H. Poppe, Electrophoresis 16 (1995) 516.
[36] R.S. Sahota, M.G. Khaledi, Anal. Chem. 66 (1994) 2374.
[37] J.L. Beckers, F.M. Everaerts, M.T. Ackermans, J. Chromatogr. 537 (1991) 407.
[38] C. Schwer, F. Lottspeich, J. Chromatogr. 623 (1992) 345.
[39] C. Schwer, B. Gas, F. Lottspeich, F. Kenndler, Anal. Chem. 65 (1993) 2108.
[40] D.F. Swile, M.J. Sepaniak, J. Liq. Chromatogr. 14 (1991) 869.
[41] T.T. Lee, E.S. Yeung, J. Chromatogr. 595 (1992) 319.
[42] H.T. Chang, E.S. Yeung, Anal. Chem. 67 (1995) 1079.
[43] A.T. Timperman, K.E. Oldenburg, J.V. Sweedler, Anal. Chem. 67 (1995) 3421.
[44] M. Albin, R. Weinberger, E. Sapp, S. Moring, Anal. Chem. 63 (1991) 417.
[45] J. Liu, Y.Z. Hsieh, D. Wiesler, M.V. Novotny, Anal. Chem. 63 (1991) 408.
[46] J. Liu, O. Shirota, M.V. Novotny, Anal. Chem. 63 (1991) 408.
[47] O. Orwar, H.A. Fishman, N.E. Ziv, R.H. Scheller, R.N. Zare, Anal. Chem. 67 (1995) 4261.
[48] Y.H. Lee, R.G. Maus, B.W. Smith, J.D. Winefordner, Anal. Chem. 66 (1994) 4142.
[49] M.P. Richard, J.H. Bettie, J. Chromatogr. 648 (1993) 459.
[50] M.M. Bushey, J.W. Jorgenson, Anal. Chem. 62 (1990) 978.
[51] J.P. Larman Jr., A.V. Lemmo, A.W. Moore Jr., J.W. Jorgenson, Electrophoresis 14 (1993) 439.
[52] H. Yamamoto, M. Manabe, T. Okuyama, J. Chromatogr. 515 (1990) 659.
[53] A.V. Lemmo, J.W. Jorgenson. J. Chromatogr. 633 (1993) 213.
[54] A.W. Moore Jr., J.W. Jorgenson, Anal. Chem. 67 (1995) 3448.
[55] A.W. Moore Jr., J.W. Jorgenson, Anal. Chem. 67 (1995) 3456.
[56] J.H. Wahl, D.R. Goodlett, H.R. Udseth, R.D. Smith, Electrophoresis 14 (1993) 448.
[57] J.H. Wahl, D.C. Gale, R.D. Smith, J. Chromatogr. 659 (1994) 217.
[58] M.A. Moseley, J.W. Jorgenson, J. Shabanowitz, D.F. Hunt, K.B. Tomer, J. Am. Soc. Mass Spectrom. 3 (1992) 289.

[59] J.A. Loo, H.K. Jones, H.R. Udseth, R.D. Smith, J. Microcol. Sep. 1 (1989) 223.
[60] J.A. Loo, H.R. Udseth, R.D. Smith, Anal. Biochem. 179 (1989) 404.
[61] I.M. Johansson, E.C. Huang, J.D. Henion, J. Zweigenbaum, J. Chromatogr. 554 (1991) 311.
[62] M.A. Moseley, L.J. Deterding, K.B. Tomer, J.W. Jorgenson, Anal. Chem. 63 (1991) 109.
[63] L.J. Deterding, C.E. Parker, J.R. Perkins, M.A. Moseley, J.W. Jorgenson, K.B. Tomer, J. Chromatogr. 554 (1991) 329.
[64] J.A. Castoro, R.W. Chiu, C.A. Monning, C.L. Wilkins, J. Am. Chem. Soc. 114 (1992) 7571.
[65] R.S. Smith, J.A. Loo, C.J. Barinaga, C.G. Edmonds, H.R. Udseth, J. Chromatogr. 480 (1989) 211.
