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Determination of limiting mobilities and dissociation constants of 21 amino acids by capillary zone electrophoresis at very low pH☆

Iva Zusková*, Alena Novotná, Kateřina Včeláková, Bohuslav Gaš

Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Prague, Czech Republic

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Abstract

The dependence of the effective electrophoretic mobility on pH of the background electrolyte was experimentally determined by capillary zone electrophoresis (CZE) for cationic forms of amino acids. The pH of the background electrolytes was in the highly acidic range, 1.6–2.6 pH units, to ensure a high degree of protonation of the amino acids. Poly(vinyl alcohol) was added to the background electrolytes to avoid possible adsorption of the analytes at the inner capillary wall. Non-linear regression of the experimental data was applied to obtain the parameters of the relevant regression functions—the actual mobilities and mixed dissociation constants corresponding to the actual ionic strength. The extended Onsager and Debye–Hückel law was used to calculate the limiting mobilities and thermodynamic dissociation constants. The comparison of the experimental electropherogram with the computer prediction by PeakMaster using the determined data is presented for the selected sample of amino acids.

Keywords: Amino acids; Capillary electrophoresis; Conductivity; Mobility; Dissociation constant

1. Introduction

Physicochemical parameters are the essential characteristics of a compound, e.g., knowledge of the dissociation constants is of fundamental importance for understanding and quantifying the acid–base behavior of the species in the electrophoretic separation processes. The pH of the separation system determines the degree of dissociation so the dissociation constants are decisive for finding the optimum pH range for the separation. When employing electromigration separation techniques, the limiting mobilities are also significant for the process of separation. There is no doubt that even rough estimates of physicochemical parameters of compounds are very helpful for finding the composition of a background electrolyte (BGE) that enables good separation. Of course, knowledge of accurate data significantly enhances the quality of the predictions.

As is well known, capillary zone electrophoresis (CZE) is an analytical technique for separation of charged analytes which move in the electric field in a capillary typically made from fused silica. Coating of the inner capillary wall with a polymer is used mainly for control of the electroosmotic flow (EOF) and suppression of the analyte–wall interactions. Two different approaches are employed for coating the inner capillary wall—dynamic coating (reversible adsorption on the capillary wall) and permanent coating. The first kind of coating is formed by water-soluble polymers (cellulose derivatives, polysaccharides) added in low concentration to the BGE. The second kind – permanent coating – is formed by molecules strongly bonded to the inner capillary wall. Among all the capillary coatings described in literature, those based on poly(vinyl alcohol) (PVA) have proven to exhibit a particularly good performance [1–6]. Extraordinary high separation efficiencies could be achieved using capillaries coated with PVA, especially for proteins [2].

Capillary zone electrophoresis can serve not only for separation of the analytes of a sample. Unlike other physicochemical methods (potentiometry, spectroscopy or conductivity measurements), CZE has proved to be an easy, fast and accurate method for determination of physicochemical parameters (dissociation constant, K_a and limiting mobility, m_{lim}) [7]. The essential advantages of CZE include the simplicity of the system and

Abbreviations: CCD, contactless conductivity detector; PVA, poly(vinyl alcohol); OH-Pro, hydroxyproline

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* Corresponding author. Tel.: +420 2 2195 1306; fax: +420 2 2491 9752.
E-mail address: zuskova@natur.cuni.cz (I. Zusková).

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the need for only minimum amounts of the analytes [8]. CZE enables simultaneous determination of both the dissociation constant and the limiting mobility [9–17] which ranks CZE as the most advantageous method for the experimental determination of these characteristics.

Hirokawa et al. [18–22] have done a great deal of work in the field of determination of mobilities and pK_a values. They utilized isotachophoresis which offers accurate results in the moderate pH regions. Recently, especially the pK_a values were determined by CZE for pharmaceutically and nutritionally important compounds [23–26]. Pospíchal determined the mobilities of various analytes by CZE as well as reviewed of the data published in the literature [27].

