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Evaluation of chromatographic versus electrophoretic behaviour of a series of therapeutical peptide hormones

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Abstract

In this work, models describing the effect of pH on chromatographic and electrophoretic behaviour for a series of polyprotic therapeutic peptide hormones were compared, taking into account the species in solution and the activity coefficients. The usefulness of the proposed equations is twofold, they permit the determination of the acidity constants in water and in the hydroorganic mobile phases used in liquid chromatography (LC) and capillary electrophoresis (CE) and can also be used for the selection of the optimum pH for the separation of mixtures of the modelled compounds. The proposed relationships allow an important reduction of the experimental data needed for the development of new separation methods. The accuracy of the proposed equations is verified by modelling the chromatographic and electrophoretic behaviour of a series of polyprotic therapeutic peptide hormones. By calculating the values of predicted resolutions, selection of the optimum pH to perform LC or CE separations of their mixtures becomes a rapid and simple process.

Keywords: Optimization; Retention prediction; Migration prediction; Ionization constants; Peptides; Hormones

1. Introduction

The recent increased use of native and synthetic peptide hormones and proteomimetics in biomedical therapy is a result of their large range of activity and specificity, usually with low toxicity and rapid metabolization [1]. For the near future, proteomic research is expected to play a major role in discovery of new peptide-based drugs, as new disease biomarkers, molecular targets for therapy and end points for therapeutic efficacy and toxicity are being described [2]. Thus, separation and analysis of peptides and peptide hormones has become increasingly important for an ever-widening range of research disciplines. Development of highly efficient and selective separation methods is necessary before overcoming the determination and characterization of peptide hormones in complex matrices. At present, liquid chromatography (LC) and capillary electrophoresis (CE) are considered orthogonal and the most useful techniques for rapid and efficient separation of a wide variety of peptides and proteins [3–5]. Furthermore, their coupling with mass spectrometry has become a powerful tool for the systematic separation, determination and characterisation of peptides and proteins in complex matrices [5–7].

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The development of rapid, efficient and selective separation methodologies requires a previous optimization of the separation conditions. Although a desired peptide separation 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. Modelling chromatographic and electrophoretic behaviour of substances can be used to predict separations using a few experimental data [8–18]. Furthermore, migration models can be used to perform physicochemical and conformational characterization of biomolecules [12,16–25].

Several semiempirical approaches have been proposed to relate chromatographic retention or electrophoretic mobility with structural parameters of peptides and proteins [26–29]. Nevertheless, their application is limited by the assumptions made in their development and there is no general rule to a priori select the appropriate relationship for each type of compound [27]. Likewise, in the separation of ionizable compounds, pH plays an important role as it determines the extent of ionization of each individual solute [8,10,30,31]. Accurate quantitative relationships between chromatographic retention or electrophoretic mobilities and pH can be very useful [8,20,32]. These models allow determination of ionization constants [16–25] and simultaneous selection of the optimum pH for the separation of complex mixtures of the modelled compounds, by resolution calculations [12,13,15–18,27,33].

In this work, general equations relating chromatographic retention or electrophoretic mobility with pH, pK_a and activity coefficients are presented for polyprotic peptide substances. Validation of the studied model is performed for a series of polyprotic amphoteric peptide hormones with therapeutic interest. The usefulness of the proposed equations is twofold. They permit the determination of the acidity constants and can also be used to establish a general model that relates the elution behaviour of the solute with pH and ionic strength. Selectivity and resolution can be easily predicted, and hence optimum separation conditions for the mixture are systematically selected on the basis of a few experimental data.

2. Experimental

2.1. Chemicals and reagents

All chemicals used in the preparation of buffers and solutions were analytical reagent grade. Acetonitrile, trifluoroacetic acid (TFA), potassium bromide, potassium hydrogenphthalate, phosphoric acid (85%), sodium hydroxide, hydrogen chloride (25%), formic acid (98%), acetic acid (glacial), diethylmalonic acid, boric acid and acetone were supplied by Merck (Darmstadt, Germany). Tris [Tris(hydroxymethyl)aminomethane] was purchased from J.T. Baker (Deventer, The Netherlands). Water with a conductivity lower than 0.05 mS cm^{-1} was obtained using a Milli-Q water purification system (Millipore, Molsheim, France). The selected peptide hormones studied were: oxytocin, bradykinin, Leu-enkephalin, Met-enkephalin, triptorelin, buserelin and eledoisin. The peptide hormones were purchased from Sigma (St. Louis, MO, USA), except buserelin which was purchased from Hoechst Ibérica (Barcelona, Spain) and triptorelin which was purchased from Lasa (Barcelona, Spain). They were stored in a freezer at −4 °C when not in use. The structures of the selected substances are shown in Table 1.

2.1.1. LC analysis

Stock solutions of peptide hormones were prepared by dissolving ~5 mg of each substance in 5 ml of water; working solutions were prepared by 10-fold dilution of the stock solution. The mixture of the studied peptide hormones was prepared daily by 100-fold dilution of the stock solution. The solvent used as mobile phase was MeCN–water (35:65), 0.1% (v/v) TFA. This percentage of organic modifier in the mobile phase was previously optimized using linear solvation energy relationships [34]. All the eluents and mobile phases 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.

2.1.2. CE analysis

Electrolyte solutions covering the pH range 2–12 were prepared at the following concentrations and adjusted with 1 *M* NaOH or 1 *M* HCl: 20 m*M*

Table 1
Structures of the studied peptide hormones. Polyprotic formulation[a]

