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Solvatochromic parameter values and pH in acetonitrile–water mixtures
Optimization of mobile phase for the separation of peptides by high-performance liquid chromatography☆

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Abstract

The proportion of the organic modifier and the pH of the mobile phase were optimized in order to separate a series of low-molecular-mass peptides by HPLC. The composition of the mobile phase was optimized by establishing relationships between retention parameters and Reichardt's E_T^N scale of solvent polarity, and between retention and the Kamlet–Taft multiparameter solvent scale of the eluent, using linear solvation energy relationships (LSER). The pH of the mobile phase was also successfully optimized by establishing relationships between the retention and the pH measured in the aqueous–organic mixture used as eluent.

Keywords: Mobile-phase composition; Linear solvation energy relationship; pH effects; Solvatochromic parameters; Peptides

1. Introduction

High-performance liquid chromatography (HPLC) separations are essential to the modern, ever-growing biotechnology industry and to research in many scientific fields, specially the life sciences. An increasing number of peptides are available for biomedical research and therapeutic applications. Pharmaceutical products require extensive purification and characterization, not only of the peptide itself but also of impurities resulting from the processing steps. For the separation and determination of peptides, HPLC offers the possibility of accomplishing a high level of purification in a minimum number of steps with minimal loss of biological activity. A further line has to be drawn between the determination and purification on a preparative scale of a peptide in an academic research laboratory and in an industrial plant. Since it is expensive, large-scale industrial purification by HPLC requires a high throughput with maximum efficiency and, consequently, the optimization of the separation process. Therefore, a detailed knowledge of the behaviour of peptides under chromatographic conditions, i.e., in contact with

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both the mobile phase and the stationary phase, is essential [1–5].

The retention behaviour of a peptide is the result of complex interactions with both the stationary and mobile phases. However, most peptide separations are still performed on ordinary, porous, silica-based stationary phases, although these materials have several disadvantages, such as low stability at high pH (>8) [5,6]. Although the stationary phase plays an active role in the separation process, most researchers have focused attention on mobile phase optimization since this is the easiest way to control retention and selectivity in reversed-phase liquid chromatography (RPLC) [7].

An increasing number of biochemical studies require the analysis of complex peptide mixtures, e.g., the analysis and characterization of brain peptides or peptide mapping of protein digests. In the pharmaceutical industry, peptide mapping is becoming an invaluable tool for controlling the purity and structural consistency of recombinant protein pharmaceuticals [8]. In order to avoid long empirical optimization procedures, a system for predicting peptide retention times is of great advantage. However, the accurate prediction of peptide retention is still a challenging task [3].

The method of linear solvation energy relationships (LSER), based on the Kamlet–Taft multiparameter scale, has been used successfully to study retention in HPLC [9–12]. The solvatochromic LSER approach of Kamlet and Taft seeks to relate retention in a fixed solute–stationary phase system to variations in characteristic properties of mobile phases such as the solvatochromic parameters π^*, α and β. The π^* parameter is used to evaluate solvent dipolarity/polarizability [13] and α and β scales evaluate solvent hydrogen bond acidity [14] and solvent hydrogen bond basicity [15] respectively.

The polarity of the mobile phase has a strong influence on solute retention in RPLC. Except in rare instances, retention in RPLC increases as the polarity of the mobile phase is increased by the addition of water. The normalized E_T^N scale of mobile phase polarity proposed by Reichardt [16] has also been used to study chromatographic retention. Dorsey and co-workers [17–19] have conclusively shown that plots of log k' versus the E_T solvatochromic parameter of the mobile phases are very often more linear than plots of log k' versus fraction of organic modifier. This relationship can be expressed as

$$\log k' = C + eE_T^N \quad (1)$$

when the E_T^N normalized parameter [16] is used instead of the $E_T(30)$ value in order to use similar units as for the other parameter. Therefore, suitable prediction of the retention for a specific solute can be achieved from the E_T^N values of the mobile phases and a few experimental data. However, Cheong and Carr [20] concluded that good correlations between retention and this single solvent parameter can be obtained only over a narrow range of solvent composition.

These approaches only allow the prediction of retention at different mobile phase compositions, and do not provide information about the pH of the mobile phase, which is important in understanding the retention process. The pH of the mobile phase is also critical for optimizing selectivity in RPLC since the degree of ionization of solutes, stationary phases and mobile phase additives may be affected by the pH [21].

The operational pH in mixed aqueous–organic solvents is usually measured assuming that the pH of the mobile phase is the same as that of the aqueous fraction, in which case errors due to the medium effects contribute to uncertainty regarding the true pH [22]. In acetonitrile–water mixtures, the influence of the co-solvent on the pH and pK_a values is substantial [22–24], and therefore for successful systematic optimization of the mobile phase pH, accurate pH measurements in the most widely used binary aqueous–organic solvent mixtures are needed.

