Prediction of chromatographic retention, pK_a values and optimization of the separation of polyphenolic acids in strawberries

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Abstract

Polyphenolic acids are a complex group of compounds that have attracted enormous attention in the last few years because of their biological properties. In this work, the proportion of organic modifier and the pH of acetonitrile–water mixtures used as mobile phases were optimized in order to separate a series of polyphenolic compounds. The linear solvation energy relationship formalism based on the single solvent polarity parameter, E_T^N, was used to predict their chromatographic behavior as a function of the percentage of acetonitrile in the eluent. Moreover, the correlation established between retention and the pH of the aqueous–organic mobile phase was used to optimize the pH of the mobile phase. The optimized mobile phase is composed of acetonitrile and formic acid buffer adjusted to pH 4.25, with 12% (v/v) acetonitrile. Also, the pK_a values of polyphenolic acids in acetonitrile–water mixtures were determined using chromatographic data, and in order to validate the optimized conditions, a series of polyphenolic compounds was studied in strawberries.
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1. Introduction

Polyphenolic acids are a complex group of compounds that have attracted enormous attention in the last few years because of their biological properties. The main reason for investigating polyphenolic compounds stems from their biological importance as secondary plant metabolites, their ecological role and anti-oxidant capacity, their physiological effects, their employment as markers in taxonomic studies,

and their properties related to food quality [1–5]. Polyphenolic compounds are widely distributed in the plant kingdom, principally in the form of by-products generated from plant metabolism. Thus they may accumulate as the end-products of two distinct biochemical pathways: the shkimate pathway, which gives rise to phenylpropanoids and cumarins, and the acetate pathway, which yields the simples phenones and several quinones. Furthermore, they may be generated through an intermediate metabolic pathway that produces flavonoids. In our work, the series of polyphenolic compounds considered included five of the most representative single phenols from the acetate pathway and four phenylpropanoids (Fig. 1).

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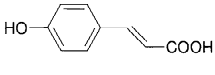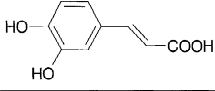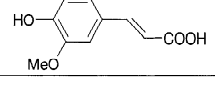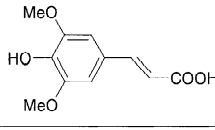*E-mail address:* barbosa@apolo.qui.ub.es (J. Barbosa).
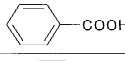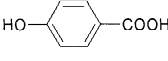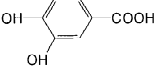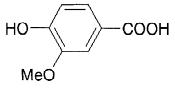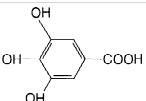
Fig. 1. Structural formulae of the polyphenolic acids studied.

The compounds studied in this work have at least two different ionizable functional groups, which means that their acid–base chemistry involves some protons (Fig. 1). pK_1 values can be associated with the carboxylic acid function and other pK values can be assigned to the phenolic acid function. The exception is benzoic acid, which has only one relevant ionizable functional group associated with the carboxylic group.

In order to establish separation methodologies and determine polyphenolic compounds in biological materials, liquid chromatography (LC) is the method of choice because of its versatility, precision and sensitivity, and results are obtained in a reasonable time. LC is the most widely used analytical technique for the analysis and separation of hydroxylated cinnamic and benzoic acid derivatives [6–11]. Op-timization of the chromatographic resolution of ionogenic solutes in LC is a task that has been actively researched [12–15]. Because of the specific ionization characteristics of these types of solutes, the two most useful optimization parameters are the pH and the percentage of organic modifier in the mobile phase.

The approach for optimizing the organic modifier concentration in the mobile phase during chromato-graphic separations has been tackled in previous works [16–20] by establishing a relationship be-tween the retention parameter and Reichardt's E_T^N scale of solvent polarity. Moreover, the pH of the mobile phase is a major factor influencing the chromatographic behavior of polyphenolic acids because they contain ionogenic functions such as carboxylic and hydroxylic groups. Their retention depends on the percentage of ionized and non-ion-ized species of each compound. Thus, knowledge of the acid–base dissociation constants of polyphenolic acids in acetonitrile–water mixtures (MeCN–water), which are usually used as the mobile phase, can help to improve the analytical method and can lead to a better understanding of the behavior of their bio-chemical solutions.