Peptide	Structure	Ionisable groups		Formula	
		Acidic (A)	Basic (B)	$H_aAB_z^z$	H_nX^z
Oxytocin	**Cys**–**Tyr**–Ile–Gln–Asn–Cys–Pro–Leu–Gly–NH_2	1 Phenol (–Tyr–)	1 Amino group (Cys–)	$HABH^+$	H_2X^+
Eledoisin	Pyr–Pro–Ser–**Lys**–**Asp**–Ala–Phe–Ile–Gly–Leu–Met–NH_2	1 Carboxylic acid (–Asp–)	1 Amino group (–Lys–)	$HABH^+$	H_2X^+
Bradykinin	**Arg**–Pro–Pro–Gly–Phe–Ser–Pro–Phe–**Arg**	1 Carboxylic acid (–Arg)	1 Amino group (Arg–) 2 Guanidine groups (Arg–/–Arg)	$HABH_3^{3+}$	H_4X^{3+}
Met-enkephalin	**Tyr**–Gly–Gly–Phe–**Met**	1 Carboxylic acid (–Met) 1 Phenol (Tyr–)	1 Amino group (Tyr–)	H_2ABH^+	H_3X^+
Leu-enkephalin	**Tyr**–Gly–Gly–Phe–**Leu**	1 Carboxylic acid (–Leu) 1 Phenol (Tyr–)	1 Amino group (Tyr–)	H_2ABH^+	H_3X^+
Triptorelin	Pyr–Pro–**His**–Trp–Ser–**Tyr**–D–Trp–Leu–**Arg**–Pro–Gly–NH_2	1 Phenol (–Tyr–)	1 Imidazol group (–His–) 1 Guanidine group (Arg)	$HABH_2^{2+}$	H_3X^{2+}
Buserelin	Pyr–Pro–**His**–Trp–Ser–**Tyr**–D–Ser (^{t}Bu)–Leu–**Arg**–Pro–NHC_2H_5	1 phenol (–Tyr–)	1 Imidazol group (–His–) 1 Guanidine group (Arg)	$HABH_2^{2+}$	H_3X^{2+}

[a] Ionizable groups are marked with bold letters.

H_3PO_4 (pH 2), 50 m*M* acetic acid–50 m*M* formic acid (pH 2.5–5), 20 m*M* diethylmalonic acid (pH 5.5–6.5), 50 m*M* Tris (pH 7–9), 50 m*M* H_3BO_3 (pH 9.5–10.5) and 10 m*M* H_3PO_4 (pH 11–12). Solutions (250 ppm) of each peptide hormone were prepared in water, containing acetone at 3% (v/v) as the electroosmotic flow (EOF) marker. A mixture containing all the peptides at 250 ppm was prepared. Samples and running electrolytes were passed through a 0.45 μm nylon filter (MSI).

2.2. Instruments

The chromatographic equipment consisted of an ISCO Model 2350 (Lincoln, NE, USA) pump with an injection valve with a 10 μl sample loop 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 Peceman AT Supermicro personal computer. A Merck LiChrospher 100 RP-18 (5 mm) column 250×4 mm I.D. was used at room temperature. The electrophoretic system consisted of a Beckman P/ACE system 5000 (Beckman Instruments, Fullerton, CA, USA). 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 3 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 was used. Electropherograms shown correspond to detection 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). pH measurements were performed with a Crison 2002 potentiometer (Crison Instruments, Barcelona, Spain), equipped with a ROSS electrode 8102 (Orion Research, Boston, MA, USA).

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 7, with sodium hydroxide using the percentage of acetonitrile previously optimized [33–37]. The LiChrospher C_{18} column was equilibrated at 1 ml/min with new mobile phase conditions for 30 min. Retention factors (k) 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 each substance. The t_R of the solutes were determined from three different in-

jections of their individual solutions at every mobile phase considered. pH measurements in the mixed mobile phase, where the chromatographic separation takes place, were made taking into account the reference pH values of primary standard buffer solutions, pH_S, for the standardization of potentiometric sensors in MeCN–water mixtures. These were assigned in previous works [38,39], in accordance with IUPAC rules [40,41] and on the basis of multiprimary standard scale, according to the US National Institute of Standards and Technology (NIST) [42].

2.3.2. Electrophoretic procedure

New capillaries were activated by flushing them at 20 p.s.i. during 20 min with 1 *M* aqueous NaOH, followed by 15 min with water and 30 min with working buffer. Between days or after a change of buffer, capillary was conditioned by rinsing successively for 5 min with water, 5 min with 0.1 *M* aqueous NaOH, 10 min with water and 20 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 0.1 *M* aqueous NaOH, 2 min of water and 2 min of buffer, in order to reequilibrate it and thereby minimize hysteresis effects. Capillary was stored overnight filled with working buffer electrolyte. Electrolyte solutions indicated in the previous section were run in sequence from low to high pH. Individual solutions of each peptide hormone were injected at each pH until the electrophoretic mobility (m_e) 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 [30,31]. Each electrophoretic mobility was obtained as the average of three replicates.

2.4. Data analysis

In order to study the influence of pH upon chromatographic and electrophoretic behaviour of ionisable substances, relationships between pH, pK_a and retention factors or electrophoretic mobilities can be adequately established [33–37]. Polyprotic compounds undergo successive ionisation equilibria when the pH of the solvent is gradually changed. The dissociation equilibria for a generic fully protonated polyprotic species H_nX^z, are:

$$\begin{aligned} &H_nX^z \Leftrightarrow H_{n-1}X^{z-1} + H^+ && K_1 \\ &\vdots \\ &H_{n-(i-1)}X^{z-(i-1)} \Leftrightarrow H_{n-i}X^{z-i} + H^+ && K_i \\ &\vdots \\ &H_{n-(n-1)}X^{z-(n-1)} \Leftrightarrow X^{z-n} + H^+ && K_n \end{aligned} \quad (1)$$

where n is the total number of ionogenic groups, z the maximum net charge, given by the protonated basic groups, and K_i is the dissociation equilibrium constant of the i^{th} dissociation step, that is given by the expression:

$$K_i = \frac{\left[H_{n-i}X^{z-i}\right] y^{z-i} a_{H^+}}{\left[H_{n-(i-1)}X^{z-(i-1)}\right] y^{z-(i-1)}} \quad (2)$$