The pH measurements in a mixed solvent can be performed as easily as in water taking into account the operational definition of pH [22,25,26]:

$$pH_X = pH_{PS} + \frac{E_{PS} - E_X}{g} \quad (2)$$

where E_X and E_{PS} denote the electromotive force (e.m.f.) measurements in cell A on the sample solution at unknown pH_X and on the

standard primary reference solution at known pH_{PS} respectively, and $g = (\ln 10)RT/F$:

reference electrode	salt bridge	sample solution at pH_X or standard buffer solution at pH_{PS} in solvent S	H^+ sensing electrode

From the point of view of practical chromatography, it is possible to measure the activity of the hydronium ion in acetonitrile–water mixtures with commercial electrodes because reference pH values of standard buffer solutions, pH_{PS}, in these solvents have been determined previously [24,27–29], and the effect of liquid junction in cells with commercial potentiometric sensors have been evaluated [12].

In this study, the proportion of the organic modifier and the pH of the aqueous–organic mobile phase were optimized in order to separate a series of ten low-molecular-mass peptides. We chose such peptides as a first step in the chromatographic study of peptides of high pharmacological and biotechnological interest. The LSER method, based either on the multiparameter π^*, α and β scale or the single solvent parameter E_T^N and the relationships with log k', was applied to the optimization of the mobile phase composition and to the prediction of the chromatographic behaviour of peptide substances. Moreover, the pH measurements in the acetonitrile–water mixtures used as mobile phases and their correlation with k' were used in the optimization of the mobile phase pH for the required separation. This relationship also made it possible to determine dissociation constants of the peptides studied in acetonitrile–water from the same retention measurements as those used for the optimization of the mobile phase pH.

2. Experimental

2.1. Chemicals and reagents

Water with a conductivity lower than 0.05 μS/cm and acetonitrile (Merck, Darmstadt, Germany) were of HPLC grade. Trifluoroacetic acid (TFA), sodium hydroxide, potassium bromide and potassium hydrogenphthalate (dried at 110°C before use) were all of analytical-reagent grade from Merck. Peptides were purchased from Sigma (St. Louis, MO, USA): L-2-amino-5-ureidovaleric acid (citrulline), N-(N-glycyl)-glycine (Gly-Gly), N-(N-glycylglycyl)glycine (Gly-Gly-Gly), N-(N-γ-L-glutamyl-L-cisteinyl)-glycine (glutathione), N-(N-L-tyrosylglycyl)glycine (Tyr-Gly-Gly), N-(N-glycylglycyl)-L-valine (Gly-Gly-Val), N-(N-glycylglycyl)-L-isoleucine (Gly-Gly-Ile), N-(N-DL-alanyl-DL-leucyl)glycine (Ala-Leu-Gly), N-(N-glycylglycyl)-L-phenylalanine (Gly-Gly-Phe). All amino acids except glycine and those which form Ala-Leu-Gly (DL-Ala-DL-Leu-Gly) have the L-configuration. In the case of DL-Ala-DL-Leu-Gly, it was possible to separate two diastereoisomers. 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.

Stock standard solutions of the peptides were prepared by dissolving ca. 50 mg of each peptide and diluting to 25 ml; working standard solutions were prepared by tenfold dilution of the stock standard solutions. A mixture of the ten peptides studied was prepared by 100-fold dilution of the stock standard solution. The solvent was 0.05% (v/v) aqueous TFA or acetonitrile–water (7:93, v/v) containing 0.05% (v/v) TFA. All the eluents and mobile phases were passed through a 0.22-μm nylon filter (MSI, Westboro, MA, USA) and degassed by sonication and the samples were passed through a 0.45-μm nylon filter (MSI).

2.2. Apparatus

The chromatographic equipment consisted of an ISCO (Lincoln, NE, USA) Model 2350 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-μm) column (250 × 4 mm I.D.) was used at room temperature.

The e.m.f. values used to evaluate the pH of the mobile phase were measured (±0.1 mV) with

a Model 2002 potentiometer (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.

2.3. Chromatographic procedure

For the optimization of the mobile phase composition, the solution was made of different acetonitrile–water mixtures containing 0.05% (v/v) trifluoroacetic acid, with the pH of the aqueous phase adjusted to 2.2 with sodium hydroxide [30–33] at several acetonitrile concentrations up to 40% (v/v). The LiChrospher C_{18} column was equilibrated with new mobile phase conditions for 30 min. All chromatograms were measured at room temperature. The flow-rate of the mobile phase was 1 ml/min and the signal was monitored at 214 nm. The hold-up time, t_0, was established for every mobile phase composition using potassium bromide solution (0.01% in water) [34]. The retention times and the capacity factors of the solutes were determined from three different injections at each mobile phase composition considered.