Recently, we presented the determination of the limiting mobilities and dissociation constants of the cationic forms of 22 amino acids by CZE in free solution [28]. To achieve conditions where the amino acids are fully protonated, the dissociation of their carboxylic groups must be suppressed as much as possible. According to published data, pK_a values of the carboxylic groups of common amino acids are around two, so only the highly acidic BGEs (pH ∼ 0) will fulfill this requirement. Unfortunately, correction of mobilities and dissociation constants for the ionic strength is more difficult when highly acidic buffers are used. In Ref. [28] we used background electrolytes covering the pH range 2.0–3.2 with constant ionic strength of 10^{-2} mol dm^{-3}. We focused on the optimization of the experimental conditions, especially on the process of effective electrophoretic mobility determination employing the double conductivity detector, which eliminates the effects of the initial migration stage. The precision of the determination of the pK_a values and limiting mobilities was very good for the univalent amino acids with pK_a values higher than two. Less satisfactory results were obtained for the other amino acids due to the fact that their protonation was less than 50%, even in the most acidic BGE.

In the present paper we concentrate on (i) improving the precision mainly of the limiting mobilities by using BGEs with pH values lower than two, (ii) suppression of the possible adsorption of the protonated amino acids onto the inner capillary wall by using capillaries dynamically coated with PVA.

2. Experimental

2.1. Chemicals

The sources of chemicals were as follows: chloroacetic acid, arginine, aspartic acid, isoleucine, lysine, threonine, tryptophane and valine, all 99% – Aldrich (Steinheim, Germany); alanine, asparagine, β-alanine, glycine, leucine, and *p*-nitrophenol, all analytical grade – Lachema (Brno, Czech Republic); cysteine, phenylalanine, glutamine, glutamic acid, histidine, hydroxyproline, methionine, proline, tyrosine and serine, all 99% – Merck (Darmstadt, Germany); poly(vinyl alcohol) – Hoechst (Frankfurt, Germany). The stock solutions of BGEs were prepared by dissolving the appropriate amount of chloroacetic acid and glycine in water. Stock solutions of individual amino acids were prepared at a concentration of 50 mmol dm^{-3}. An aqueous solution of *p*-nitrophenol in 20 mmol dm^{-3} concentration was used as the EOF marker. All running BGEs contained PVA in 0.2% (v/v) concentration to prevent the adsorption of the amino acids at the inner capillary wall. Water used for preparation of all the solutions was purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

All experiments were carried out with the ^{3D}CE equipment (Agilent Technologies, Waldbronn, Germany) with fused silica capillaries (50.0 cm total length, 25 μm I.D., 375 μm O.D.; CaCo Silica Tubing & Optical Fibres, Bratislava, Slovakia). The instrument was equipped with a built-in photometric diode array detector (DAD) and a laboratory made contactless conductivity detector (CCD) of our construction [29] with a double detection cell. The CCD cells were positioned approximately at 30 cm from the inlet and their distance (14.71 mm) was precisely determined by the procedure described in [28]. The detection window for the DAD light beam was positioned 41.5 cm from the inlet end of the capillary. The separation capillary and the CCD detector cells were thermostated at 25 °C. A separation voltage of 20 kV (current inside the separation capillary was lower than 30 μA) and a hydrodynamic injection of 10 mbar s were used in all CZE experiments. In order to condition the inner capillary wall, the following flushing process was performed prior to the first application of a new capillary: 30 min with 0.1 mol dm^{-3} NaOH, three times with water for 3 min. A voltage of 20 kV was applied for 30 min to the capillary filled with the running BGE to stabilize the EOF. Prior to each run, the capillary was flushed with the running buffer. A laboratory pH meter (PHM 220, Radiometer, Denmark) was employed to measure the pH of the BGEs. The computer program ChemStation (Agilent Technologies) was used for data collection and processing. The computer program PeakMaster [30,31] was employed for finding the suitable composition of BGEs. Origin 6.1 (OriginLab Corporation, Northampton, MA, USA) was used for fitting and graphical depiction of the experimental results.