In Eq. (2), the effect of ionic strength (I) upon dissociation constants has been taken into account, considering the activity coefficients of the solutes, y. The activity coefficients are obtained, according to IUPAC rules [41], from the Debye–Hückel equation, $\log y = -z^2 A\sqrt{I}/(1 + a_0 B\sqrt{I})$, where A and B are the Debye–Hückel constants, a_0 is the ion size parameter in the solvent mixture and I is the ionic strength. In another way, apparent dissociation constants can be defined as,

$$K_i' = \frac{y^{z-(i-1)}}{y^{z-i}} \cdot K_i \quad (3)$$

The retention factor, k, of a polyprotic compound, H_nX^z, coexisting in the form of various species at a given pH, is a weighted average of the retention factors, k_i, of the individual species [43], according to the molar fraction, x_i, of these species in the mobile phase. Thus the overall observed k values for the peptide hormones can be given as:

$$k = \sum_{i=0}^{n} x_{H_{n-i}X^{z-i}} k_{H_{n-i}X^{z-i}} \quad (4)$$

In the same way, the effective electrophoretic mobility, m_e, is a function of the mobility and the molar fraction, x_i, of the individual species [18–23]:

$$m_e = \sum_{i=0}^{n} x_{H_{n-i}X^{z-i}} m_{H_{n-i}X^{z-i}} \quad (5)$$

Replacing the molar fraction by its expression the following equations can be obtained:

$$k = \sum_{i=0}^{n} \frac{\left[H_{n-i}X^{z-i}\right]}{\sum_{i=0}^{n}\left[H_{n-i}X^{z-i}\right]} \cdot k_{H_{n-i}X^{z-i}} \quad (6)$$

$$m_e = \sum_{i=0}^{n} \frac{\left[H_{n-i}X^{z-i}\right]}{\sum_{i=0}^{n}\left[H_{n-i}X^{z-i}\right]} \cdot m_{H_{n-i}X^{z-i}} \quad (7)$$

Eqs. (6) and (7) can be rewritten as a function of dissociation constants, dividing numerator and denominator by an appropriate concentration $\left[H_{n-r}X^{z-r}\right]$. Thus, the overall observed k and m_e values for the compounds considered can be given as:

$$k = \frac{\sum_{i=0}^{r-1} \frac{a_{H^+}^{r-i}}{\prod_{j=i+1}^{r} K_j'} k_{H_{n-i}X^{z-i}} + k_{H_{n-r}X^{z-r}} + \sum_{i=r+1}^{n} \frac{\prod_{j=r+1}^{i} K_j'}{a_{H^+}^{i-r}} k_{H_{n-i}X^{z-i}}}{\sum_{i=0}^{r-1} \frac{a_{H^+}^{r-i}}{\prod_{j=i+1}^{r} K_j'} + 1 + \sum_{i=r+1}^{n} \frac{\prod_{j=r+1}^{i} K_j'}{a_{H^+}^{i-r}}} \quad (8)$$

$$m_e = \frac{\sum_{i=0}^{r-1} \frac{a_{H^+}^{r-i}}{\prod_{j=i+1}^{r} K_j'} m_{H_{n-i}X^{z-i}} + m_{H_{n-r}X^{z-r}} + \sum_{i=r+1}^{n} \frac{\prod_{j=r+1}^{i} K_j'}{a_{H^+}^{i-r}} m_{H_{n-i}X^{z-i}}}{\sum_{i=0}^{r-1} \frac{a_{H^+}^{r-i}}{\prod_{j=i+1}^{r} K_j'} + 1 + \sum_{i=r+1}^{n} \frac{\prod_{j=r+1}^{i} K_j'}{a_{H^+}^{i-r}}} \quad (9)$$

Furthermore, considering that $a_{H^+}^{n} = 10^{-npH}$ and that $K_i' = 10^{-pK_i'}$, Eqs. (8) and (9) can be rearranged more conveniently as a function of pH to give

$$k = \frac{\sum_{i=0}^{r-1} 10^{\left[-(r-i)pH + \sum_{j=i+1}^{r} pK_j'\right]} k_{H_{n-i}X^{z-i}} + k_{H_{n-r}X^{z-r}} + \sum_{i=r+1}^{n} 10^{\left[(i-r)pH - \sum_{j=r+1}^{i} pK_j'\right]} k_{H_{n-i}X^{z-i}}}{\sum_{i=0}^{r-1} 10^{\left[-(r-i)pH + \sum_{j=i+1}^{r} pK_j'\right]} + 1 + \sum_{i=r+1}^{n} 10^{\left[(i-r)pH - \sum_{j=r+1}^{i} pK_j'\right]}} \quad (10)$$

$$m_e = \frac{\sum_{i=0}^{r-1} 10^{\left[-(r-i)pH + \sum_{j=i+1}^{r} pK_j'\right]} m_{H_{n-i}X^{z-i}} + m_{H_{n-r}X^{z-r}} + \sum_{i=r+1}^{n} 10^{\left[(i-r)pH - \sum_{j=r+1}^{i} pK_j'\right]} m_{H_{n-i}X^{z-i}}}{\sum_{i=0}^{r-1} 10^{\left[-(r-i)pH + \sum_{j=i+1}^{r} pK_j'\right]} + 1 + \sum_{i=r+1}^{n} 10^{\left[(i-r)pH - \sum_{j=r+1}^{i} pK_j'\right]}} \quad (11)$$

where r can take any value higher than zero and less or equal to the maximum net charge z. Eqs. (10) and (11) are general expressions that can predict retention factors and electrophoretic mobilities of ionogenic substances as a function of pH when several data pairs of k–pH or m_e–pH are available. Particular equations can be obtained from general equations for each peptide hormone, taking into account the relevant dissociation constants over the pH range studied.

In the case of chromatographic analysis with ODS columns, only pK associated to the C-terminal carboxylic group of Met-enkephalin, Leu-enkephalin and bradykinin and the pK associated to the imidazol group of triptorelin and buserelin are within the pH range studied. Oxytocin has functional groups that are not within this pH range. The equations deduced in each particular case are summarized in Table 2.

In the case of electrophoretic data, further simplifications to Eq. (11) are made when in the pH range studied the r value can be taken as z, because the species considered is the zwitterionic form $H_{n-z}X$ that has no net charge, and hence its mobility is assumed to be nil [18–23]. Moreover, in the pH range studied, the dissociation equilibrium due to the presence of the guanidine group is never determined, because the guanidine pK_a is usually higher than 12. The corresponding equations are shown in Table 2.