In order to study the influence of the eluent pH on the chromatographic separation, the mobile phase was adjusted to different pH values, from 2.2 to 6.0, with sodium hydroxide. The pH was measured in the mixed mobile phase, where the chromatographic separation takes place, taking into account the reference pH values of primary standard buffer solutions, pH_{PS}, for the standardization of potentiometric sensors in acetonitrile–water mixtures. This was assigned in previous work [24,27–29], in accordance with IUPAC rules [26] and on the basis of the multi-primary standard scale according to the National Institute of Standard and Technology (NIST) [25]. In this study we used potassium hydrogenphthalate (0.05 mol/kg) as a primary standard buffer reference solution in the acetonitrile–water mixtures studied and a commercial combination pH electrode [12].

3. Results and discussion

The logarithms of the capacity factor values (log k') were obtained at different percentages of acetonitrile for the peptides studied (Table 1). The mobile phases assayed were acetonitrile–water (3:97), (5:95), (7:93), (10:90), (12.5:87.5), (15:85), (20:80), (25:75), (30:70) and (40:60, v/v). Capacity 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 given for each mobile phase composition studied in Table 1, and t_R is the retention time of each peptide for each mobile phase. Table 1 also gives relative standard deviations (R.S.D.) of the log k' values.

To optimize the composition of the mobile phase, the Kamlet–Taft solvatochromic parameters characteristic of the mobile phase (π^*, α and β) and Reichard's E_T^N polarity scale (known for the whole range of composition of acetonitrile–water) were related to the capacity factors of the peptides to be separated using the LSER methodology. Values of π^* [35], α [36] and β [37] together with E_T^N values [38] for all the acetonitrile–water mixtures used were obtained by interpolating literature values and are given in Table 2.

The LSER approach, when applied to chromatographic processes, correlates a general solute property (SP) such as logarithm of the capacity factor with parameters of the solute and both the mobile and stationary phases [39]:

$$\log k' = (SP)_0 + M(\delta_s^2 - \delta_m^2)\bar{V}_2/100 + S(\pi_s^* - \pi_m^*)\pi_2^* + A(\beta_s - \beta_m)\alpha_2 + B(\alpha_s - \alpha_m)\beta_2 \quad (3)$$

where k' is the chromatographic capacity factor, $(SP)_0$ is the intercept of the regression equation, $\bar{V}_2$ is the molar volume of the solute, δ^2 is the square of the Hildebrand solubility parameter (a measure of the work required to produce a cavity of unit volume in the solvent) and π^*, α and β are the Kamlet–Taft solvatochromic parameters. Subscripts s and m refer to the stationary and mobile phase, respectively, and superscript 2 refers to the solute properties. The values of M, S, A and B are the coefficients for this equation

Table 1
Logarithms of the capacity factors of the peptides studied and the hold-up time at various percentages of acetonitrile (AN) in the mobile phase, with R.S.D. (%) in parentheses

AN (%)	Citrulline	Gly-Gly	Gly-Gly-Gly	Glutathione	Tyr-Gly-Gly	Gly-Gly-Val	Gly-Gly-Ile	Ala-Leu-Gly (I)	Ala-Leu-Gly (II)	Gly-Gly-Phe	KBr
40	−0.715	−0.597	−0.640	−0.607	−0.496	−0.464	−0.351	−0.351	−0.351	−0.296	1.66
	(2.1)	(1.9)	(1.9)	(0.1)	(0.1)	(0.1)	(0.1)	(1.8)	(1.8)	(0.1)	
30	−0.691	−0.573	−0.602	−0.573	−0.436	−0.338	−0.231	−0.276	−0.206	−0.115	1.72
	(3.4)	(1.8)	(0.1)	(1.8)	(1.7)	(1.8)	(3.5)	(1.9)	(2.1)	(3.1)	
25	−0.663	−0.553	−0.571	−0.535	−0.380	−0.261	−0.106	−0.198	−0.100	0.064	1.75
	(1.9)	(0.1)	(1.8)	(1.7)	(1.7)	(1.9)	(0.1)	(2.2)	(3.4)	(1.5)	
20	−0.648	−0.518	−0.534	−0.472	−0.246	−0.186	0.078	−0.060	0.080	0.319	1.78
	(1.8)	(0.1)	(0.1)	(1.7)	(0.1)	(2.2)	(0.1)	(0.1)	(0.1)	(0.7)	
15	−0.590	−0.475	−0.482	−0.366	−0.156	−0.005	0.352	0.160	0.349	0.624	1.79
	(0.1)	(0.1)	(0.1)	(1.7)	(0.1)	(0.1)	(0.3)	(2.0)	(0.9)	(0.9)	
12.5	−0.556	−0.442	−0.449	−0.238	−0.097	0.106	0.510	0.299	0.514	0.821	1.80
	(0.1)	(0.1)	(1.6)	(0.1)	(3.5)	(1.9)	(1.2)	(0.1)	(0.2)	(0.4)	
10	−0.533	−0.425	−0.425	−0.201	0.073	0.254	0.684	0.475	0.718	1.007	1.81
	(1.7)	(1.6)	(0.1)	(2.1)	(2.9)	(1.0)	(1.4)	(1.2)	(0.8)	(0.4)	
7	−0.475	−0.403	−0.379	−0.073	0.301	0.452	0.934	0.711	0.989	1.258	1.82
	(2.8)	(1.6)	(0.1)	(4.4)	(0.7)	(0.4)	(1.0)	(0.1)	(0.1)	(0.1)	
5	−0.452	−0.396	−0.367	0.038	0.505	0.586	1.102	0.886	1.190	1.453	1.84
	(0.1)	(0.1)	(1.6)	(6.0)	(0.2)	(1.2)	(0.6)	(1.2)	(0.2)	(0.2)	
3	−0.406	−0.389	−0.345	0.162	0.695	0.662	1.190	1.042	1.345	–	1.86
	(0.1)	(0.1)	(0.1)	(1.1)	(3.0)	(3.4)	(0.3)	(1.1)	(0.4)		

