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pK_a values of peptides in aqueous and aqueous–organic media Prediction of chromatographic and electrophoretic behaviour

V. Sanz-Nebot*, I. Toro, F. Benavente, J. Barbosa

Department of Analytical Chemistry, University of Barcelona, Diagonal 647, 08028 Barcelona, Spain

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Abstract

In the present work, models describing the effect of the pH on the chromatographic and electrophoretic behaviour for polyprotic peptides were compared. The proposed models can be simultaneously used for determination of dissociation constants and selection of the optimum pH for the separation of peptides, in water and acetonitrile–water mixtures widely used in liquid chromatography and in capillary electrophoresis. The models use the pH value measured in the acetonitrile–water mixture instead of the pH value in water and take into account the effect of the activity coefficients. They permit the determination of the acidity constants in the aqueous and hydro–organic mobile phase from chromatographic retention and electrophoretic migration measurements, respectively. The values obtained by both proposed techniques agree with the potentiometric values previously determined. The suitability of the proposed models for predicting chromatographic and electrophoretic behaviour of compounds studied from a limited number of experimental data was also compared. The separation between solutes by both techniques in a complex mixture can be easily predicted, making simple and rapid pH selection to achieve optimum separation.

Keywords: Dissociation constants; Mobile phase composition; Relation prediction; Buffer composition; pH effects; Optimization; Peptides

1. Introduction

The use of synthetic peptides in pharmaceutical research and in human and veterinary medicine is widespread. In this way, peptide and protein separations have been extensively explored, especially when high efficiency and resolution are desirable [1–4]. Novel applications in this field are continuously described, whereas the major drawbacks of the technique are tried to be solved on its way to maturity [4–8]. In the near future, the combined advances of biotechnology and proteomic research must lead to an increasing activity in the discovery and synthesis of new biologically active peptides [9], which is necessary for the development of new separation and characterization technologies. At present, liquid chromatography (LC) and capillary electrophoresis (CE) are orthogonal techniques for peptide and protein separation and identification [1–6], improvement of hyphenated techniques [10,11] must increase their value also in other fields.

The development of rapid, efficient and selective separation methods requires optimization of separation conditions. Although a desired peptide sepa-

*Corresponding author. Tel.: +34-93-402-9083; fax: +34-93-402-1233.

E-mail address: vsanz@apolo.qui.ub.es (V. Sanz-Nebot).

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ration may be obtained by trial and error, this may take many attempts with subsequent loss of time and final peptide yield, and could be a particular problem when only limited amounts of sample are available. We can minimize the total number of attempts by using experimental data to select the best conditions and by making use of accurate quantitative relationships able to predict elution of peptides under different separation conditions.

The optimization of chromatographic and electrophoretic separation of ionogenic solutes is a task that has been actively researched [12–15]. Due to the specific acid–base characteristics of ionogenic solutes, the two most useful optimization parameters are the pH and the organic modifier concentration.

The use of non-aqueous solvents in general, and the binary water–organic solvent system in particular, extends the range of aqueous CE applications. This provides a more versatile electrophoretic separation, since it is possible to work in a medium with different dielectric constants, polarities, densities, viscosities and acid–base properties [5,16,17]. Other advantages of non-aqueous CE are improved solubility of analytes with low solubility in pure aqueous buffer solutions, and low operating currents when voltage is applied [18]. As a result, less Joule heat is produced under non-aqueous CE conditions than in buffered aqueous systems, allowing much higher electrical field strengths than those currently used in CE [19].

The inclusion of the pH as an additional optimization parameter raises several problems [20]. The pH of the mobile phase is usually taken to be the same as that of the aqueous fraction. However, the pK_a values of the acids used to prepare the buffers, change with the solvent composition [21,22] so does the pH of the buffer [23,24]. Sometimes the pH is measured after mixing the buffer with the organic modifier [25]. But even in this instance, the potentiometric system is usually calibrated with aqueous standards, and the measured pH is not the true pH of the mobile phase. Additionally, the proposed models do not consider the effect of the activity coefficients. This effect can be neglected in water, which has a high dielectric constant, but it may be considerable in acetonitrile–water mixtures [21,22].

pH measurements in acetonitrile–water, the most widely used mobile phase, can be performed in a manner similar to that in water [26–28], taking into account the pH values previously assigned to primary standard buffer solutions in acetonitrile–water mixtures [23,24] according to the National Institute of Standards and Technology (NIST) multiprimary standard scale [26]. Also, in compliance with IUPAC rules [28,29], the activity coefficients of the species in acetonitrile–water mixtures can be calculated from the ionic strength through the classical Debye–Hückel equation [29].

Several semi-empirical approaches have been proposed to relate chromatographic retention or electrophoretic mobility with structural parameters of proteins and peptides, but their application is limited by the assumptions made in their development [30–33]. Furthermore, there is no general rule to a priori select the appropriate relationship for each type of compound [31]. Likewise, in the separation of ionizable compounds, pH plays an important role as it determines the extent of ionization of each individual solute [15]. Accurate quantitative relationships between chromatographic retention and electrophoretic mobility, respectively and pH, can be very useful [12,34,35].

In this way, modelling the chromatographic and electrophoretic behaviour is a key tool to predict separations, avoiding long and tedious separation optimizations performed by trial and error procedures. Furthermore, these retention or migration models can be used to perform physico–chemical and conformational characterization of biomolecules like acidity constants [31,36–38].

The activity of many biological molecules depends on the presence of charged groups. Consequently, the dissociation constant can be a key parameter for understanding and quantifying chemical phenomena or biological activity. Thus, the passage of many drugs into cells and across other membranes is a function of pH in the internal environment and the pK_a of the drug [35].