3. Results and discussion

The retention factor values, k, for the series of peptide hormones considered at different pH values of the mobile phase are shown in Fig. 1. Relative standard deviation lower than 5% for the k values

Table 2
Electrophoretic and chromatographic models for the studied peptide hormones

Peptide	H_nX^z	
	Chromatographic models (Eq. (10))	
Bradykinin	H_4X^{3+}	$k = \frac{10^{(pK_1'-pH)}k_{H_4X^{3+}} + k_{H_3X^{2+}}}{10^{(pK_1'-pH)} + 1}$
Met-enkephalin Leu-enkephalin	H_3X^{+}	$k = \frac{10^{(pK_1'-pH)}k_{H_3X^{+}} + k_{H_2X}}{10^{(pK_1'-pH)} + 1}$
Triptorelin Buserelin	H_3X^{2+}	$k = \frac{10^{(pK_1'-pH)}k_{H_3X^{2+}} + k_{H_2X^{+}}}{10^{(pK_1'-pH)} + 1}$
	Electrophoretic models (Eq. (11))	
Eledoisin	H_2X^{+}	$m_e = \frac{10^{-2pH}m_{H_2X^{+}} + 10^{-(pK_1'+pK_2')}m_{X^{-}}}{10^{-2pH} + 10^{-(pK_1'+pH)} + 10^{-(pK_1+pK_2')}}$
Oxytocin[a]	H_3X^{2+}	$m_e = \frac{10^{(pK_1'+pK_2'-2pH)}m_{H_3X^{2+}} + 10^{(pK_2'-pH)}m_{H_2X^{+}} + 10^{(pH-pK_3')}m_{X^{-}}}{10^{(pK_1'+pK_2'-2pH)} + 10^{(pK_2'-pH)} + 1 + 10^{(pH-pK_3')}}$
Bradykinin	H_4X^{3+}	$m_e = \frac{10^{(pK_1'-pH)}m_{H_4X^{3+}} + m_{H_3X^{2+}} + 10^{(pH-pK_2')}m_{H_2X^{+}}}{10^{(pK_1'-pH)} + 1 + 10^{(pH-pK_2')}}$
Met-enkephalin Leu-enkephalin	H_3X^{+}	$m_e = \frac{10^{(pK_1'-pH)}m_{H_3X^{+}} + 10^{(pH-pK_2')}m_{HX^{-}} + 10^{(2pH-pK_2'-pK_3')}m_{X^{2-}}}{10^{(pK_1'-pH)} + 1 + 10^{(pH-pK_2')} + 10^{(2pH-pK_2'-pK_3')}}$
Triptorelin[a] Buserelin[a]	H_4X^{3+}	$m_e = \frac{10^{(pK_1'+pK_2'+pK_3'-3pH)}m_{H_4X^{3+}} + 10^{(pK_2'+pK_3'-2pH)}m_{H_3X^{2+}} + 10^{(pK_3'-pH)}m_{H_2X^{+}}}{10^{(pK_1'+pK_2'+pK_3'-3pH)} + 10^{(pK_2'+pK_3'-2pH)} + 10^{(pK_3'-pH)} + 1}$

[a] One extra basic group is considered in addition to marked groups on Table 1. Explanation is given in the text.

were obtained. The percentage of acetonitrile in the hydro–organic mixture used as mobile phase, 35% (v/v) acetonitrile, was optimized using the solvatochromic parameter E_T^N as solvent descriptor [44] and taking into account that log k values and E_T^N solvent parameters correlate linearly [34,37]. The octadecylsilica (ODS) stationary phase used may only be used in the pH range 2–7, so it was not possible to study the retention of peptide hormones 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 associated to carboxylic C-terminal groups and to imidazol groups of histidine residues are relevant, Table 1.

The retention of Met-enkephalin, Leu-enkephalin and bradykinin is high at low pH values (Fig. 1), where the compound exists as a charged cation and forms an ionic pair with TFA anions; when pH increases, the k value decreases, levels off at the isoelectric point pH and stays constant; this decrease in the chromatographic retention could be explained due to the equilibrium between the double charged zwitterionic and neutral forms being displaced to the first one. In the case of oxytocin, no variation of chromatographic retention with pH was observed because in the pH range studied, oxytocin was not involved in acid–base equilibria. k values of tri-

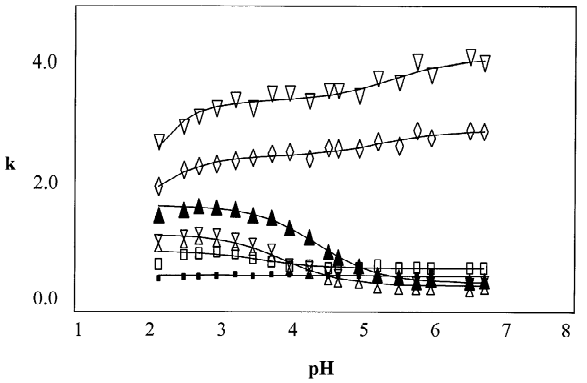

Fig. 1. Experimental (points) and predicted (lines) retention factor, *k*, vs. pH of the mobile phase for the peptide hormones: ▽, buserelin; ◊, triptorelin; ▲, Leu-enkephalin; ⧖, Met-enkephalin; □, bradykinin; ■, oxytocin. Experimental conditions: MeCN–water (35:65, v/v), 0.1% TFA and adjusting pH values up to 7 with sodium hydroxide.

ptorelin and buserelin slightly increase due to the low dissociation of their protonated basic imidazol groups when pH increases.

Although the effects of solute ionization on retention are known, the theoretical interpretation of this phenomenon is hampered by the lack of a rigorous treatment of protolytic equilibria in hydro-organic mixtures. Here, a general equation that describes the chromatographic retention as a function of pH of the mobile phase and of activity coefficients, has been deduced (Eq. (10)). Particular formulations of this equation have been derived, taking into account the number and type of ionogenic groups present in each molecule and involved in the pH range studied (Table 2). Experimental pairs *k*–pH for each peptide hormone have been fitted to the corresponding equation and good correlations have been observed between experimental and predicted values ($r>0.99$) as it is shown in Fig. 1.

In the same way, the m_e values for the series of peptide hormones considered have been plotted against pH of the electrolyte solutions as can be seen in Fig. 2. The electrophoretic migration behaviour of the studied peptide hormones can be described as a function of pH and pK_a' by general Eq. (11). Particular formulations of Eq. (11) are deduced, taking into account the number and type of ionogenic groups present in each molecule and involved in the pH range studied (Table 2). The existence of the zwitterionic species, with net charge zero, is considered in all cases, except for bradykinin, which never exists as these species over the pH range studied.