2.3. Regression functions

We used a series of BGEs with practically constant ionic strength which allowed us to express the effective electrophoretic mobility as the function of only one variable—pH. The experimentally obtained dependence is fitted by the appropriate regression function. Schematically, the dissociation equilibria of amino acid A can be described as follows

$$(A^{2+} \rightleftarrows)\ A^{+} \rightleftarrows A^{0} \rightleftarrows A^{-} \rightleftarrows (A^{2-})$$
$$+\ H^{+} \qquad +\ H^{+} \qquad +\ H^{+} \qquad +\ H^{+}$$

We employ three groups of regression functions derived in our previous paper [28] according to the type of the amino acid:

a) simple amino acids containing one basic and one acidic group (Ala, Asn, Cys, Gln, Gly, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, hydroxyproline (OH-Pro), β-Ala; for abbrevi-

ations see Table 2)

$$m_{\mathrm{eff}} = \frac{m_{\mathrm{A}^+}}{1 + 10^{\mathrm{pH}-\mathrm{p}K_{\mathrm{a},1}^{\mathrm{mix}}}} \quad (1)$$

(note, that the second acidic group of Cys (–SH) and of Tyr (–OH) is not dissociated in the acidic pH region),

b) basic amino acids containing two basic and one acidic groups (Arg, Lys, His)

$$m_{\mathrm{eff}} = \frac{m_{\mathrm{A}^{2+}} 10^{\mathrm{p}K_{\mathrm{a},1}^{\mathrm{mix}}-\mathrm{pH}} + m_{\mathrm{A}^+}}{1 + 10^{\mathrm{p}K_{\mathrm{a},1}^{\mathrm{mix}}-\mathrm{pH}}}, \quad (2)$$

c) acidic amino acids containing one basic and two acidic groups (Asp, Glu)

$$m_{\mathrm{eff}} = \frac{m_{\mathrm{A}^+} 10^{\mathrm{p}K_{\mathrm{a},1}^{\mathrm{mix}}-\mathrm{pH}} - m_{\mathrm{A}^-} 10^{\mathrm{pH}-\mathrm{p}K_{\mathrm{a},2}^{\mathrm{mix}}}}{10^{\mathrm{p}K_{\mathrm{a},1}^{\mathrm{mix}}-\mathrm{pH}} + 10^{\mathrm{pH}-\mathrm{p}K_{\mathrm{a},2}^{\mathrm{mix}}} + 1}, \quad (3)$$

where m_{eff} stands for the effective electrophoretic mobility of the species at the pH of the BGE, m_{A^+}, $m_{\mathrm{A}^{2+}}$ and m_{A^-} are the actual mobilities of the charged forms of the amino acid. The values $\mathrm{p}K_{\mathrm{a},1}^{\mathrm{mix}}$ and $\mathrm{p}K_{\mathrm{a},2}^{\mathrm{mix}}$ describe the negative logarithm of the mixed dissociation constants [28]. In contrast to the previous paper [28] where a special regression function for His was presented, the use of a BGE with a very low pH permits the addition of His ($\mathrm{p}K_{\mathrm{a},2}$(imino group) ≅ 6) into the group b) of amino acids.

2.4. Ionic strength correction

The thermodynamic (K_{a}) and mixed ($K_{\mathrm{a}}^{\mathrm{mix}}$) dissociation constants of the ionic form of an amino acid are interconnected by the activity coefficient, γ_i. The extended form of the Debye–Hückel formula [32] with a linear correction term served for the calculation of the activity coefficient of the ith ion

$$\log \gamma_i = \frac{-Az_i^2\sqrt{I}}{1 + Ba\sqrt{I}} + CI, \quad (4)$$

where I is the ionic strength of the BGE, z_i the charge number, A and B are constants characteristic for the solvent and temperature (for aqueous solutions and 25 °C, we took $A = 0.5085$ $(\mathrm{mol\,dm^{-3}})^{-1/2}$ and $B = 3.286\,\mathrm{nm^{-1}}$ $(\mathrm{mol\,dm^{-3}})^{-1/2}$), where a stands for the effective ion diameter and C is the empirical constant. The product Ba is often approximated by the value of 1.5 $(\mathrm{mol\,dm^{-3}})^{-1/2}$ and $C = 0.1z_i^2$ [33].

The dependence of the actual mobility of ions in a binary electrolyte on the ionic strength of the BGE is expressed by the Onsager law [34,35], which describes the relation between the actual mobility of the ion and its limiting mobility, m_{lim}. Here, we use the equation for the actual mobility of cation m_+, with charge number z_+, in the following form

$$m_+ = m_{\mathrm{lim},+} - \left(m_{\mathrm{lim},+}z_+|z_-|B_1\frac{q}{1+\sqrt{q}} + B_2 z_+\right) \times \frac{\sqrt{I}}{1 + Ba\sqrt{I}}, \quad (5)$$