Table 2
Solvatochromic parameter values for the acetonitrile–water mixtures

AN (%)	E_T^N	π^*	α	β
40	0.83	1.00	0.95	0.38
30	0.86	1.06	1.01	0.35
25	0.88	1.09	1.04	0.34
20	0.90	1.11	1.08	0.34
15	0.92	1.14	1.12	0.35
12.5	0.94	1.15	1.14	0.36
10	0.95	1.15	1.16	0.37
7	0.96	1.16	1.19	0.39
5	0.97	1.17	1.21	0.41
3	0.98	1.17	1.24	0.43

and are independent of the solutes and, if the model were rigorously correct, they should be independent of the phases [20].

When a system with a fixed pair of solute and stationary phase is considered, and assuming the invariance of the properties of the stationary phase with changes in the mobile phase composition [20] and the relationship between δ_m^2 and the Kamlet–Taft solvatochromic parameters of the mobile phase [20,38], Eq. 3 can be simplified to

$$\log k' = (\log k')_0 + s\pi_m^* + b\alpha_m + a\beta_m \quad (4)$$

where $(\log k')_0$ depends on the parameters of the stationary phase and the solute, s, a and b are the correlation coefficients which depend on the solute parameters and π_m^*, α_m and β_m are the Kamlet–Taft solvatochromic parameters of the mobile phase [12,20].

Table 3 shows the multiparametric relationships between the capacity factors of the peptides and the solvatochromic parameters of the mobile phase studied in accordance with Eq. 4. Log k' correlates well with the solvatochromic parameters π^*, α and β, since the average correlation coefficient (r) was higher than 0.99 using simple linear regression for the data sets examined here.

The relationship found between the chromatographic parameter log k' and the properties of the eluent mixtures π^*, α and β allow us to predict the chromatographic retention of the peptides studied for any composition of the eluent system. For this purpose, the capacity factors and the separation factors for the substances studied were calculated for the different compositions of the eluent system using the LSER relationships obtained. From these values, we can predict that the best chromatographic separation (in which the separation factors for the peptides studied are highest) takes place when the acetonitrile content in the mobile phase ranges from 7 to 10% (v/v), although the complete separation of Gly-Gly, Gly-Gly-Gly and citrulline was not achieved under these conditions. In the case of DL-Ala-DL-Leu-Gly, it was possible to separate two diastereoisomers owing to their different chromatographic properties; we chose a mobile phase containing 7% (v/v) of

Table 3
Relationships between log k' for the peptides studied and π^*, α and β solvatochromic parameters of the eluent system in the interval studied using the LSER approach

Substance	Multiparameter relationship	r
Citrulline	$\text{Log } k' = -2.32 + 0.65\pi^* + 0.46\alpha + 1.35\beta$	0.998
Gly-Gly	$\text{Log } k' = -1.76 + 0.62\pi^* + 0.38\alpha + 0.46\beta$	0.987
Gly-Gly-Gly	$\text{Log } k' = -2.02 + 0.46\pi^* + 0.71\alpha + 0.60\beta$	0.996
Glutathione	$\text{Log } k' = -4.80 + 1.75\pi^* + 0.87\alpha + 4.27\beta$	0.999
Tyr-Gly-Gly	$\text{Log } k' = -3.40 - 3.56\pi^* + 5.45\alpha + 3.47\beta$	0.995
Gly-Gly-Val	$\text{Log } k' = -6.53 + 2.61\pi^* + 1.61\alpha + 5.10\beta$	0.996
Ala-Leu-Gly (1)	$\text{Log } k' = -6.85 + 1.08\pi^* + 3.32\alpha + 5.95\beta$	0.997
Ala-Leu-Gly (2)	$\text{Log } k' = -8.46 + 2.07\pi^* + 3.76\alpha + 6.48\beta$	0.998
Gly-Gly-Ile	$\text{Log } k' = -8.93 + 3.92\pi^* + 2.31\alpha + 6.39\beta$	0.997
Gly-Gly-Phe	$\text{Log } k' = -7.38 - 1.42\pi^* + 7.38\alpha + 3.86\beta$	0.999

acetonitrile because of the more advantageous separation factor values.