In MeCN–water mixtures, the influence of the co-solvent on the pH is substantial and, therefore, for successful systematic optimization of the mobile phase, accurate pH measurements in these solvent mixtures are required to understand the retention behavior of the compound investigated. pH measure-ments in MeCN–water, the most widely used mobile phase, are based upon the operational definition of pH [21,22]:

$$pH_x = pH_s + \frac{E_s - E_x}{k_g} \tag{1}$$

where the unknown pH of solution x, pH_x, is related to the pH of a standard reference solution, pH_s, and the e.m.f. values of the potentiometric cell containing the standard, E_s, and the unknown solution, E_x. k_g must be used for practical measurements, usually carried out in cells with glass electrodes, and corre-sponds to the practical slope of the E versus pH function. Thus, the availability of standard buffer solutions of known pH, in the desired solvent mixture previously established [23,24], is the key in

order to make pH measurements in these hydro-organic media [20,21]. Although there are several publications related to the dissociation constants of phenolic acids in water [25–28], there are no data on the pK_a values of polyphenolic acids in MeCN–water, which is the most widely used mobile phase for the separation of these compounds using liquid chromatography.

In this study, the proportion of organic modifier and the pH of the hydro-organic mobile phase were optimized in order to separate a series of nine polyphenolic acids. The relationships between the retention parameters of the compounds and Reichardt's E_T^N scale of solvent polarity were used to optimize the proportion of organic modifier in the mobile phase [16–20], and the relationships between the capacity factor and the pH measured in the different MeCN–water mixtures were used to optimize the pH of the mobile phase. In this work, the pK_a values of the studied polyphenolic acids were also determined using chromatographic data for mixtures with up to 30% (v/v) MeCN. Moreover, in order to validate the optimized conditions, a series of polyphenolic compounds were studied in strawberries.

2. Experimental

2.1. Chemicals and reagents

Analytical reagent grade chemicals were used unless otherwise indicated. Water, with a conductivity of <0.05 μS cm^{-1}, and MeCN (Merck) were of HPLC grade. Sodium hydroxide (Merck), potassium hydrogen phthalate (dried at 110 °C before use, Fluka), formic acid (Panreac) and potassium bromide (Merck) were used. Gallic acid, *p*-hydroxybenzoic acid, protocatechuic acid, vanillic acid, benzoic acid, ferulic acid, *p*-coumaric acid, sinapinic acid and caffeic acid were purchased from Sigma and used without further purification (Fig. 1). Stock standard solutions of the phenolic acids were prepared in water at concentrations of approximately 200 mg l^{-1}. Working solutions were diluted with the corresponding mobile phase to 10 mg l^{-1}. The solutions

were passed through a 0.45 μm nylon filter membrane (MSI) before injection. Stock standard solutions were stored in a freezer and diluted working solutions were stored at 4 °C in the dark.

2.2. Apparatus

The chromatographic equipment consisted of an ISCO pump (Model 2350; Lincoln, NE, USA) with an injector valve with a 20-μl sample loop, and a variable-wavelength absorbance detector (V^4, ISCO) was used when working with 10% (v/v) MeCN–water. The system operates at 280 nm for hydroxybenzoic acids and at 320 nm for hydroxycinnamic acids. The chromatographic system was controlled by ChemResearch Chromatographic Data Management Controller software (version 2.4) running on a personal computer. Also, a chromatographic system consisting of a Shimadzu Model LC 10 ADVP pump with an autoinjector (SIL 10 AD VP) and a diode-array detector system (SPDM 10 A DAD) was used for studies with 20 and 30% (v/v) MeCN–water binary mixtures. This equipment has a column oven (CTO 10 AVP) and a degasser system (DGU 14 A). For strawberry samples, we used a chromatographic system consisting of an Agilent technologies 1100 Series Model liquid chromatograph, a Hewlett-Packard diode-array detector and degasser system with a quartet pump.