In contrast to LC experiments using ODS columns, CE experiments using bare fused-silica capillaries permit to study the electrophoretic migration behaviour of the peptide hormones in a wide pH range between 2 and 12. Therefore, electrophoretic equations summarized in Table 2 are more complex than relations obtained to explain their chromatographic behaviour in the pH range 2–7. Eq. (11) for oxytocin, triptorelin and buserelin, was deduced considering an additional basic group. The plot of mobility vs. pH for these substances shows an unexpected increase at strong acidic pH value (Fig. 2), that has been interpreted as an additional very acidic proton binding site common to the three hormones, as was observed by Castagnola et al. [45] in water–trifluoroethanol buffers.

Experimental pairs m_e–pH for each peptide have been fitted to the corresponding equation shown in Table 2. Good correlations have been observed between experimental and predicted values ($r>0.99$) as shown in Fig. 2. Considering the additional basic group for oxytocin, triptorelin and buserelin the concordance between experimental and predicted curves was also excellent.

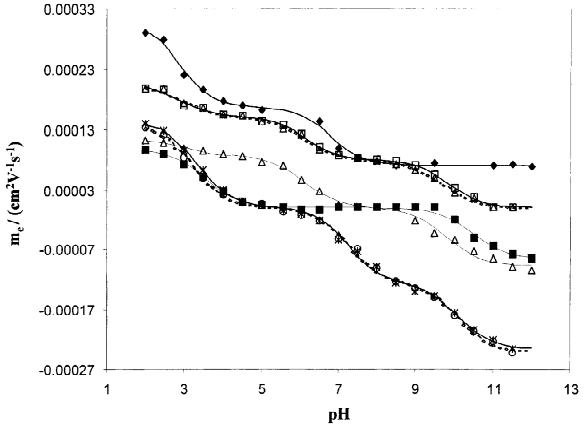

Fig. 2. Experimental (points) and predicted (lines) electrophoretic mobility vs. pH of the running electrolyte for bradykinin (♦), triptorelin (△), buserelin (□), Met-enkephalin (○), Leu-enkephalin (*), oxytocin (▲) and eledoisin (■).

Once the validity of Eqs. (10) and (11) is verified, only a few experimental data pairs m_e–pH and k–pH are necessary to predict the m_e and k values of the studied substances at any buffer pH. The predicted chromatographic retentions and electrophoretic mobilities for all the studied peptide hormones are plotted against buffer pH in Figs. 1 and 2, respectively. Symbols stand for the experimental data and solid lines indicate the predicted k and m_e values obtained using only a few experimental data pairs. Selection of only a few experimental data means a great saving in analysis time.

Figs. 1 and 2 can be used to perform a systematic and rapid selection of the optimum separation conditions, by selecting the appropriate pH to obtain the greatest differences between retention factors or electrophoretic mobilities of adjacent peaks. However, it is preferable to predict other parameters that quantitatively describe the extent of separation of the analytes present in the mixtures. In this way, predicting resolution (R_s) between adjacent peaks is the best way to evaluate separation between critically adjacent peaks, because efficiency and selectivity are simultaneously taken into account. In the case of chromatographic separations predicted resolution can be calculated from the expression:

$$R_s = \underbrace{\frac{\sqrt{N}}{4}}_{\text{efficiency}} \cdot \underbrace{\left(\frac{\alpha - 1}{\alpha}\right) \cdot \left(\frac{k_2}{1 + k_2}\right)}_{\text{selectivity}} \qquad (12)$$

where N is the number of theoretical plates, α the predicted selectivity ($\alpha = k_i/k_j$) and k the predicted retention factors calculated using equations in Table 2. In a similar way, predicted resolution can be expressed as follows for the electrophoretic separations:

$$R_s = \underbrace{\frac{N^{1/2}}{4}}_{\text{efficiency}} \cdot \underbrace{\frac{(m_1 - m_2)}{(m_{avg} + m_{EOF})}}_{\text{selectivity}} \qquad (13)$$

where m_i is the predicted electrophoretic mobility of the solutes obtained by equations of Table 2, m_{avg} is the average of m_i values, m_{EOF} is the mobility of electroosmotic flow, that can be evaluated from the study of m_{EOF} vs. pH (data not shown) and N the number of theoretical plates. Similar N values for all the peaks present in the mixture are desirable to perform accurate resolution estimations with Eqs. (12) and (13). Here, average typical values of 5000 and 15 000 theoretical plates have been selected for chromatographic and electrophoretic resolution calculations, respectively.

The k and m_e predicted values have then been used to calculate resolution for solute adjacent pairs, considering the changes in migration orders that can be observed in Figs. 1 and 2 [18]. Plotting predicted resolution over the pH range studied gives more information about the separation of the overall mixture (Figs. 3 and 4), and permits selection of a suitable pH in order to achieve the best separation, or the selection of various pH ranges when resolution is not fully achieved at a single pH. In Fig. 3, points stand for experimental resolution and solid lines indicate predicted chromatographic resolution values obtained using Eq. (12) and retention factors estimated using equations in Table 2. Good concordance between both sets of resolution values has been observed in all cases confirming the accuracy of chromatographic behaviour prediction with equations in Table 2, and suitability of Eqs. (12) and (13) to estimate resolution. Thus, according to variation of predicted resolution vs. pH (Fig. 3) and Fig. 1, a good chromatographic separation in a reasonable retention time can be obtained in a pH range between 3 and 3.5. Fig. 5 shows the chromatogram of the

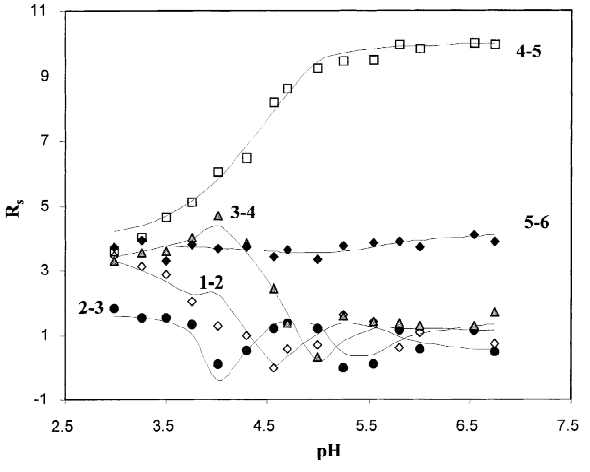

Fig. 3. Chromatographic resolution (R_s) between adjacent peaks vs. pH of mobile phase. Solid lines indicate predicted R_s values and points stand for experimental R_s data.