where B_1 and B_2 are constants, which for aqueous solutions at 25 °C acquire values of $B_1 = 0.7817$ $(\mathrm{mol\,dm^{-3}})^{-1/2}$ and $B_2 = 3.138 \times \mathrm{m^2\,V^{-1}\,s^{-1}}$ $(\mathrm{mol\,dm^{-3}})^{-1/2}$, the product Ba has the same meaning and value as in Eq. (4), and q is defined by

$$q = \frac{z_+|z_-|}{z_+ + |z_-|}\frac{m_{\mathrm{lim},+} + m_{\mathrm{lim},-}}{|z_-|m_{\mathrm{lim},+} + z_+m_{\mathrm{lim},-}}, \quad (6)$$

where z_- and $m_{\mathrm{lim},-}$ are the charge number and the limiting mobility of the anion of the BGE, respectively. The limiting mobility from Eq. (5) for unsymmetrical electrolytes (for symmetrical electrolytes $q = 1/2$) must be obtained by iteration. The validity of Eq. (5) has been verified for ionic strengths up to 100 mmol dm^{-3} [36].

3. Results and discussion

3.1. Experimental conditions

In spite of the fact that the separation is performed in a very narrow capillary, thermal phenomena play a significant role in the determination of the ionic mobilities. To reduce the unfavorable effects of Joule heating we applied a running voltage of 20 kV and a rather narrow capillary of 25 μm I.D.

The migration velocity of the analyte is not constant during the entire run, as the composition of the injected zone is different from that of the BGE and the initial part of the capillary is not thermostated. Hence, the calculation of the analyte velocity just from migration time and the migration distance from the inlet end of the capillary to the detector is not accurate enough. We employed the double contactless conductivity detector with two conductivity cells (distance of the cells is 14.71 mm) located inside the thermostated part of the cassette. The effective electrophoretic mobility of the analytes is determined from the time difference in the signals in both detection cells.

3.2. Composition of BGE

The buffer constituents—chloroacetic acid ($\mathrm{p}K_{\mathrm{a}} = 2.87$ [19]) and glycine ($\mathrm{p}K_{\mathrm{a}} = 2.35$ [19]) were chosen due to their $\mathrm{p}K_{\mathrm{a}}$ values to cover the pH region of 1.6–2.6 units with sufficient buffering capacity. The PeakMaster computer program [30,31] was employed to calculate the composition of the series of these BGEs with various pH and nearly constant ionic strength of 30 mmol dm^{-3}. As the $\mathrm{p}K_{\mathrm{a}}$ values of the cationic forms of most of the amino acids of interest are in the range of about two, the BGE pH used allows a significant protonation of the compounds. The calculated compositions of the stock solution of the buffers components are given in Table 1. The final running buffer solutions were prepared by mixing of the stock buffer solutions and PVA solution (1% v/v) in the ratio of 4:1. The resulting concentration of PVA in the running BGEs was 0.2% (v/v) and the average ionic strength was 25 mmol dm^{-3}.

Table 1
Composition of the stock solution of BGE components at constant ionic strength ($I = 30$ mmol dm^{-3})

c (mmol dm^{-3})		pH_{theor}	pH_{exp}
Chloroacetic acid	Glycine		
500.0	1.0	1.603	1.62
328.0	14.5	1.801	1.81
221.0	25.5	1.994	1.98
148.0	37.5	2.203	2.19
103.5	52.0	2.408	2.38
77.5	71.0	2.598	2.59

pH_{theor} is pH calculated by PeakMaster, pH_{exp} is measured pH.

3.3. Determination of limiting mobilities and dissociation constants of the cationic forms of the amino acids

On the basis of computer simulation by PeakMaster (the database of pK_a and m_{lim} was taken from [28]), several amino acids were collected into different groups and run together by CZE (Lys + Pro; Arg + Asp; Asn + β-Ala + Glu + Tyr; Gln + Val + Ala + His; Phe + Met + Ser + Thr; Trp + Ile; Leu + Cys + OH-Pro). Three subsequent runs were carried out at each pH, fresh BGE was used for each run. The concentration of the amino acids in the samples was 4 mmol dm^{-3} (except for the first two groups where the concentration was 8 mmol dm^{-3}). The electrophoretic mobility of the EOF was monitored in each experiment and was used to calculate the effective electrophoretic mobilities of the analytes.