In this work models describing the effect of pH on retention in LC and CE were compared. The suggested models use the pH value in the acetonitrile–water mixture used as mobile phase, instead of pH value in water and take into account the effect of the activity coefficients. The model has been tested for a series of peptides in acetonitrile–water (7:93, v/v) for LC and in water and acetonitrile–water

(12.5:87.5, v/v) mixtures for CE. The usefulness of the proposed equations is twofold. They permit the determination of the acidity constants in water and in the hydro–organic mobile phases, and also can be used, to establish a general model that relates the elution behaviour of the solute with the significant mobile phase properties: composition, pH and ionic strength [37,39]. The advantages of using LC and CE to determine accurate thermodynamic pK_a values of compounds are numerous: the use of these techniques requires small amounts of sample at low solute concentration and the procedure does not require solute measurement or titrant concentration, like potentiometric techniques, but only retention or migration times. Calculations are independent of solutes impurities, since impurities can be separated from the solutes of interest [40,41]. Moreover, CE permits pK_a determination in aqueous solution without difficulties [41] which is not the case for LC, in which the retention could be unsuitable without the addition of an organic modifier [14].

2. Experimental

2.1. Chemical and reagents

Water with a conductivity lower than 0.05 μS/cm was obtained using a Milli-Q water purification system (Millipore, Molsheim, France); acetonitrile (Merck, Darmstadt, Germany) was LC grade. Trifluoroacetic acid (TFA), phosphoric acid (85%), sodium hydroxide, hydrochloric acid (25%), formic acid (98%), acetic acid (glacial), acetone, potassium bromide and potassium hydrogen phthalate were supplied by Merck. Tris [tris(hydroxymethyl)-aminomethane] was purchased from J.T. Baker (Deventer, The Netherlands). All reagents were analytical grade. The peptides used in this study were purchased from Sigma (St. Louis, MO, USA) and are: citrulline, Gly–Gly, Gly–Gly–Gly, Tyr–Gly–Gly, Gly–Gly–Val, Gly–Gly–Ile, Ala–Leu–Gly and Gly–Gly–Phe. In the case of Ala–Leu–Gly, it was possible to separate two diastereoisomer mixtures named Ala–Leu–Gly (1) and Ala–Leu–Gly (2). Citrulline, Gly–Gly and Gly–Gly–Gly were kept at room temperature and the remainder were stored in a freezer at 0°C when not in use.

2.1.1. Chromatographic analysis

Mobile phases of 7% (v/v) of acetonitrile containing 0.05% TFA were prepared varying the pH of the mobile phase from 2 to 6.5 with 1 *M* NaOH. Stock solutions of the peptides were prepared by dissolving approximately 10 mg of each peptide and diluting to 5 ml; working solutions were prepared by 10-fold dilution of the stock solution. The mixtures of the peptides studied was prepared by 100-fold dilution of the stock solution in the mobile phase.

2.1.2. Electrophoretic analysis

A buffer containing 50 m*M* acetic acid–50 m*M* formic acid was prepared to cover the acidic pH range (2.5–4.5 approximately), adjusting to the appropriate pH with 1 *M* NaOH. For the determination of the electrophoretic mobility of the fully protonated peptides a 20 m*M* phosphate buffer was used [pH 2.11 and pH 2.20 in water and in 12.5% (v/v) MeCN, respectively]. A 50 m*M* Tris solution was the operating buffer in the basic pH range (7–9.5 approximately), adjusting to the appropriate pH with 1 *M* HCl. When working in the 12.5% (v/v) MeCN medium, the background solvent was prepared by mixing water and MeCN in the appropriate amounts to obtain a stock solvent solution. All the buffers and solutions used in the 12.5% (v/v) MeCN medium study were prepared using this stock solvent. 150 μ*M* peptide solutions were separately prepared in water, containing acetone at 3% (v/v) as the electroosmotic flow (EOF) marker [15]. A mixture containing all the peptides at 150 μ*M* was prepared.

All the eluents and mobile phases used for the chromatographic and electrophoretic methods were passed through a 0.22-μm nylon filter (MSI, Westboro, MA, USA) and degassed by sonication. The samples were passed through a 0.45-μm nylon filter (MSI).

2.2. Instrumental

2.2.1. Chromatographic apparatus

The chromatographic equipment consisted of an ISCO Model 2350 (Lincoln, NE, USA) pump with a 10-μl injection valve and a variable-wavelength V^4 absorbance detector (ISCO) operating at 214 nm. The chromatographic system was controlled by

ChemResearch Chromatographic Data Management System Controller software (ISCO) running on a personal computer. A Merck LiChrospher 100 RP-18 (5 μm) column 250×4 mm I.D. was used at room temperature.

2.2.2. Electrophoretic apparatus

A Beckman P/ACE system 5000 (Beckman Instruments, Fullerton, CA, USA) was used in all the electrophoretic experiments. A 57 cm×75 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was inserted in a capillary cartridge, thermostated to 25°C (±0.1°C). Samples were injected hydrodynamically at 0.5 p.s.i. for 4 s (1 p.s.i.=6894.76 Pa). Experiments were conducted under normal polarity, applying a voltage of 25 kV during electrophoretic separations. The detection window was placed at 50 cm from the inlet of the capillary. A photodiode array detector allows monitoring studied peptides at 195 nm. All data were recorded and analyzed by a computer program supplied by Beckman (P/ACE Station 1.0 with Golden System interface).