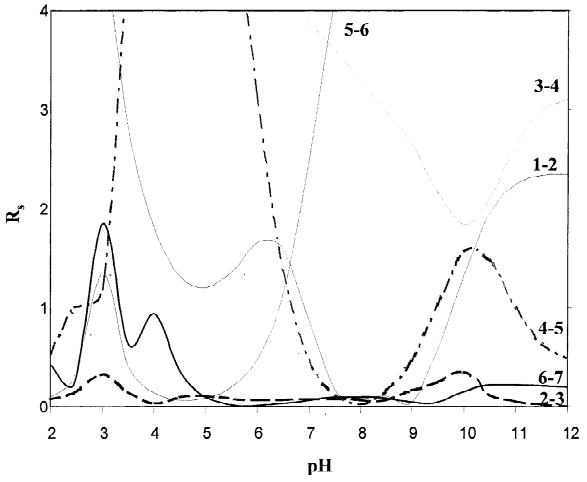

Fig. 4. Predicted electrophoretic resolution (R_s) between adjacent peaks vs. pH of the running electrolyte.

overall peptide mixture at pH 3.3 (35% (v/v) MeCN, 0.1% TFA).

On the other hand, this peptide mixture cannot be fully resolved by CE at only one pH, because only partial separations for the pairs triptorelin/buserelin and Met-enkephalin/Leu-enkephalin can be obtained, and not at the same pH (Figs. 2 and 4). Thus, two pH ranges must be selected to obtain the best separations in each case, one very narrow around pH 2.85 and another wider around pH 10.00. The experimental electropherograms at pH 2.85 and 10 are shown in Fig. 6a and b, respectively. At pH 2.85 all the pairs are separated, excepting triptorelin and buserelin that comigrate. At pH 10.00 triptorelin and buserelin are partially resolved, while the pair Met-enkephalin and Leu-enkephalin comigrates. Table 3 summarizes experimental and predicted values (Eq. (13)) of the electrophoretic resolution at pH 2.60, 2.85 and 10 and it shows a good concordance between both sets of values.

The separations obtained by both methodologies show different selectivities, even around the same pH values (Figs. 5 and 6a). In general, this orthogonality between the partitioning mechanism in LC and the charge-to-mass ratio in CE separations can be used to obtain combined separations with enhanced selectivities [46].

Various authors [20,21,47,48] have remarked on the advantages of the LC and CE methodologies for evaluating the ionization constants of substances. Small quantities of compounds are required, poor water-solubility is not a serious drawback and the purity of the substance is not a critical factor if

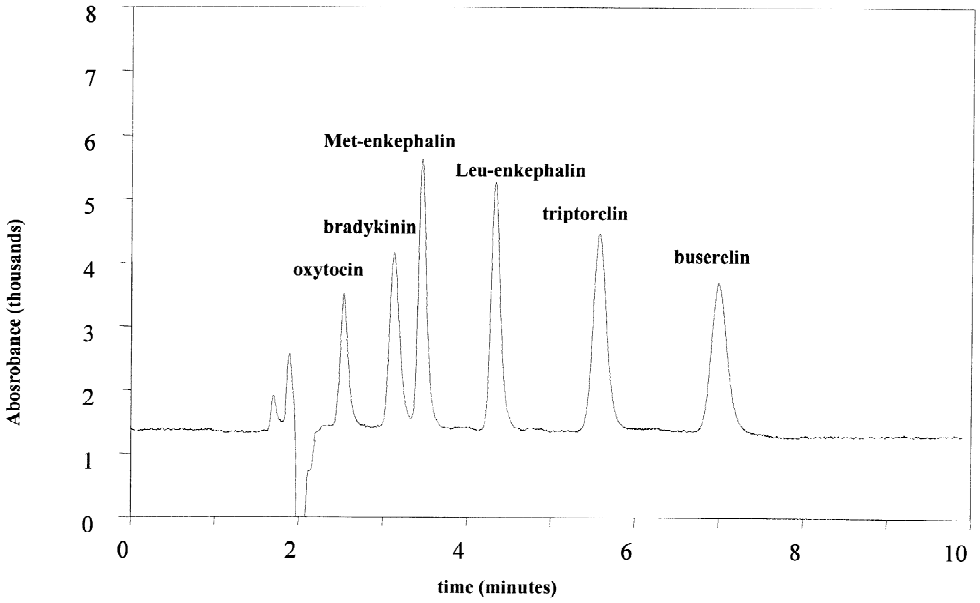

Fig. 5. Chromatogram of peptide hormones at pH 3.3 (35% MeCN, 0.1% TFA).