A LiChrosphere 100 RP-18 column (Merck) (250×4 mm I.D., 5 μm) was used at ambient temperature. The e.m.f. measurements used to evaluate the pH of the mobile phase were performed using a Model 2002 potentiometer ($\pm$0.1 mV) (Crison Instruments, Barcelona, Spain) with an Orion 8102 Ross combination pH electrode (Orion Research, Boston, MA, USA). A Mettler-Toledo MA 235 pH/ion analyser with a Hanna HI 1332 combination pH electrode was also used. All solutions were thermostated externally at 25$\pm$0.1 °C. The electrode was stabilized in the appropriate MeCN–water mixture prior to e.m.f. measurement. The measurements were performed in triplicate to ensure stability and reproducibility of the potentiometric system. Potassium hydrogenphthalate solutions (0.05 mol kg^{-1}) dissolved in the appropriate MeCN–water medium were used as primary standard buffer references [23,24].

2.3. Chromatographic procedure

Throughout this study, the mobile phases assayed were MeCN–water (10:90, 20:80 and 30:70, v/v) with 0.1% formic and diethylmalonic acids. In recent studies, different kinds of buffers were used [29], but better peak symmetry and resolution were observed when using formic acid. In this work, at low pH values, up to pH 5.5, the buffer capacity of formic acid is sufficient for adjusting the pH, while at higher pH values, diethylmalonic acid was preferred because of its appropriate pK_a values. The pH of the mobile phase was adjusted to between 3 and 7 with sodium hydroxide. The flow-rate was maintained at 1 ml min^{-1}. For each compound and for every mobile phase composition and pH considered, the retention time values, t_R, were determined from three different injections. Retention factors were calculated as $k = (t_R - t_m)/t_m$, where t_m is the retention time of potassium bromide (hold-up time). The retention factors were established for each mobile phase composition and pH studied.

2.4. Data analysis

Theoretical models describing the dependence of the retention factor, k, on the pH of the mobile phase, using reversed-phase sorbents, can be derived by considering pH values in the mobile phase, the activity coefficients and by taking into account the ionization equilibria of the compounds. The capacity factor of an ionizable solute can be expressed by considering that the observed capacity factor, k, is a weighted average of the k of the ionic and neutral forms of the solute [30] according to the molar fractions of these forms in the mobile phase. As an octadecylsilica (C_{18}) stationary phase was used, only pH values in the acidic region were studied, and therefore only the protolytic equilibrium corresponding to the pK_1 values of the polyphenolic compounds are relevant. The overall observed k for these compounds can be given as:

$$k = x_{HA} \cdot k_{HA} + x_{A^-} \cdot k_{A^-} \tag{2}$$

where k_{HA} and k_{A^-} are the capacity factors of the solute in the non-ionized and ionized form, respec-

tively, and x_i is the molar fraction of the species. Substituting the terms x_i, Eq. (2) can be written as:

$$k = \frac{[HA]k_{HA} + [A^-]k_{A^-}}{[HA] + [A^-]} \tag{3}$$

The protolytic equilibrium of the studied polyphenolics, as monoprotic acids in the pH range studied, is governed by the thermodynamic dissociation constant:

$$K_a = \frac{[A^-]\gamma a_{H^+}}{[HA]} \tag{4}$$

By substituting Eq. (4) into Eq. (3) we obtain:

$$k = \frac{k_{HA} + k_{A^-}Ka/\gamma a_{H^+}}{1 + Ka/\gamma a_{H^+}} \tag{5}$$

Eq. (5) can also be written as:

$$k(1 + Ka/\gamma a_{H^+}) = k_{HA} + k_{A^-}Ka/\gamma a_{H^+} \tag{6}$$

Plots of $k(1 + Ka/\gamma a_{H^+})$ vs. $Ka/\gamma a_{H^+}$ suggest the possibility of making predictions of k from just two experimental measurements of k for each compound and the pK_a values. The molar activity coefficients, γ, were calculated using the classical Debye–Hückel expression:

$$\log \gamma = \frac{-AI^{1/2}}{1 + a_0BI^{1/2}} \tag{7}$$

where A and B are the values of the Debye–Hückel constants and a_0 is the ion size parameter in MeCN–water mixtures [18]. The ionic strength, I, of the mobile phase used can be calculated from charge and mass balances at each mobile phase composition, the analytical concentration of the acid in the mobile phase and the pH values and activity coefficients, involving the use of an iterative calculation [31,32].