2.2.3. Capillary treatment

All capillary rinses were performed at high pressure (20 p.s.i.). New capillaries were activated flushing them during 20 min with aqueous 1 *M* NaOH, followed by 15 min with water and 30 min with working buffer. Between days or after a change of buffer, capillary was conditioned rinsing successively for 5 min with water, 5 min with aqueous 0.1 *M* NaOH, 15 min with water and 30 min with buffer. Both activation and conditioning methods, include, after the last flush with buffer, 15 min of 25 kV application. It was empirically demonstrated that this final step accelerates capillary equilibration. Between runs, the capillary is successively rinsed with 2 min of aqueous 0.1 *M* NaOH, 3 min of water and 5 min of buffer, in order to reequilibrate it and thereby minimize hysteresis effects. The capillary was stored overnight filled with working buffer electrolyte.

2.2.4. pH measurements

The electromotive force (e.m.f.) values used to evaluate the pH of the mobile phase were measured with a potentiometer (±0.1 mV) Model 2002 (Crison Instruments, Barcelona, Spain) using an Orion 8102 ROSS combination pH electrode (Orion Research, Boston, MA, USA). All solutions were thermostated externally at 25±0.1°C. The electrodes were stabilized in the appropriate acetonitrile–water mixtures before the e.m.f. measurements, which were performed in triplicate to ensure potentiometric system stability.

To calibrate the potentiometric system in hydro–organic media, solutions of reference standards in these media were used. The pH value of these reference solutions, pH_S was previously assigned [23,24], in accordance with IUPAC rules [28,29] and on the basis of the NIST multiprimary standard scale [26].

2.3. Experimental procedures

2.3.1. Chromatographic procedure

In order to study the influence of the eluent pH on the chromatographic separation, the mobile phase was adjusted to different pH values, from 2 to 6.5, with sodium hydroxide at 7% (v/v) of MeCN. This percentage was chosen because it corresponds to the optimum composition of the mobile phase for the separation of the studied peptides. The mobile phase optimization was performed previously [42] using the solvatochromic parameter E_T^N as solvent descriptor and taking into account that log k values and E_T^N solvent parameter correlate linearly [42].

Retention factors were calculated from $k=(t_R-t_0)/t_0$, where t_0 is the retention time of the potassium bromide (hold-up time) which is established for each mobile phase composition and pH studied, and t_R is the retention time of peptides. The flow-rate of the mobile phase was maintained at 1 ml/min. Each retention time was obtained as the average of three replicates and the corresponding relative standard deviations are lower than 2%.

2.3.2. Electrophoretic procedure

In order to study the influence of the eluent pH on the electrophoretic separation, the mobile phase was adjusted to different pH values from 2 to 9.5 in water and in aqueous–MeCN (87.5:12.5, v/v) mixtures. Individual solutions of peptides were injected at each pH until the electrophoretic mobility was constant. m_e values were calculated as the difference between the apparent mobility of each peptide and the

mobility of acetone used as neutral marker [15]. Each electrophoretic mobility was obtained as the average of three replicates. Relative standard deviations lower than 2% for the m_e values were obtained.

2.3.3. pH measurement procedure

The pH was measured in the mixed mobile phase, where the chromatographic and electrophoretic separation takes place, taking into account the reference pH values of primary standard buffer solutions, pH_S, for the standardization of potentiometric sensors in acetonitrile–water mixtures. The knowledge of pH_S values allows one to perform pH measurements in a mixed solvent as easily as in water taking into account the operational definition of pH [21,28]:

$$pH_X = pH_S + \frac{E_S - E_X}{k_g} \quad (1)$$

where E_X and E_S denote the e.m.f. measurements on the sample solution at unknown pH_X and on the standard primary reference solution at known pH_S, respectively, and $k_g = (\ln 10)RT/F$. In this study we used potassium hydrogenphthalate (0.05 mol/kg) or phosphate (0.03043 mol/kg disodium hydrogenphosphate, 0.008695 mol/kg potassium dihydrogenphosphate) as primary standard buffer reference solution in the acetonitrile–water mixtures studied [27].

The molar activity coefficient, y, was calculated using the classical Debye–Hückel expression:

$$\log y = \frac{-AI^{1/2}}{1 + a_0 BI^{1/2}} \quad (2)$$

where A and B are the Debye–Hückel constants and a_0 is the ion size parameter in the solvent mixture [29,40]. The ionic strength, I, of the mobile phases used can be easily calculated over the entire range of pH explored [37].

3. Results and discussion

The retention factor values, k, in MeCN–water (7:93, v/v) mixtures, for the series of peptides studied at every mobile phase pH considered, are shown in Fig. 1 [42]. The peptides studied here usually have two relevant functional groups. pK_a values in the acid range can be associated with

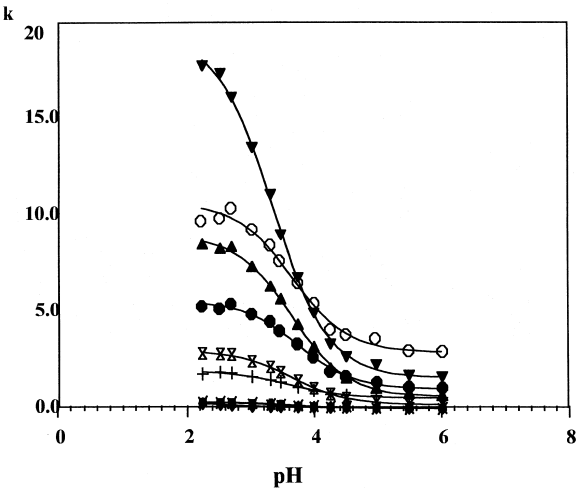

Fig. 1. Plots of retention factors, k, versus pH for Gly–Gly–Phe (▼), Ala–Leu–Gly (2) (○), Gly–Gly–Ile (▲), Ala–Leu–Gly (1) (●), Gly–Gly–Val (×), Tyr–Gly–Gly (+), Gly–Gly–Gly (x), Gly–Gly (■), citrulline (♦) in MeCN–water (7:93, v/v). Symbols stand for the experimental data and solid lines indicate the best non-linear regression fits.