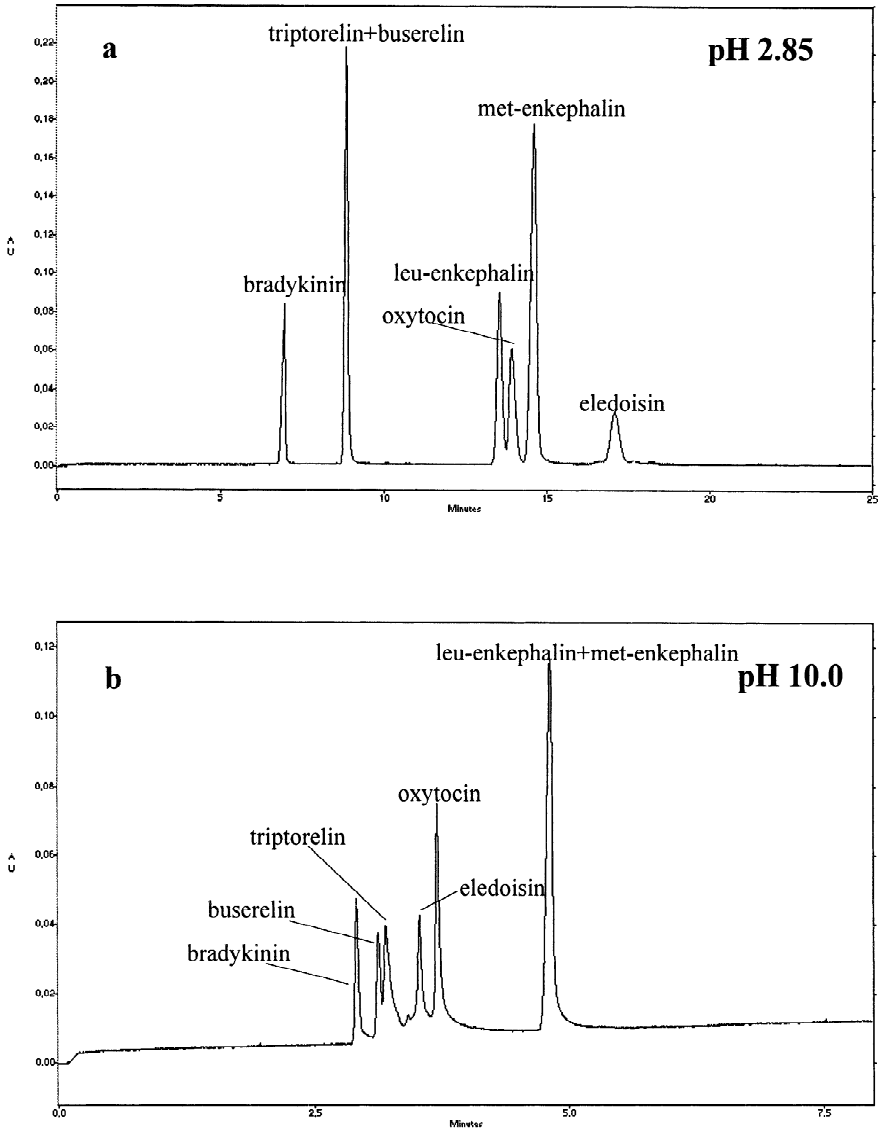

Fig. 6. Electropherograms of a mixture containing all the studied peptides at different pH values.

impurities can be separated from the target analyte on the columns. pK_a values of the studied substances can be calculated from the measured k or m_e values and the corresponding pH using Eqs. (10) and (11) [18,22,23], taking into account the activity coefficients of the species involved in each acid–base equilibrium (Eq. (3)). pK_a values are summarised in Table 4 together with the few values reported in the literature [49–51]. The values obtained by LC methodology are higher than the CE values because they have been determined in acetonitrile–water mixtures with an acetonitrile percentage of 35% (v/v). They cannot be compared with literature data because hydro–organic media values are not available. On the other hand, CE has proved suitable to perform pK_a determinations in aqueous solutions, which is not the case for LC, where retention could be unsuitable without the addition of an organic modi-

Table 3
Experimental and predicted resolution values between adjacent peaks

	pH 2.61		pH 2.85		pH 10.00	
	R_s exp.	R_s pred.	R_s exp.	R_s pred.	R_s exp.	R_s pred.
Bradykinin/triptorelin	6.01	6.09	5.21	5.22	1.26	1.34
Triptorelin/buserelin	0	0.21	0	0.24		
Buserelin/Leu-enkephalin	7.32	7.30	8.57	8.18		
Leu-enkephalin/Met-enkephalin	1.22	1.23				
Met-enkephalin/oxytocin	0.31	0.42				
Oxytocin–eledoisin	2.01	2.14				
Leu-enkephalin/oxytocin			0.93	0.99		
Oxytocin/Met-enkephalin			1.17	1.08		
Met-enkephalin/eledoisin			2.05	1.91		
Buserelin/triptorelin					0.42	0.34
Triptorelin/eledoisin					1.79	1.84
Eledoisin/oxytocin					1.32	1.40
Oxytocin/Leu-enkephalin					5.47	5.25
Leu-enkephalin/Met-enkephalin					0	0.15

Table 4
pK_a values of the studied peptide hormones

Peptide		35% (v/v) MeCN, LC (34)	0% (v/v) MeCN, CE (23)	0% (v/v) MeCN, Microtitration (49)	0% (v/v) MeCN, CE (50)	0% (v/v) MeCN, (51)
Eledoisin	1		3.48			
	2		10.54			
Oxytocin	1		2.98			
	2		6.04			
	3		9.84			
Bradykinin	1	3.21	2.68			
	2		6.66			
Triptorelin	1	4.71	2.80			
	2		5.93			
	3		9.63			
Buserelin	1	5.11	2.66			
	2		5.92			
	3		9.76			
Met-enkephalin	1	3.96	3.17	3.45	3.52	3.20
	2		7.30	7.36		7.70
	3		10.30	10.36		10.30
Leu-enkephalin	1	4.38	3.31	3.69	3.69	3.20
	2		7.29	7.40		7.70
	3		10.34	10.34		10.30

fier. The electrophoretic aqueous pK_a values obtained for Met-enkephalin and Leu-enkephalin are similar to the ones reported by Castagnola et al. [49,50], and also to the average values given by Shields [51]. The pK_a values obtained by CE for the structurally-related pairs triptorelin/buserelin and Met-enkephalin/Leu-enkephalin are very similar. However, the values obtained by LC show great differences, suggesting that elution behaviour of these closely related pairs is influenced by a certain kind of non-hydrophobic, hydrogen bonding or ionic interaction with the stationary phase. Thus, they will be easily resolved by LC, although the pK_a values obtained by this methodology would be less rigorous than the electrophoretic ones. Likewise, the electrophoretic separation of these mixtures would be only possible when differences due to their size are predominant, which in this case occurs when they are fully charged, at very acidic pH values. Several experiments are currently being developed in our laboratory using new LC stationary phases and coated capillaries to avoid undesired analyte–stationary phase or analyte–capillary wall interactions that negatively affect pK_a determinations. Furthermore, these investigations may be used to obtain stationary phases or coated capillaries with improved separation selectivities.

In conclusion, the suitability of the chromatographic and electrophoretic models studied to determine dissociation constants and to predict chromatographic and electrophoretic behaviour of peptide hormones 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 selection to achieve optimum separations.