[66] E.D. Lee, J.D. Henion, T.R. Covey, J. Chromatogr. 458 (1989) 313.
[67] K. Tsuji, L. Baczynskyi, B.E. Bronson, Anal Chem. 64 (1992) 1864.
[68] R. Kostiainen, E. Lasonder, W. Bloemhoff, P.A. van Veelen, G.W. Welling, A.P. Bruin, Biol. Mass Spectrom. 23 (1994) 346.
[69] J.F. Banks Jr., J. Chromatogr. A 712 (1995) 245.
[70] M.T. Davis, D.C. Stahl, S.A. Hefta, T.D. Lee, Anal Chem. 67 (1995) 4549.
[71] A.J. Tomlinson, S. Naylor, J. Liq. Chromatogr. 18 (1995) 3591.
[72] J.T. Edsall and J. Wyman, Biophysical Chemistry, Academic Press, New York, 1958.
[73] C.R. Cantor and P.R. Shimmel, Biophysical Chemistry. Part III: The Behaviour of Biological Macromolecules, Freeman and Company, San Francisco, CA, 1980.
[74] M. Castagnola, D.V. Rossetti, L. Cassiano, F. Misiti, L. Pennacchietti, B. Giardina, I. Messana, Electrophoresis 17 (1996) 1925.
[75] M. Castagnola, L. Cassiano, I. Messana, G. Nocca, R. Rabino, D.V. Rossetti, B. Giardina, J. Chromatogr. B 656 (1994) 87.
[76] J. Gao, F.A. Gomez, R. Härter, G.M. Whitesides, Proc. Natl. Acad Sci. USA 91 (1994) 12027.
[77] J.A. Cleveland Jr., C.L. Martin, S.J. Gluck, J. Chromatogr. A 679 (1994) 167.
[78] J.W. Jorgenson, K.D. Lukacs, Anal. Chem. 53 (1981) 1298.
[79] M. Castagnola, I. Messana and D.V. Rossetti, in P.G. Righetti (Editor), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, New York, 1996, Ch. 8, p. 239.
[80] H.J. Issaq, G.M. Janini, I.Z. Atamna, G.M. Muschik, J. Lukszo, J. Liq. Chromatogr. 15 (1992) 1129.
[81] B.J. Compton, E.A. O'Grady, Anal. Chem. 63 (1991) 2579.
[82] S.K. Basak, M.R. Ladisch, Anal. Biochem. 226 (1995) 51.
[83] K.S. Chae, A.M. Lenhoff, Biophys. J. 68 (1995) 1120.
[84] P.D. Grossman, J.C. Colburn, H.H. Lauer, Anal. Biochem. 179 (1989) 28.
[85] E.C. Rickard, M.M. Strohl, R.G. Nielsen, Anal. Biochem. 197 (1991) 197.
[86] B.J. Compton, J. Chromatogr. 559 (1991) 357.
[87] N. Chen, L. Zang, Y.K. Zhang, Chromatographia 37 (1993) 429.
[88] A. Cifuentes, H. Poppe, J. Chromatogr. 680 (1994) 321.
[89] V.J. Hilser Jr., G.D. Worosila, S.E. Rudnick, J. Chromatogr. 630 (1993) 329.
[90] M.A. Survay, D.M. Goodall, S.A.C. Wren, R.C. Rowe, J. Chromatogr. 636 (1993) 81.
[91] B. Skoog, A. Wichman, Trends Anal. Chem. 5 (1986) 82.
[92] K. Takeo, J. Chromatogr. A 698 (1995) 89.
[93] T.C. Bøg-Hansen, Anal. Biochem. 56 (1973) 480.
[94] N.H.H. Heegard, F.A. Robey, Anal. Chem. 64 (1992) 2479.
[95] Y.H. Chu, L.Z. Avila, H.A. Biebuyck, G.M. Whitesides, J. Org. Chem. 58 (1993) 648.
[96] Y.H. Chu, L.Z. Avila, J. Gao, G.M. Whitesides, Accounts Chem. Res. 28 (1995) 461.
[97] J. Wyman, D.W. Allen, J. Polymer Sci. 7 (1951) 499.