carboxylic acid function and pK_a values in the basic range can be assigned to the protonated amino groups dissociation [43]. Thus, peptides can be considered as typical zwitterion forming compounds. However, the applied octadecylsilica (ODS) stationary phase may only be used in the pH range 2–7, so it was no possible to study the retention of peptides as typical ampholytes, because correlation between k values and the pH of the mobile phase cannot be obtained over the entire range of pH. Thus, from a chromatographic point of view, with the widely used ODS stationary bonded phase, only the protolytic equilibria corresponding to pK_a values in the acid range are relevant. In this way, the pK_a value, corresponding to the dissociation of the terminal carboxylic group has been considered. The retention of peptides is high at low pH values, Fig. 1, where the peptide exists as a single charged cation. When pH increases, the k value decreases and levels off at isoelectric point pH and stays constant, Fig. 1.

Although the effect of solute ionization on retention is known, the theoretical interpretation of this phenomenon is hampered by the lack of a rigorous treatment of protolytic equilibria in hydro–organic mixtures. In doing this, pK_a values, the pH, the ionic

strength, and the mean ionic activity coefficient, must be determined at each mobile phase composition studied after mixing the aqueous solutions with the organic modifier.

An equation which describes retention factor as a function of pH of the mobile phase, considering the activity coefficient, and accounts for every peptide equilibria that influences the retention in ODS columns, can be derived [35] taking into account that the ionization of peptides in the mobile phase takes place according to the following equilibrium:

$$H_2Z^+ \Leftrightarrow HZ + H^+ \quad K_{a1}$$

where H_2Z^+ is the protonated form and HZ the zwitterionic form of peptides.

Equations that relate the retention of a compound in LC columns with the pH of the eluent, consider that the observed retention factor, *k*, is a weighted average of the *k* of the ionic and neutral forms of the solute [20,35,37], according to the molar fractions of these forms in the mobile phase. The overall observed, *k*, for peptides can be given as:

$$k = x_{H_2Z^+} k_{H_2Z^+} + x_{HZ} k_{HZ} \tag{3}$$

where $k_{H_2Z^+}$ and k_{HZ} are the retention factors and $x_{H_2Z^+}$ and x_{HZ} the molar fraction of the protonated and zwitterionic forms of peptides, respectively, that can be written as:

$$x^+_{H_2Z} = \frac{a_{H^+}}{a_{H^+} + K_{a1} y} \tag{4}$$

$$x_{HZ} = \frac{K_{a1} y}{a_{H^+} + K_{a1} y} \tag{5}$$

By replacing Eqs. (4) and (5) in Eq. (3):

$$k = \frac{k^+_{H_2Z} a_{H^+} + k_{HZ} K_{a1} y}{a_{H^+} + K_{a1} y} \tag{6}$$

or

$$k = \frac{k_{H_2Z^+} \dfrac{a_{H^+}}{K_{a1} y} + k_{HZ}}{\dfrac{a_{H^+}}{K_{a1} y} + 1} \tag{7}$$

The pK_a values of the substances studied were determined from the experimental *k* values, Fig. 1, the pH data and the calculated *y* values, by a non-linear least-squares fit of the data to Eq. (7) and are shown in Table 1. The activity coefficient, *y*, is obtained according to IUPAC rules [28,29] from the Debye–Hückel equation. In Fig. 1, symbols stand for the experimental data and solid lines indicate the best non-linear regression fits for each peptide, using Eq. (7). The agreement between both values is good, with correlation coefficients of 0.999.

In order to study the influence of pH on the electrophoretic behaviour of substances, we can relate pH, pK_a and electrophoretic mobility. The established relationships are based on the principle that a solute has its maximum electrophoretic mobility when it is fully ionized, has no electrophoretic mobility in its neutral form, and has an intermediate mobility in the pH region surrounding its pK_a [15,38,41]. Thus, the electrophoretic mobility, m_e, of a substance is a function of the mobility and the molar fraction, x_i, of its species. In the case of the studied peptide substances, the first dissociation constant is related to the concentration of protonated $(H_2Z)^+$ and undissociated (HZ) species [37,44]. Thus, effective mobility can be expressed as follows:

$$m_e = x_{H_2Z^+} m_{H_2Z^+} + x_{HZ} m_{HZ} \tag{8}$$

where the term corresponding to the HZ species is considered nil, because the HZ species has no charge and migrates with the electroosmotic flow. Assuming that the mobility of the fully protonated species is $m_{H_2Z^+} = m_a$ and replacing the molar fraction by its expression for ampholytes, the electrophoretic mobility, m_e, can therefore be obtained:

$$m_e = \frac{a_{H^+} m_a}{a_{H^+} + K_{a1} y} \tag{9}$$

Expressions similar to Eq. (9) can be deduced in basic media [44], that permit electrophoretic determination of the second dissociation constant of the studied peptides. Thus, considering that the electrophoretic mobility is maximum for Z^- species, the expression for mobility in the pH region surrounding the pK_{a2} is $m_e = x_{Z^-} m_b$, where $m_{Z^-} = m_b$ is the electrophoretic mobility of the fully deprotonated species, Z^-, whose sign is opposite to that of the mobility of the fully protonated form, H_2Z^+. Replac-

Table 1
pK_a values determined from potentiometric, chromatographic and electrophoretic method for the peptides studied in water and hydro–organic mixtures