The usefulness of such equations is two-fold. They define the equilibrium that influences the sorption of organic acids in LC and they permit prediction of the elution behavior as a function of the minimum number of measurements. That is, the k values can be predicted as a function of pH if K_a are known [Eq. (6)], and also, from k values at different pH, the pK_a values can be determined [Eq. (5)].

2.5. Extraction and hydrolysis of the sample

Two procedures were tested in order to obtain the best extraction of polyphenolic compounds from strawberries. In the first, the strawberries were crushed in a food processor. The berry sample (5 g) was diluted with purified water to 15 ml, and 25 ml methanol and 10 ml of 6 mol l^{-1} HCl were added (final HCl concentration 1.2 mol l^{-1}). The mixture was refluxed for 16 h at 85 ± 5 °C. The extract was allowed to cool and was then filtered through a 0.45 μm filter prior to injection (20 μl) into the HPLC system [33]. In the second method, we used hydrolysis at low temperature in complete darkness as optimal conditions for the extraction procedure. This a priori condition is justified by the fact that phenolic compounds exhibit great sensitivity to light and high temperature because they induce phenolic decomposition [2]. The sample (5 g of pulp or 5 ml of juice) was rinsed with 25 ml of methanol into a bottle. Ascorbic acid (80 mg), dissolved in 15 ml purified water, was added as antioxidant. To this mixture was added 10 ml of 6 mol l^{-1} HCl by careful mixing and the solution was sonicated for 2 min. The mixture was shaken in a water bath in the dark at 35 °C. After 16 h, the extract was allowed to cool and filtered prior to injection (20 μl).

3. Results and discussion

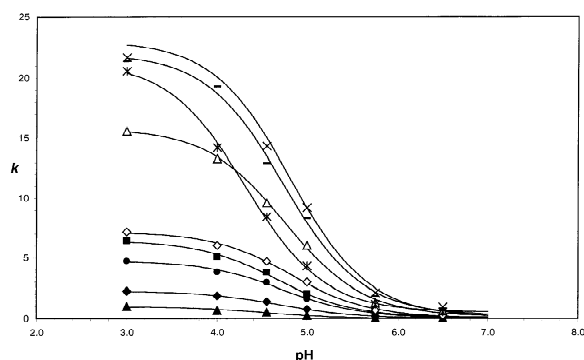Retention factor values, k, for the polyphenolic compounds studied were obtained experimentally in

Fig. 2. Plot of the chromatographic capacity factor, k, of the studied phenolic acids vs. the pH of the mobile phase for 10% (v/v) MeCN: (▲) gallic acid, (♦) protocathechuic acid, (●) *p*-hydroxybenzoic acid, (■) vanillic acid, (◊) caffeic acid, (△) *p*-coumaric acid, (*) benzoic acid, (–) ferulic acid, (×) sinapinic acid. The solid lines indicate the retention factors predicted by Eq. (5).

MeCN–water mixtures with 10, 20 and 30% (v/v) acetonitrile in the mobile phases and at pH 3.5, 4, 4.5, 5, 5.5 and 7. As an example, the retention factor values obtained for MeCN–water mobile phases with 20% (v/v) acetonitrile are given in Table 1.