The entire sets of the experimentally obtained effective mobilities at different pH were fitted with the appropriate regression function, Eqs. (1)–(3), according to the type of the particular amino acid. For the first type of the amino acids – containing one basic and one acidic group – we employed the regression function (Eq. (1)) with two fitted parameters: the actual mobility of the univalent cation, m_{A^+}, and the mixed dissociation constant of the relevant carboxylic group, $pK_{a,1}^{mix}$. The regression function for the basic amino acids and for His (Eq. (2)) in addition includes the actual mobility of the bivalent cation, $m_{A^{2+}}$, as the third fitted parameter.

For the acidic amino acids containing two carboxylic groups the regression function (Eq. (3)) has four parameters, but only parameters m_{A^+} and $pK_{a,1}^{mix}$ refer to the cationic forms. The values related to the anionic forms of these amino acids, m_{A^-} and $pK_{a,2}^{mix}$, respectively, were taken as input data. According to [19], the limiting mobilities and thermodynamic dissociation constant for Asp $m_{lim,A^-} = 30.1 \times 10^{-9}$ m^2 s^{-1} V^{-1} and p$K_{a,2} = 3.90$ and for Glu $m_{lim,A^-} = 27.0 \times 10^{-9}$ m^2 s^{-1} V^{-1} and p$K_{a,2} = 4.32$ were recalculated (see Eqs. (4) and (5)) to the ionic strength of the running BGEs used ($I = 25$ mmol dm^{-3}). The values of $m_{A^-} = 25.2 \times 10^{-9}$ m^2 s^{-1} V^{-1} and $pK_{a,2}^{mix} = 3.84$ for Asp and the values of $m_{A^-} = 22.2 \times 10^{-9}$ m^2 s^{-1} V^{-1} and $pK_{a,2}^{mix} = 4.26$ for Glu were then used in the fitting procedure. A typical graphical depiction of the dependence of the effective electrophoretic mobility on the BGE pH is shown in Fig. 1.