Peptide	pK_a	0% MeCN		7% MeCN		12.5% MeCN	
		CE method	Bibliography	LC method	Potentiometric method	CE method	Potentiometric method
Gly–Gly	1	3.08 (0.04)	3.14	3.35 (0.06)	3.18 (0.01)	3.33 (0.02)	3.33
	2		8.04			8.32 (0.02)	8.30
Gly–Gly–Ile	1	3.26 (0.07)		3.59 (0.02)	3.54 (0.03)	3.53 (0.02)	3.55
	2		8.09			8.11 (0.01)	8.09
Gly–Gly–Gly	1	3.22 (0.02)	3.30	3.57 (0.06)	3.50 (0.02)	3.41 (0.02)	3.46
	2		7.96			8.08 (0.01)	8.09
Tyr–Gly–Gly	1	3.18 (0.02)		3.54 (0.05)	3.45 (0.01)	3.36 (0.02)	3.46
	2					7.41 (0.04)	7.45
Gly–Gly–Val	1	3.23 (0.02)		3.59 (0.02)	3.47 (0.02)	3.50 (0.02)	3.54
	2		8.12			8.15 (0.02)	8.08
Ala–Leu–Gly 1	1	3.34 (0.01)		3.71 (0.04)	3.57 (0.02)	3.56 (0.03)	3.62
	2					8.13 (0.02)	8.12
Ala–Leu–Gly 2	1	3.27 (0.01)		3.61 (0.05)	3.57 (0.02)	3.46 (0.03)	3.62
	2					8.30 (0.02)	8.12
Gly–Gly–Phe	1	3.06 (0.01)		3.30 (0.02)	3.21 (0.01)	3.33 (0.05)	3.33
	2		8.04			8.01 (0.02)	8.02
Citrulline	1	2.30 (0.04)	2.40	2.37 (0.11)	2.57 (0.01)	2.60 (0.06)	2.39
	2		9.69			9.44 (0.03)	9.58

ing x_{Z^-} by its expression for ampholytes, the following equation for mobility in basic media is obtained:

$$m_e = \frac{K_{a2} m_b}{K_{a2} + a_{H^+} y} \quad (10)$$

The electrophoretic migration behaviour of these peptides can be described by Eqs. (9) and (10), which at a given pH, relate electrophoretic mobilities to the corresponding mobilities of the fully protonated or deprotonated species, m_a or m_b, respectively, and the dissociation constants, pK_{a1} or pK_{a2}, taking into account the activity coefficients effect. The electrophoretic mobilities for all the studied peptides obtained in water and in MeCN–water (12.5:87.5, v/v) mixtures were plotted against the buffer pH in Fig. 2A and B, respectively. Symbols stand for the experimental data and solid lines indicate the best non-linear regression fits for each peptide, using Eqs. (9) and (10). The agreement between both values is good, with correlation coefficients of 0.999. From this regression analysis, dissociation constants for each peptide were calculated in addition to m_a and m_b values.

In this way, models described by Eqs. (7), (9) and (10), that were established using data pairs pH–k or pH–m_e, respectively, and ionic strength over the whole pH range in water and MeCN–water mixtures, allow pK_a determinations. pK_a values determined for the studied peptides using the non-linear regression method, Eqs. (7), (9) and (10), are shown in Table 1, with their respective standard deviations. Moreover, Table 1 summarizes the pK_a values previously determined by potentiometric techniques [43].

In general, the values obtained by LC methodologies at 7% (v/v) MeCN are slightly higher than the ones determined by potentiometric techniques. This fact can be explained taking into account the non-

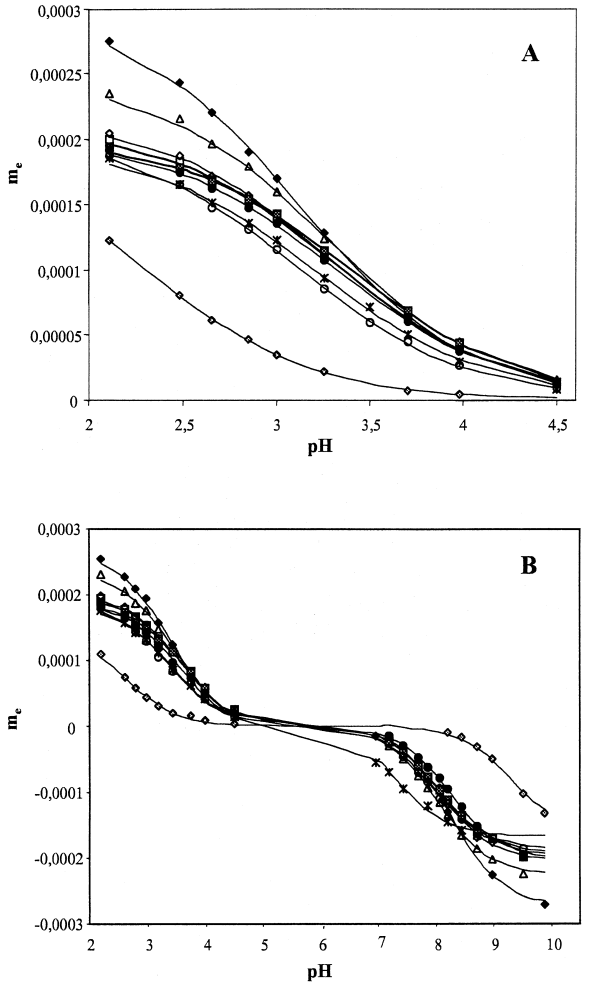

Fig. 2. Plots of electrophoretic mobilities, m_e, versus pH for Gly–Gly (♦), Gly–Gly–Gly (△), Gly–Gly–Val (◇), Gly–Gly–Ile (□), Ala–Leu–Gly (1) (▩), Ala–Leu–Gly (2) (●), Gly–Gly–Phe (○), Tyr–Gly–Gly (✱) and citrulline (◈). Symbols stand for the experimental data and solid lines indicate the best non-linear regression fits.

hydrophobic interactions of the compound studied with the residual silanol groups that remain intact after chemical modification of the silica surface in the used column. These interactions increase the retention factor of peptides and, therefore, the obtained pK_a values are higher than potentiometric ones.