[98] J. Wyman, Adv. Prot. Chem. 19 (1964) 223.
[99] J.T. Edsall and H. Gutfreund, Biothermodynamics: The Study of Biochemical Processes at Equilibrium, Wiley, New York, 1983, p. 39.
[100] K.L. Rundlett, D.W. Armstrong, J. Chromatogr. A 721 (1996) 173.
[101] D.J. Winzor, J. Chromatogr. A 696 (1995) 160.
[102] A.V. Hill, J. Physiol. (London) 40 (1910) iv.
[103] E. Di Cera, S.J. Gill, J. Wyman, Proc. Natl Acad. Sci. USA 85 (1988) 449.
[104] N.H.H. Heegard, F.A. Robey, J. Immunol. Meth. 166 (1993) 103.
[105] F.A. Gomez, J.K. Chen, Y.H. Chu, G.M. Whitesides, Anal. Chem. 66 (1994) 1785.
[106] K. Takeo, Lectins 2 (1982) 583.
[107] Y.H. Chu, W.J. Lees, A. Stassinopoulos, C.T. Walsh, Biochemistry 33 (1994) 10616.
[108] L.Z. Avila, Y.H. Chu, E.C. Blossey, G.M. Whitesides, J. Med. Chem. 36 (1993) 126.
[109] J. Oravcová, B. Böhs, W. Lindner, J. Chromatogr. B 677 (1996) 1.
[110] P. Sun, A. Hoops, R.A. Hartwick, J. Chromatogr. B 661 (1994) 335.
[111] N.H.H. Heegaard, O.J. Bjerrum, Anal. Biochem. 195 (1991) 319.
[112] R. Kuhn, R. Frei, M. Christen, Anal. Biochem. 218 (1994) 131.
[113] H.A. Fishman, O. Orwar, R.H. Schellen, R.N. Zare, Proc. Natl. Acad. Sci. USA 92 (1995) 7877.
[114] K. Haupt, F. Roy, M.A. Vijayalakshmi, Anal. Biochem. 234 (1996) 149.
[115] R.A. Mosher, Electrophoresis 11 (1990) 765.
[116] T. Kornfelt, A. Vinther, G.N. Okafo, P. Camilleri, J. Chromatogr. A 726 (1996) 223.
[117] R. Kuhn, D. Riester, B. Fleckenstein, K.H. Wiesmüller, J. Chromatogr. A 716 (1995) 371.
[118] T. Ohara, A. Shibukawa, T. Nakagawa, Anal. Chem. 67 (1995) 3520.
[119] Y. Walbroehl, J.W. Jorgenson, J. Chromatogr. 315 (1984) 135.
[120] Y. Walbroehl, J.W. Jorgenson, Anal. Chem. 58 (1986) 479.
[121] R.S. Sahota, M.G. Khaledi, Anal. Chem. 66 (1994) 1141.
[122] I. Bjørnsdottir, S.H. Hansen, J. Chromatogr. A 711 (1995) 313.

[123] M. Chiari, E. Kenndler, J. Chromatogr. A 716 (1995) 303.
[124] G.M. Janini, K.H. Chan, J.A. Barnes, G.M. Muschik, H.J. Issaq, Chromatographia 35 (1993) 497.
[125] M. Castagnola, L. Cassiano, I. Messana, M. Paci, D.V. Rossetti, B. Giardina, J. Chromatogr. A 735 (1996) 271.
[126] J.W. Nelson, N.B. Kallenbach, Biochemistry 28 (1989) 5256.
[127] M.D. Bursh, L.M. Gierasch, J. Biol. Chem. 265 (1990) 3851.
[128] A.R. Van Buuren, H.J.C. Berendson, Biopolymers 33 (1993) 1159.
[129] J. Gao, F.A. Gomez, R. Härter, G.M. Whitesides, Proc. Natl. Acad Sci. USA 91 (1994) 12027.
[130] A.W. Moore Jr., J.W. Jorgenson, Anal Chem. 67 (1995) 3464.