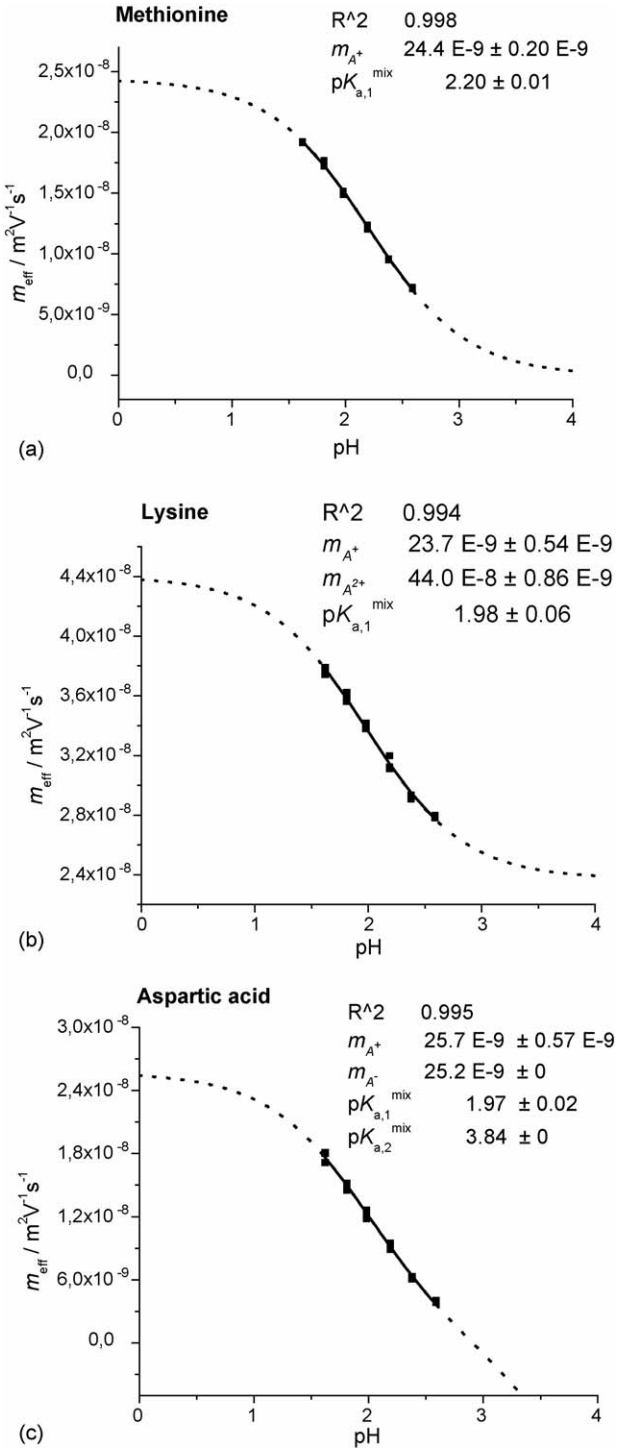

Fig. 1. Effective electrophoretic mobilities as a function of the pH of the BGE for (a) Met; (b) Lys; (c) Asp. The curves were fitted according to Eqs. (1)–(3).

The relevance of the above fitting process is based on the assumption that the actual mobilities depend only on the ionic strength of the BGEs. This assumption may not be exactly fulfilled in BGEs with significant concentration of hydroxonium ions, which exhibit the so-called mixing effects, as shown by Onsager and Fuoss [37]. As we verified by PeakMaster, which utilizes the Onsager–Fuoss model, the deviations are negligible.

Table 2
Cationic actual mobilities, m_+ and mixed pK_a^{mix} values at ionic strength of 25 mmol dm^{-3}, cationic limiting mobilities, m_{lim} and thermodynamic pK_a values of common amino acids

Amino acid	Abbreviation	Actual mobility, m_+ ($\times10^{-9}$ m^2 s^{-1} V^{-1})	pK_a^{mix}	Limiting mobility, m_{lim} ($\times10^{-9}$ m^2 s^{-1} V^{-1})	pK_a
Alanine	Ala	30.8 ± 0.4	2.31 ± 0.02	35.9 ± 0.4	2.25 ± 0.02
Arginine	Arg	49.6 ± 1.4	1.76 ± 0.05	61.0 ± 1.5	1.58 ± 0.04
		20.7 ± 0.4	–	25.5 ± 0.4	–
Asparagine	Asn	27.0 ± 0.3	2.16 ± 0.01	32.0 ± 0.3	2.10 ± 0.01
Aspartic acid	Asp	25.7 ± 0.6	1.97 ± 0.02	30.6 ± 0.7	1.90 ± 0.03
Cysteine	Cys	28.8 ± 0.3	1.93 ± 0.01	33.8 ± 0.3	1.87 ± 0.01
Glutamic acid	Glu	23.8 ± 0.3	2.20 ± 0.01	28.7 ± 0.2	2.14 ± 0.01
Glutamine	Gln	24.7 ± 0.2	2.16 ± 0.01	29.6 ± 0.2	2.10 ± 0.01
Histidine	His	36.9 ± 0.4	2.20 ± 0.06	47.5 ± 0.4	2.01 ± 0.07
		22.0 ± 0.6	–	26.8 ± 0.8	–
Isoleucine	Ile	24.3 ± 0.2	2.36 ± 0.01	29.2 ± 0.2	2.30 ± 0.01
Leucine	Leu	24.3 ± 0.2	2.33 ± 0.01	29.2 ± 0.2	2.26 ± 0.01
Lysine	Lys	44.0 ± 0.9	1.98 ± 0.06	55.1 ± 1.0	1.79 ± 0.06
		23.7 ± 0.5	–	28.6 ± 0.5	–
Methionine	Met	24.4 ± 0.2	2.20 ± 0.01	29.3 ± 0.2	2.13 ± 0.01
Phenyalanine	Phe	22.6 ± 0.3	2.20 ± 0.01	27.4 ± 0.3	2.13 ± 0.01
Proline	Pro	30.7 ± 0.9	1.91 ± 0.02	35.8 ± 0.9	1.85 ± 0.02
Serine	Ser	28.4 ± 0.3	2.20 ± 0.01	33.4 ± 0.3	2.13 ± 0.01
Threonine	Thr	25.5 ± 0.2	2.20 ± 0.01	30.4 ± 0.2	2.14 ± 0.01
Tryptophan	Trp	20.8 ± 0.3	2.37 ± 0.02	25.6 ± 0.3	2.31 ± 0.02
Tyrosine	Tyr	21.5 ± 0.2	2.24 ± 0.01	26.3 ± 0.2	2.18 ± 0.01
Valine	Val	26.0 ± 0.3	2.27 ± 0.01	30.9 ± 0.3	2.21 ± 0.01
Hydroxyproline	OH-Pro	27.7 ± 0.6	1.84 ± 0.02	32.7 ± 0.6	1.78 ± 0.02
β-Alanine	β-Ala	33.4 ± 0.1	3.49 ± 0.01	38.5 ± 0.1	3.42 ± 0.01

Temperature 25 °C.