In contrast, values obtained in water by CE are similar to potentiometric values. Only slight differences in some peptides can be observed. These differences could also be explained by the presence of residual silanols groups in the inner surface of the capillary. The specific interaction between positively charged peptides and the fused-silica capillary wall diminishes the electrophoretic mobility of peptides, resulting in slightly lower pK_a values than the potentiometric ones. Citrulline has an exceptional behaviour due to its stronger acid character.

pK_{a2} values determined by CE in 12.5% MeCN media, are very similar to potentiometric ones. In basic media, amino group of peptides becomes deprotonated diminishing interactions with the capillary wall. Differences between electrophoretic and potentiometric values are positive as well as negative, therefore, they are probably reflective of the random error of the procedure, not of specific interactions.

Finally, the slightly differences observed between pK values obtained from LC, CE and potentiometric methods, demonstrate the suitability of these techniques for pK_a determination. In general, as can be seen in Table 1, the acid dissociation constants for deprotonation of carboxylic acid and protonated amino groups decrease as the solvent become enriched in the organic component. These variations of pK_a values are lower than expected because of the preferential solvation of water in MeCN–water mixtures [45,46] and have been interpreted in previous works.

Moreover, note that pK_a of both Ala–Leu–Gly diastereoisomers can be differentiated by LC and CE. This makes both techniques specially attractive for pK_a determinations of diastereoisomers, because potentiometry is not able to differentiate between them [43].

On the other hand, Eq. (7) can be written in a linearized form:

$$\boxed{k\left(\frac{a_{H^+}}{K_{a1}y}+1\right)} = k_{H_2Z^+}\ \boxed{\frac{a_{H^+}}{K_{a1}y}} + k_{HZ} \qquad (11)$$

When pK_a values of substance are known, plots of the terms in the boxes can be used in order to optimize the pH of the mobile phase. Fig. 3 shows

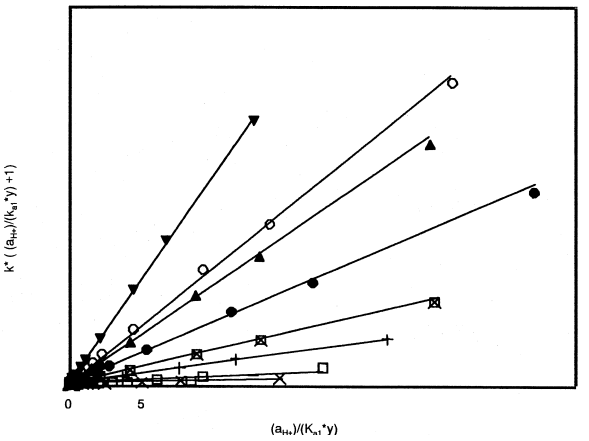

Fig. 3. Plots of experimental (points) and predicted with linear models (lines) $k[(a_{H^+})/(K_{a1}y)+1]$ versus $(a_{H^+})/(K_{a1}y)$ in MeCN–water (7:93, v/v). Symbols: (▼) Gly–Gly–Phe, (○) Ala–Leu–Gly (2), (▲) Gly–Gly–Ile, (●) Ala–Leu–Gly (1), (×) Gly–Gly–Val, (+) Tyr–Gly–Gly, (□) Gly–Gly–Gly, (x) Gly–Gly and (♦) citrulline.

these plots for the peptides studied. From these it is also possible to obtain k values of the species of the peptides from the intercept and slope. In accordance with Eq. (11), all present good linearities. Once the linearity has been verified only two experimental measurements of k for each compound considered at two different pH values are enough for predicting k values at any pH of the mobile phase, and then, for optimizing the chromatographic separation.

In the same way, Eqs. (9) and 10 obtained by the electrophoretic method, also can be written in a linearized form, respectively:

$$\boxed{\frac{1}{m_e}} = \frac{1}{m_a} + \frac{1}{m_a} \cdot \boxed{\frac{K_{a1}y}{a_{H^+}}} \tag{12}$$

and

$$\boxed{\frac{1}{m_e}} = \frac{1}{m_b} + \boxed{\frac{a_{H^+}y}{K_{a2}}} \cdot \frac{1}{m_b} \tag{13}$$

When pK_a values of substance are known, plots of the boxed terms can be used, in order to optimize the pH of the electrolyte solutions. Fig. 4 shows these