The experimental log k' values were also related to the E_T^N solvent parameter according to Eq. 1, as shown in Fig. 1. All the peptides showed similar elution profiles. Log k' and E_T^N correlate linearly over the whole experimental range of acetonitrile contents studied, but there are two straight lines with different slopes, which intersect roughly at an acetonitrile content of 20% (v/v). These two straight lines could be explained by taking into account the structural features of acetonitrile–water mixtures, described by Marcus and Migron [40], which show three regions. On the water-rich side there is a region in which the structure of the water molecules remains more or less intact; the acetonitrile molecules gradually occupy the cavities between them with little disruption of the water structure. The limit of acetonitrile molar fraction, x_{AN}, beyond which the acetonitrile molecules can no longer be accommodated within the cavities of the water structure, varies with the method applied, but is ≥ 0.10, that is, ca. 15–20% (v/v) of acetonitrile. In the middle range of compositions, the acetonitrile–water mixtures show microheterogeneity; hence there is a preference of a given water molecule for other water molecules rather than acetonitrile molecules. The same can be said of the preference of acetonitrile molecules for being in the vicinity of a given acetonitrile molecule. At $x_{AN} \geq 0.75$ the number of water clusters is low and water–acetonitrile interactions, which could be discounted in the middle range, now become important.

In this study, we worked in two structural regions of acetonitrile–water mixtures and, as a result, obtained the two straight lines with different slopes, which change in the region where the acetonitrile–water mixtures show microheterogeneity. In accordance with structural features, in acetonitrile–water mixtures containing $\leq$15–20% (v/v) of acetonitrile, the interactions of acetonitrile molecules with other molecules are minimal and hence their effect on retention changes is high, as shown in Fig. 1. In the microheterogeneity region, acetonitrile molecule interactions lead to a smaller influence of acetonitrile molecules on retention variations. Therefore, the slope of log k' versus E_T^N is small in this region.

The use of Eq. 1 involves an important reduc-

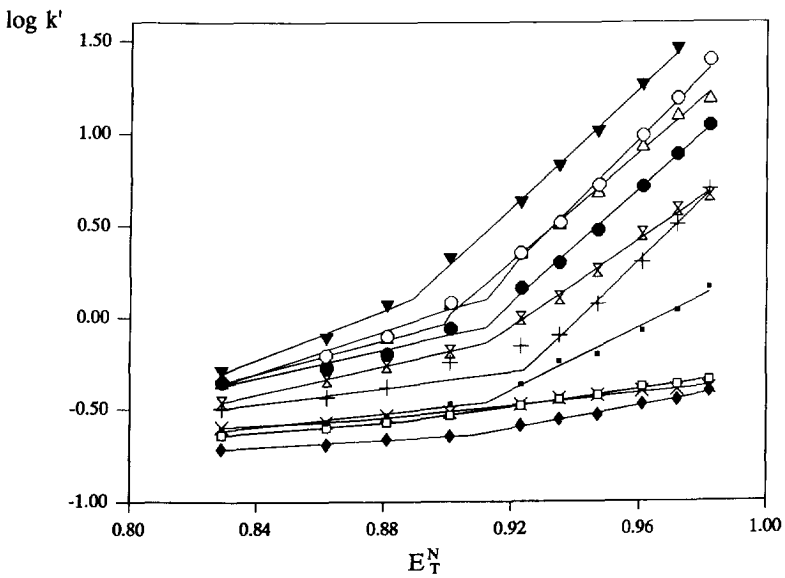

Fig. 1. Plots of log k' values of the peptides studied versus E_T^N parameters of the mobile phase. ◆ = Citrulline; × = Gly-Gly; □ = Gly-Gly-Gly; ■ = glutathione; + = Tyr-Gly-Gly; ⧖ = Gly-Gly-Val; ● = Ala-Leu-Gly I; △ = Gly-Gly-Ile; ○ = Ala-Leu-Gly (II); ▼ = Gly-Gly-Phe.

tion in experimental work in the separation of complex peptide mixtures. The verified linearity of the log k' values of peptides vs. E_T^N values would be very useful for practical reasons concerning the large number of peptides that could exist in a given analysis. Hence this behaviour could be extrapolated to other peptides and only two measurements of k' values for each compound at two different mobile phase compositions could be sufficient for the mobile phase optimization for other mixtures of peptides.

Fig. 1 indicates that good chromatographic separations can be obtained for the peptides studied when the concentration of acetonitrile in the mobile phase <10% (v/v). From the experimental data obtained by injecting the mixture of the ten peptides using mobile phases with acetonitrile contents of 7% and 10% (v/v), we chose 7% (v/v) because of the better resolution values for the peptides which were difficult to separate (citrulline, Gly-Gly and Gly-Gly-Gly).