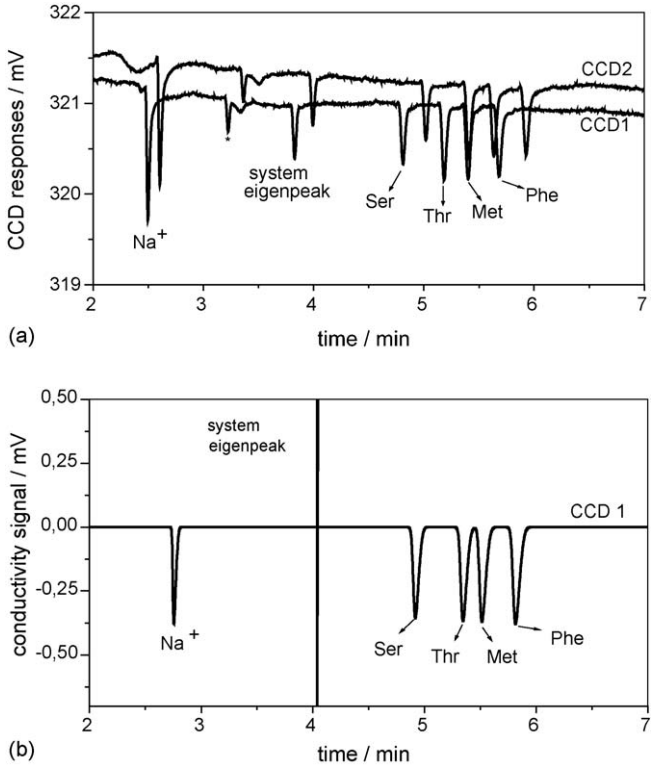

Fig. 2. CZE separation of Ser, Thr, Met and Phe (4 mmol dm^{-3}). BGE: 400 mmol dm^{-3} chloroacetic acid and 0.8 mmol dm^{-3} glycine (pH 1.62). Capillary inner diameter, 25 μm; distance of the CCD cells, 14.71 mm; total capillary length, 50.0 cm; injection, 10 mbar s; separation voltage, +20 kV; temperature, 25 °C. (a) Experimental run (peak indicated by the asterisk was not identified); (b) computer simulation.

Table 2 summarizes the determined cationic actual mobilities, m_+, and pK_a^{mix} values. Also given are the corrected limiting mobilities, m_{lim}, and thermodynamic dissociation constants pK_a of all examined amino acids. Notice that Gly was not examined because it serves as the BGE constituent. It can be seen that the standard deviation of the limiting mobilities for most of the examined amino acids is smaller than 2% and for the pK_a values the standard deviation is better than 1%. The exceptions are the mobilities and related pK_a values of the bivalent forms of Arg, His and Lys, and of Asp, where the standard deviation reaches 3%.

As we expected, using BGEs with the very low pH of 1.6–2.6 allowed us to achieve smaller standard deviations of the fitted parameters than those described in [28]. The determined limiting mobilities exhibit somewhat higher values. Their correctness is based mainly on the fact that the low pH values of the running buffers allowed the higher protonation of amino acids. The exactly same values of the limiting mobilities were obtained in both systems for β-alanine due to its $pK_a = 3.4$.

The limiting mobilities and pK_a values from Table 2 were inserted into the database of PeakMaster [31] and were tested by simulating the electropherograms obtained at low pH. In all cases a satisfactory agreement with the real experimental runs was obtained. An example is given in Fig. 2.

4. Conclusion

The correctness of the physicochemical data used in simulation tools for optimization of BGEs in CZE is a basic

requirement. We present here a method for a reliable and accurate determination of the limiting mobilities and dissociation constants of the cationic forms of amino acids. CZE performed in the highly acidic pH range enabled us to reach the degree of protonation higher than 50% for all amino acids.

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