References

[1] D.J. Anderson, B. Guo, Y. Xu, L.M. Ng, L.J. Kricka, K.J. Skogerboe, D.S. Hage, L. Schoeff, J. Wang, L.J. Sokoll, D.W. Chan, K.M. Ward, K.A. Davis, Anal. Chem. 69 (1997) 165R.
[2] P. Cutler, H. Birrell, M. Haran, W. Man, B. Neville, S. Rosier, M. Skehel, I. White, Biochem. Soc. Trans. 27 (1999) 555.
[3] V. Kasicka, Electrophoresis 20 (1999) 3084.
[4] H.J. Isaaq, Electrophoresis 22 (2001) 3629.
[5] C.K. Larive, S.M. Lunte, M.Z. Zhong, M.D. Perkins, G.S. Wilson, G. Gokulrangan, T. Williams, F. Afroz, C. Schoneich, T.S. Derrick, C.R. Middaugh, S. Bogdanowich-Knipp, Anal. Chem. 71 (1999) 389R.
[6] J. Ding, P. Vouros, Anal. Chem. 71 (1999) 378A.
[7] A. Vonbrocke, G. Nicholson, E. Bayer, Electrophoresis 22 (2001) 1251.
[8] P.J. Schoenmakers, R. Tijssen, J. Chromatogr. A 656 (1993) 577.
[9] J.A. Lewis, D.C. Lommen, W.D. Raddatz, J.W. Dolan, R. Snyder, I. Molnar, J. Chromatogr. 592 (1992) 183.
[10] C.F. Poole, S.K. Poole, Chromatography Today, Elsevier, Amsterdam, 1991.
[11] D.R. Baker, Capillary Electrophoresis, Wiley, New York, 1995.
[12] S.C. Smith, M.G. Khaledi, Anal. Chem. 65 (1994) 193.
[13] V.J. Dolník, J. Chromatogr. A 744 (1996) 115.
[14] A.G. McKillop, R.M. Smith, R.C. Rowe, S.A.C. Wren, Anal. Chem. 71 (1999) 497.
[15] J.L. Miller, D. Shea, M.G. Khaledi, J. Chromatogr. A 888 (2000) 251.
[16] R. Bergés, V. Sanz-Nebot, J. Barbosa, J. Chromatogr. A 869 (2000) 27.
[17] D. Barrón, E. Jiménez-Lozano, J. Barbosa, Anal. Chim. Acta 415 (2000) 83.
[18] V. Sanz-Nebot, F. Benavente, I. Toro, J. Barbosa, J. Chromatogr. A 921 (2001) 69.
[19] J.L. Beckers, F.M. Evaerts, M.T. Ackermans, J. Chromatogr. 537 (1991) 407.
[20] Y. Ishihama, Y. Oda, N.J. Asakawa, Pharm. Sci. 83 (1994) 1500.
[21] S.J. Gluck, K.P. Steele, M.H. Benko, J. Chromatogr. A 745 (1996) 117.
[22] D. Barrón, E. Jiménez-Lozano, A. Irles, J. Barbosa, J. Chromatogr. A 871 (2000) 381.
[23] V. Sanz-Nebot, F. Benavente, I. Toro, J. Barbosa, Electrophoresis 22 (2001) 4333.
[24] Ph. Schmitt, T. Poiger, R. Simon, D. Freitag, A. Kettrup, A.W. Garrison, Anal. Chem. 69 (1997) 2559.
[25] M.T. Hearn, H.H. Keah, R.I. Boysen, I. Messana, F. Misiti, D.V. Rosetti, B. Giardina, M. Castagnola, Anal. Chem. 72 (2000) 1964.
[26] I. Messana, D.V. Rosetti, L. Cassiano, F. Misiti, B. Giardina, M. Castagnola, J. Chromatogr. B 699 (1997) 149.
[27] A. Cifuentes, H. Poppe, Electrophoresis 18 (1997) 2362.
[28] N.J. Adamson, E.C. Reynolds, J. Chromatogr. B 699 (1997) 133.
[29] C.T. Mant, N.E. Zhou, R.S. Hodges, J. Chromatogr. 476 (1989) 363.
[30] J.P. Landers, in: 2nd ed, Handbook of Capillary Electrophoresis, CRC Press, Boca Raton, FL, 1997.
[31] D.R. Baker, Capillary Electrophoresis, Wiley, New York, 1995.
[32] P. Janos, J. Chromatogr. A 834 (1999) 3.
[33] J. Barbosa, I. Toro, V. Sanz-Nebot, J. Chromatogr. A 823 (1998) 497.

[34] V. Sanz-Nebot, I. Toro, J. Barbosa, J. Chromatogr. A 870 (2000) 335.
[35] J. Barbosa, R. Bergés, V. Sanz-Nebot, J. Chromatogr. A 823 (1998) 411.
[36] J. Barbosa, I. Toro, R. Bergés, V. Sanz-Nebot, J. Chromatogr. A 915 (2001) 85.
[37] J. Barbosa, R. Bergés, V. Sanz-Nebot, Chromatographia 51 (2000) 417.
[38] J. Barbosa, V. Sanz-Nebot, Fresenius J. Anal. Chem. 353 (1995) 148.
[39] J. Barbosa, I. Marqués, D. Barrón, V. Sanz-Nebot, Trends Anal. Chem. 18 (1999) 543.
[40] S. Rondinini, P.R. Mussini, T. Mussini, Pure Appl. Chem. 59 (1987) 1549.
[41] T. Mussini, A.K. Covington, P. Longhi, S. Rondinin, Pure Appl. Chem. 57 (1985) 865.
[42] F.G.K. Bauke, R. Naumann, C. Alexander-Weber, Anal. Chem. 65 (1993) 3244.
[43] Cs. Horváth, W. Melander, I. Molnár, Anal. Chem. 49 (1977) 142.
[44] C. Reichardt, Solvents and Solvent Effects in Organic Chemistry, VCH, Weinheim, 1988.
[45] M. Castagnola, D.V. Rosetti, F. Misiti, L. Casiano, B. Giardina, I. Messana, J. Chromatogr. A 792 (1997) 57.
[46] V. Sanz-Nebot, F. Benavente, J. Barbosa, J. Chromatogr. A 950 (2002) 99.
[47] P. Chaminade, A. Baillet, D. Ferrier, B. Bourguignon, D.L. Massart, Anal. Chim. Acta 280 (1993) 93.
[48] J.E. Hardcastle, I. Jano, J. Chromatogr. B 717 (1998) 39.
[49] M. Castagnola, L. Cassiano, I. Messana, G. Nocca, R. Rabino, D.V. Rosetti, B. Giardina, J. Chromatogr. B 656 (1994) 87.
[50] M. Castagnola, D.V. Rosetti, L. Cassiano, F. Misiti, L. Pennachietti, B. Gieradina, I. Messana, Electrophoresis 17 (1996) 1925.
[51] E.C. Rickard, M.M. Strohl, R.G. Nielsen, Anal. Biochem. 197 (1991) 197.