[131] S. Ma, F. Kálmán, A. Kálmán, F. Thunecke, C. Horváth, J. Chromatogr. A 716 (1995) 167.
[132] V.J. Hilser, G.D. Worosila, E. Freire, Anal. Biochem. 208 (1993) 125.
[133] K.R. Skelsey, M.M. Bushey, J. Chromatogr. Sci. 34 (1996) 85.
[134] K.A. Cobb, M.V. Novotny, Anal. Biochem. 200 (1992) 149.
[135] X. Huang, W.T. Kok, J. Chromatogr. A 716 (1995) 347.
[136] J. Gao, M. Mrksich, F.A. Gomez, G.M. Whitesides, Anal Chem. 67 (1995) 3093.
[137] N. Adamson, P.F. Riley, E.C. Reynolds, J. Chromatogr. 646 (1993) 391.
[138] J.F. Dawson, M.P. Boland, C.F.B. Holmes, Anal. Biochem. 220 (1994) 340.
[139] P. Fadden, T.A.J. Haystead, Anal. Biochem. 225 (1995) 81.
[140] C. Chiesa, R.A. O'Neill, C.G. Horváth and P.J. Oefner, in P.G. Righetti (Editor), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, New York, 1996, Ch. 9, p. 277.
[141] S. Hoffstetter-Khun, A. Paulus, E. Gassmann, H.M. Widmer, Anal Chem. 63 (1991) 1541.
[142] W. Nashabeh, Z. El Rassi, J. Chromatogr. 536 (1991) 31.
[143] R.S. Rush, P.L. Derby, T.W. Strickland, M.F. Rohde, Anal Chem. 65 (1993) 1834.
[144] T.P. Patel, J. Bruce, A. Merry, C. Bigge, M. Wormald, A. Jaques, R.B. Parekh, Biochemistry 32 (1993) 679.
[145] T.P. Patel, R.B. Parekh, Meth. Enzymol. 230 (1994) 57.
[146] J. Amano, A. Kobata, Meth. Enzymol. 179 (1989) 261.
[147] S.W. Yuen, L.R. Zieske, I.M. Zaidi, D. Fu and R.A. O'Neill, in J.W. Crabb (Editor), Techniques in Protein Chemistry V, Academic Press, San Diego, CA, 1994, p. 275.
[148] A. Kobata, Anal. Biochem. 100 (1979) 1.
[149] P. Ferranti, A. Malorni, P. Pucci, S. Fanali, A. Nardi, L. Ossicini, Anal. Biochem. 194 (1991) 1.
[150] M. Castagnola, I. Messana, L. Cassiano, R. Rabino, D.V. Rossetti, B. Giardina, Electrophoresis 16 (1995) 1492.
[151] L. Licklider, W.G. Kuhr, Anal. Chem. 66 (1994) 4400.
[152] N.A. Guzman, in P.G. Righetti (Editor), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, New York, 1996, Ch. 4, p. 101.
[153] R.J. Krueger, T.R. Hobbs, K.A. Mihal, L. Tehrani, M.G. Zeece, J. Chromatogr. 543 (1991) 451.
[154] A. Vinther, K. Adelhorst, O. Kirk, Electrophoresis 14 (1993) 486.
[155] F. Mulholland, S. Movahedi, G.R. Hague, T. Kasumi, J. Chromatogr. 636 (1993) 63.
[156] J.A. Boutin, P. Henning, P.H. Lambert, S. Bertin, L. Petit, J.P. Mahieu, B. Serkiz, J.P. Volland, J.L. Fauchère, Anal. Biochem. 234 (1996) 126.
[157] R.S. Rush, P.L. Derby, T.W. Strickland. M.F. Rohnde, Anal. Chem. 65 (1993) 1834.
[158] A. Manz, D.J. Harrison, E. Verpoorte and H.M. Widmer, in P.R. Brown and E. Grushka (Editors), Advances in Chromatography, Marcel Dekker, New York, 1993, p. 1.
[159] S.C. Jacobson, L.B. Koutny, R. Hergenroder, A.W. Moore Jr., J.M. Ramsey, Anal. Chem. 66 (1994) 3472.