Further direct reduction of Eq. 4 is not possible because the remaining Kamlet–Taft solvatochromic parameters measure different solvent effects, and linear correlations between them have not been demonstrated. However, the difference in β values between water and acetonitrile is small [37,40], and the β values are constant over most of the composition range [40]. Therefore, the β_m term in Eq. 4 can be included in the independent term, and taking into account the linear correlation between E_T^N and α and π^* demonstrated for 40 media: $E_T^N = 0.009 + 0.415\pi^* + 0.465\alpha$ [41], Eq. 4 can be reduced to Eq. 1.

The apparently contradictory results of Dorsey and co-workers [17–19] and Cheong and Carr [20] could be explained by taking into account that the E_T^N single-parameter scale is an accurate descriptor of the strength of the mobile phase in RPLC only if all the above conditions are met, so Eq. 1 can be used. The Dorsey and co-workers' results were obtained with a large number of solutes, but almost all lay in the microheterogeneity region of acetonitrile–water mixtures ($0.1 \leq x \leq 0.75$). The same can be said of the results presented in previous studies [42,43] where the chromatographic behaviour of series of steroids were studied in a range of acetonitrile–water mixtures containing 40–70% (v/v) acetonitrile. Plots of log k' values of the steroids studied there versus the E_T^N values showed one straight line because all the data were obtained in one of the structural regions of acetonitrile–water mixtures, namely the microheterogeneity region.

In contrast, Cheong and Carr [20] studied the relationships between log k' and E_T using acetonitrile–water systems with a wide range of acetonitrile contents and their results were obtained in two structural regions of acetonitrile–water mixtures. Thus, correlations between measures of solvent–solute interactions and solvent–solvent interactions can change if the molecular structure changes. This effect was also observed in a previous study [12] using a range of acetonitrile–water mixtures containing 5–30% (v/v) acetonitrile, where plots of log k' values of the quinolones studied versus the E_T^N parameters values showed two straight lines with different slopes, which intersected roughly at 15% (v/v) acetonitrile. The same can be said of the results in this study, in which two structural regions were examined. The study of higher percentages of acetonitrile with these peptides is of no practical interest, since the resultant k' values are subject to high errors owing to the low retention, and there are difficulties in defining the column void volume.

In order to study the influence of the pH of the mobile phase on the chromatographic retention, k' values for the peptides studied and the t_0 values at different pHs of the mobile phase were determined from three different injections at each mobile phase pH considered, and the results are given in Table 4, which also includes the R.S.D.s associated with the k' values, showing good reproducibility. NIST recommends choosing a standard reference solution with a pH_{PS} value as close as possible to the unknown pH_X [25,27]. We used a standard reference solution, pH_{PS}, of potassium hydrogen phthalate and a commercial combination pH electrode, since this pH_{PS} was previously determined in acetonitrile–water mixtures [27] and good accuracy and precision were obtained for pH values up to 7

Table 4
Values of the capacity factors of the peptides studied and the hold-up time at various pHs of the mobile phase, with R.S.D. (%) in parentheses

pH	Citrulline	Gly-Gly	Gly-Gly-Gly	Glutathione	Tyr-Gly-Gly	Gly-Gly-Val	Gly-Gly-Ile	Ala-Leu-Gly (I)	Ala-Leu-Gly (II)	Gly-Gly-Phe	KBr
2.24	0.34	0.41	0.43	0.83	1.87	2.93	8.60	5.37	10.19	17.76	1.82
	(0.8)	(0.6)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.4)	
2.52	0.29	0.39	0.41	0.71	1.96	2.90	8.37	5.25	9.92	17.37	1.82
	(0.9)	(0.1)	(0.6)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	
2.70	0.25	0.38	0.40	0.67	1.92	2.88	8.46	5.49	10.42	16.15	1.81
	(2.7)	(0.7)	(0.7)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	
3.02	0.20	0.33	0.36	0.60	1.73	2.53	7.44	4.97	9.35	13.57	1.82
	(1.3)	(0.8)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	
3.31	0.18	0.28	0.32	0.47	1.57	2.24	6.40	4.58	8.55	11.15	1.82
	(1.4)	(0.9)	(0.1)	(0.1)	(0.2)	(0.1)	(0.1)	(0.1)	(0.1)	(0.6)	
3.46	0.18	0.25	0.30	0.41	1.46	1.97	5.76	4.10	7.66	9.06	1.81
	(0.1)	(1.0)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.5)	
3.74	0.17	0.21	0.23	0.27	1.19	1.52	4.44	3.42	6.57	6.86	1.81
	(1.6)	(1.3)	(1.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	
3.99	0.16	0.18	0.21	0.21	1.02	1.14	3.28	2.74	5.49	5.02	1.80
	(0.1)	(1.4)	(1.3)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	
4.25	0.15	0.17	0.18	0.26	0.82	0.85	2.19	2.00	4.15	3.41	1.80
	(1.7)	(1.6)	(0.1)	(0.1)	(0.1)	(0.4)	(0.1)	(0.1)	(0.1)	(0.1)	
4.50	0.15	0.16	0.17	0.14	0.78	0.65	1.67	1.75	3.89	2.77	1.80
	(1.7)	(1.7)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.2)	(0.1)	
4.97	0.14	0.11	0.11	0.05	0.77	0.43	1.15	1.46	3.71	2.36	1.83
	(0.1)	(0.1)	(2.3)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	
5.49	0.13	0.10	0.10	0.01	0.66	0.32	0.89	1.21	3.10	1.82	1.81
	(2.0)	(0.1)	(2.6)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	
6.01	0.13	0.10	0.09	0.00	0.65	0.30	0.77	1.17	3.02	1.73	1.81
	(1.9)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	