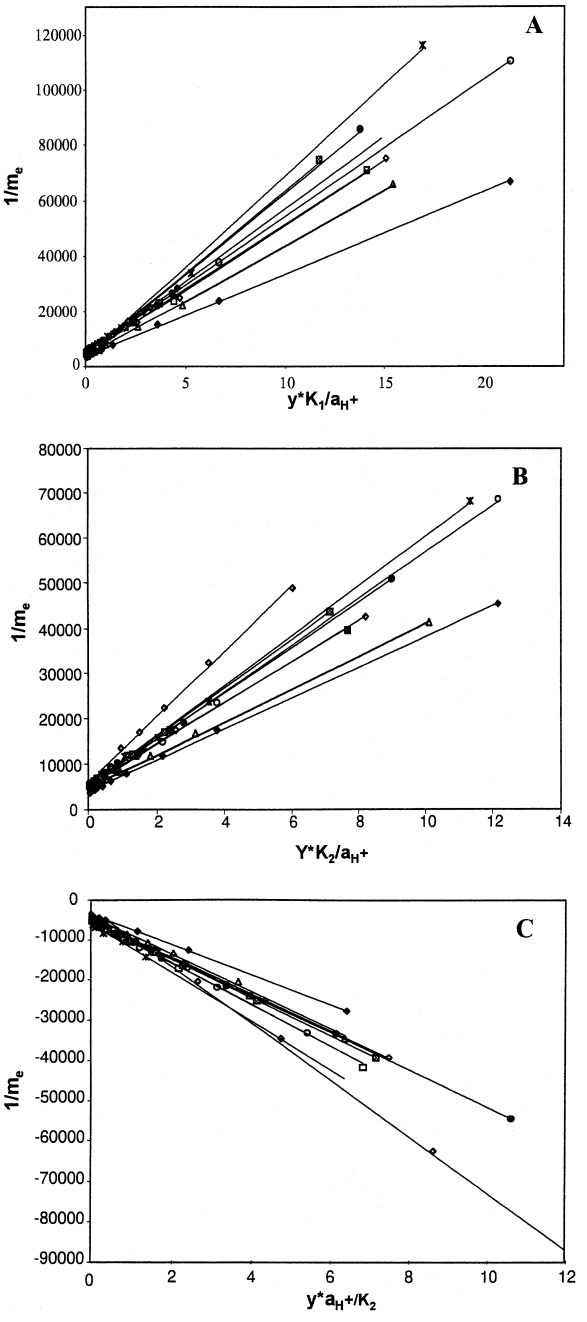

Fig. 4. Plots of experimental (points) and predicted with linear models (lines)$1/m_e$ values versus (A) $K_{a1}y/a_H^+$ in water, (B) $K_{a1}y/a_H^+$ in MeCN–water (12.5:87.5, v/v) and (C) $K_{a1}y/a_H^+$ in MeCN–water (12.5:87.5, v/v) for Gly–Gly (♦), Gly–Gly–Gly (△), Gly–Gly–Val (◇), Gly–Gly–Ile (□), Ala–Leu–Gly 1 (▩), Ala–Leu–Gly 2 (●), Gly–Gly–Phe (○), Tyr–Gly–Gly (✱), citrulline (◈).

plots for each peptide in water and in 12.5% (v/v) MeCN in acidic (Fig. 4A and B) and basic conditions (Fig. 4C). In accordance with Eqs. (12) and (13), all present good linearities. As in the LC analysis, once the linearity has been verified, only two experimental measurements of m_e–pH for each compound are sufficient for predicting their electrophoretic behaviour and for optimizing their separation.

The k and m_e predicted values have been then used to calculate selectivity and resolution for solute adjacent pairs in the usual way [37,38]. In Figs. 5 and 6 the lines indicate theoretical values obtained from two measurements by compound using Eqs. (11), (12) and (13). The points of Fig. 5 are α values obtained from experimental data. Thus it appears clear that two measurements of retention by compound are sufficient to predict accurately the optimum pH of the mobile phase if pK_a values are known. From Figs. 5 and 6, optimal separation conditions can be obtained Fig. 5 indicates that good chromatographic separation can be obtained for the peptides considered in the series of peptides studied

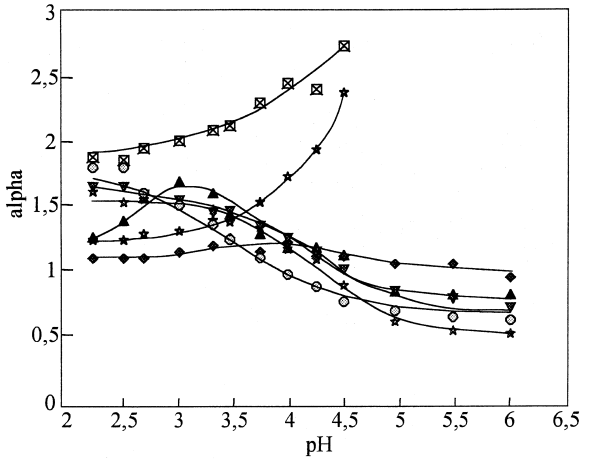

Fig. 5. Plots of experimental (points) and predicted with non-linear LC model (lines) selectivities for a mixture of the studied peptides versus pH, in MeCN–water (7:93, v/v). (⊠) Gly–Gly–Gly/Gly–Gly, (♦) Gly–Gly–Val/Tyr–Gly–Gly, (✱) Ala–Leu–Gly 1/Gly–Gly–Val, (▼) Gly–Gly–Ile/Ala–Leu–Gly 1, (★) Ala–Leu–Gly 2/Ala–Leu–Gly 1 and (○) Gly–Gly–Phe/Ala–Leu–Gly 2.

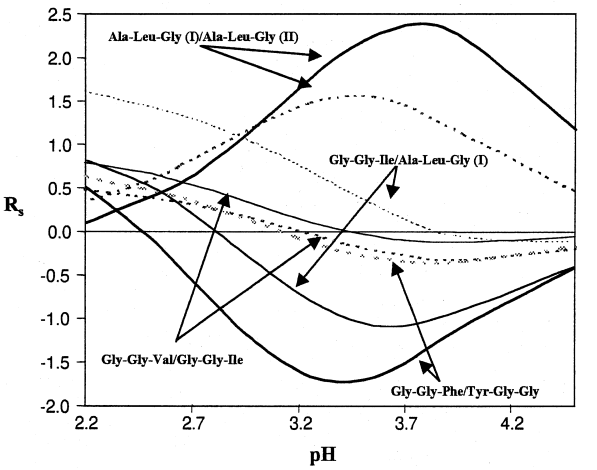

Fig. 6. Plots of predicted resolutions for some pairs of peptides versus pH, in water (solid lines) and in MeCN–water (12.5:87.5, v/v) (dashed lines).

in acetonitrile–water (7:93, v/v) mixtures [41] and at a pH value of the hydro–organic mixtures between 2.5 and 3. Fig. 7A shows a chromatogram of the separation of the nine substances studied at pH 2.8

According to Fig. 6 optimum electrophoretic separation of all peptides presents in the mixture are achieved in the pH range between 2.5 and 2.7 in the aqueous and aqueous–organic media studied. In general, in electrophoretic separations MeCN addition results in a loss of resolution. Fig. 7B and C show electropherograms of the separation of the nine substances studied at pH 2.6 in water and in acetonitrile–water (12.5:87.5, v/v) mixtures.