using this pH_{PS}. In addition, rapid stabilization of the potentiometric system was observed, as shown in a previous paper [12]. pH measurements in aqueous–organic mobile phase used permit the interpretation of chromatographic results without extrapolation of pH values from aqueous solutions. pH and pK_a values show deviations from a linear dependence on the composition of the mixtures because of preferential solvation [23,24]. If a solute interacts with one of the solvents more strongly than with the other, then the solute is preferentially solvated by the former [40].

From plots of k' values for the peptides studied versus pH of the acetonitrile–water eluent system (7:93, v/v) shown in Fig. 2, we predicted that the optimum separation for the peptides studied could be achieved at pH of the mobile phase between 2.25 and 3. By injecting the mixture of the ten peptides with mobile phase pH adjusted to 2.25, 2.59, 2.80 and 3.07, we achieved the best separation at pH 2.80, with good resolution in all cases except between Gly-Gly and Gly-Gly-Gly, which were not resolved under these conditions. The elution order was citrulline, Gly-Gly + Gly-Gly-Gly, glutathione, Tyr-Gly-Gly, Gly-Gly-Val, Ala-Leu-Gly (I), Gly-Gly-Ile, Ala-Leu-Gly (II) and Gly-Gly-Phe, where Ala-Leu-Gly (I) represents the first diastereoisomer and Ala-Leu-Gly (II) the second diastereoisomer separated from DL-Ala-DL-Leu-Gly. Fig. 3 shows the chromatogram of the separation of nine peptides with acetonitrile–water (7:93, v/v) containing 0.05% (v/v) trifluoroacetic acid as the mobile phase at pH 2.80, the optimum conditions which allow the separation of this series of low-molecular-mass peptides using RPLC.

Particularly useful for the analysis of complex mixtures and for a further approach to purification on a preparative scale is a detailed knowledge of the behaviour of peptides under chromatographic conditions. Hence the determination of pK_a values for the peptides studied in the aqueous–organic phase used for eluent system control could be essential. As the pH values of the seven primary standard reference solutions have been determined previously in acetonitrile–

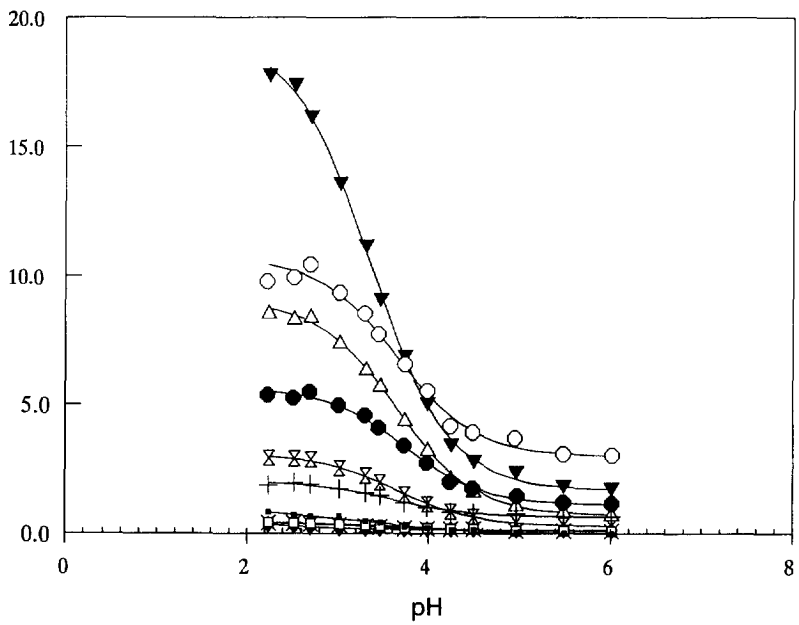

Fig. 2. Plots of k' values of the peptides studied versus mobile phase pH. ◆ = Citrulline; × = Gly-Gly; □ = Gly-Gly-Gly; ■ = glutathione; + = Tyr-Gly-Gly; ⧖ = Gly-Gly-Val; ● = Ala-Leu-Gly I; △ = Gly-Gly-Ile; ○ = Ala-Leu-Gly (II); ▼ = Gly-Gly-Phe.

water mixtures [24,27,28], pH can be measured in these mixtures as easily as in water, using Eq. 2. These data will provide a method for the determination of pK_a values for the substances studied in the eluent system used assuming the theory applied by Horváth et al. [44] on chromatographic retention and using the same chromatographic data needed for pH optimization.

The non-polar stationary bonded phase used, octadecylsilica (ODS), can only be used in the pH range 2–7, so it was only possible to study the first dissociation equilibrium of all the peptides (except glutathione) because correlations between k' and the pH of the mobile phase cannot be obtained over the entire range of pH. For these first equilibria, the chromatographic retention (k') decreases with increase in pH (Fig. 2), suggesting that there is an intermediate form of peptides mainly in zwitterionic form [44,45]. For all the peptides studied, a pK_a close to 3.5 was obtained from the chromatographic data, graphically or by means of a data analysis program. The values obtained were successfully compared in a previous study [46] with pK_1 values determined in the same acetonitrile–water mixtures by the potentiometric method recommended by IUPAC [23,26].

The verified linearity of the log k' values of peptides vs. E_T^N values in the practical range of acetonitrile–water compositions permits, in many cases, mobile phase optimization from only two log k' values, using Eq. 1. On the other hand, if the pK_a values are known, two measurements of k' are sufficient for pH optimization. This model can also be very useful for the optimization of gradient separation since it predicts the chro-

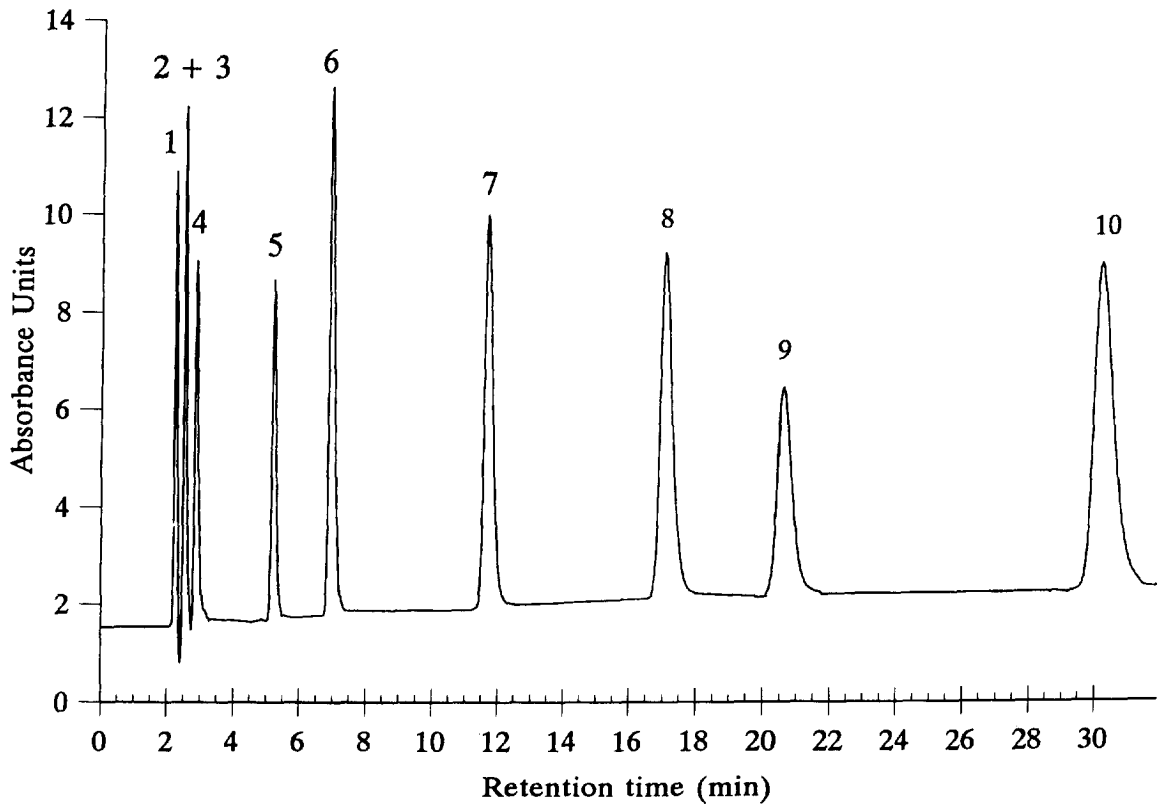

Fig. 3. Separation of citrulline (1), Gly-Gly + Gly-Gly-Gly (2 + 3), glutathione (4), Tyr-Gly-Gly (5), Gly-Gly-Val (6), Ala-Leu-Gly (I) (7), Gly-Gly-Ile (8), Ala-Leu-Gly (II) (9) and Gly-Gly-Phe (10) with a mobile phase of acetonitrile–water (7:93, v/v) containing 0.05% (v/v) trifluoroacetic acid adjusted to pH 2.8 with sodium hydroxide.

matographic behaviour of peptides at different percentages of acetonitrile and at different pHs of the mobile phase.

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