The analysis time obtained for two methodologies is similar and around 25 min. Likewise, the optimum pH for the best separation of the series of peptide compounds studied is similar (pH 2.8 in the chromatographic separation and pH 2.6 in the electrophoretic separation) by both techniques. The selectivities differ, as was expected, because the retention behaviour is based on different mechanisms and in consequence the elution order for the studied compounds is not the same. In this way, citrulline was the first substance eluted by LC and the last substance eluted by CE.

In conclusion, the suitability of the chromato-

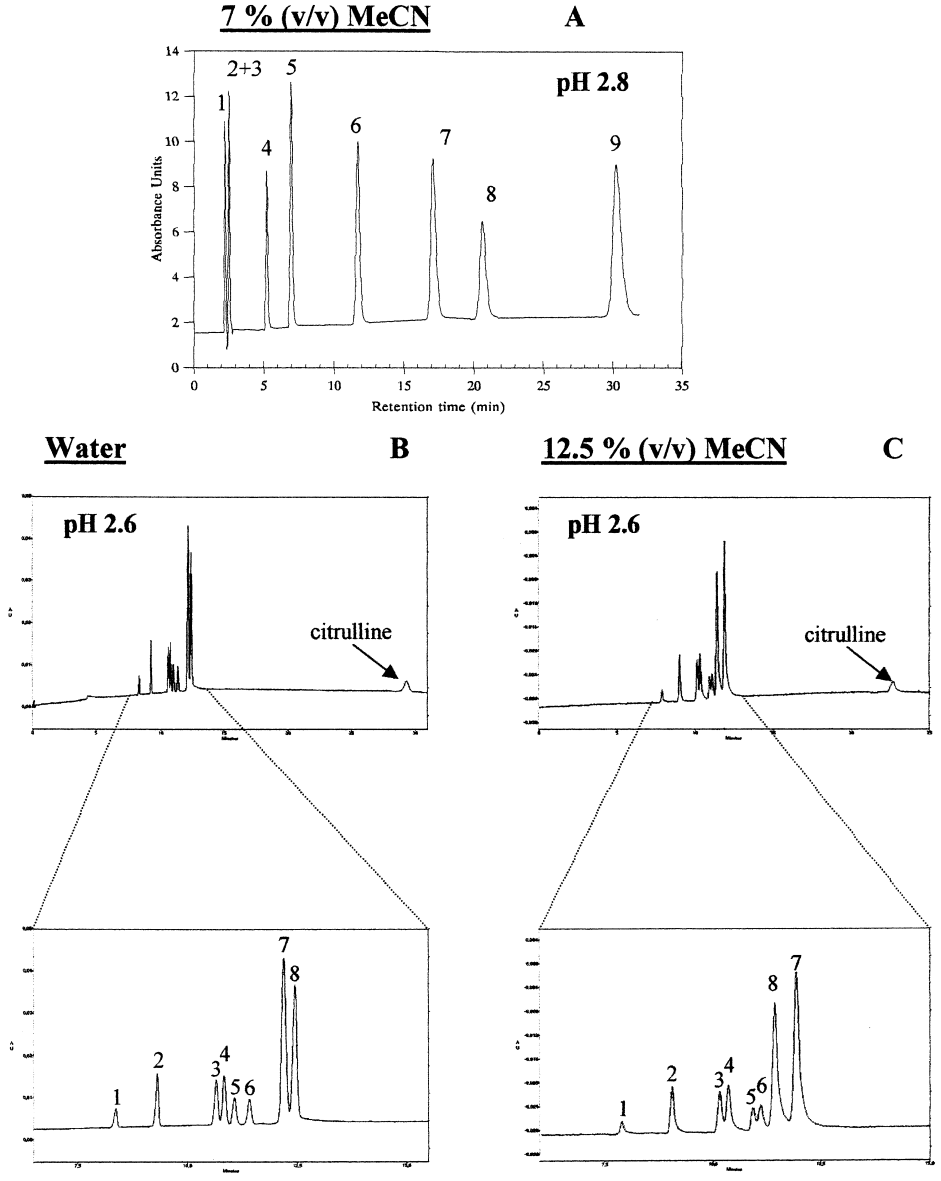

Fig. 7. Chromatogram of a mixture of the studied peptides with a mobile phase of 0.05% TFA, (A) MeCN–water (7:93, v/v). Electropherograms at 25 kV of a mixture of the studied peptides in 50 m*M* formic acid–50 m*M* acetic acid buffer (B), water (C) MeCN–water (12.5:87.5, v/v). (1) Citrulline, (2) Gly–Gly, (3) Gly–Gly–Gly, (4) Gly–Gly–Val, (5) Tyr–Gly–Gly, (6) Ala–Leu–Gly 1, (7) Gly–Gly–Ile, (8) Ala–Leu–Gly 2 and (9) Gly–Gly–Phe.

graphic and electrophoretic studied models to the determination of dissociation constants and to predict chromatographic and electrophoretic behaviour of peptides from a limited number of experimental data has been shown. In a parallel way, separation between solutes in a complex mixture can be easily

predicted, making a simple and rapid pH selection to achieve optimum separations.

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