The retention behavior of all studied compounds can be described by Eq. (5). At a given pH, this equation relates the retention factor of the ionic and neutral forms of the solute and the dissociation constant, taking into account the effect of the ionic strength. As an example, Fig. 2 shows the retention factors for 10% MeCN–water for all compounds plotted versus the pH of the mobile phase. The symbols indicate experimental data and the solid

Table 1

Retention factors, k, of polyphenolic acids at various pH in a mobile phase consisting of an acetonitrile–water (20:80, v/v) mixture

Compound	k					
	pH 3.5	pH 4.0	pH 4.5	pH 5.0	pH 5.5	pH 7.0
Gallic acid	0.494 (0.003)	0.46 (0.01)	0.454 (0.002)	0.13 (0.01)	0.154 (0.002)	0.018 (0.002)
Protocathechuic acid	0.940 (0.001)	0.83 (0.01)	0.747 (0.002)	0.534 (0.002)	0.458 (0.002)	0.042 (0.004)
p-Hydroxybenzoic acid	1.764 (0.002)	1.580 (0.003)	1.445 (0.001)	1.026 (0.003)	0.606 (0.003)	0.095 (0.004)
Vanillic acid	2.026 (0.001)	1.779 (0.002)	1.62 (0.06)	1.107 (0.001)	0.65 (0.01)	0.060 (0.004)
Caffeic acid	1.940 (0.003)	1.79 (0.04)	1.672 (0.001)	1.175 (0.003)	0.694 (0.002)	0.130 (0.002)
p-Coumaric acid	3.904 (0.001)	3.67 (0.03)	3.39 (0.05)	2.399 (0.001)	1.389 (0.001)	0.252 (0.003)
Benzoic acid	7.095 (0.004)	6.0 (0.1)	4.85 (0.04)	2.789 (0.003)	1.39 (0.01)	0.286 (0.003)
Ferulic acid	4.68 (0.02)	4.43 (0.02)	4.073 (0.003)	2.85 (0.01)	1.633 (0.002)	0.315 (0.002)
Sinapinic acid	4.47 (0.06)	4.253 (0.002)	3.880 (0.004)	2.65 (0.02)	1.44 (0.08)	0.335 (0.003)

Values in parentheses are the standard deviations.

Table 2
pK_{a1} values of polyphenolic acids obtained from chromatographic measurements

Compound	Percentage of acetonitrile (v/v)		
	10% Fitted Eq. (5)	20% Fitted Eq. (5)	30% Fitted Eq. (5)
Gallic acid	4.52 (0.10)	4.81 (0.28)	5.04 (0.05)
Protocathechuic acid	4.69 (0.05)	5.10 (0.07)	5.27 (0.08)
p-Hydroxybenzoic acid	4.74 (0.06)	5.12 (0.06)	5.43 (0.15)
Vanillic acid	4.66 (0.05)	5.08 (0.07)	5.32 (0.04)
Caffeic acid	4.83 (0.05)	5.17 (0.05)	5.33 (0.08)
p-Coumaric acid	4.77 (0.03)	5.18 (0.04)	5.43 (0.09)
Benzoic acid	4.33 (0.03)	4.76 (0.05)	4.98 (0.12)
Ferulic acid	4.75 (0.05)	5.17 (0.04)	5.33 (0.06)
Sinapinic acid	4.83 (0.11)	5.13 (0.04)	5.36 (0.07)

Values in parentheses are the standard deviations.

lines the best non-linear regression fits for each compound using Eq. (5). The correlation between the experimental k values of the solutes studied over the whole experimental pH range was good, as shown in Fig. 2. Similar results were obtained for the other percentages of MeCN studied.

Knowledge of the dissociation constants in the hydro-organic media used as mobile phases can be very useful for explaining the chromatographic behavior of the analytes [12,13]. This is one of the reasons why determination of the pK_a values in hydro-organic media is recommended by IUPAC. Various authors [34–36] have reported the advantages of the LC method for evaluating the ionization constants of compounds: small quantities of compounds are required, poor water solubility is not a

serious drawback, and the samples need not be pure. In order to obtain pK_a values of the compounds by chromatographic measurements, Eq. (5) for each compound was verified experimentally and the pK_1 values of the compounds studied were determined from the experimental k values, the pH measurements and the calculated activity coefficient values. The obtained pK_a values, for all percentages of MeCN studied, are listed in Table 2 and were calculated by a non-linear least-squares fit of the data using the programme NLREG [37]. Data in parentheses are the standard deviations. Also, the obtained retention factor values for the neutral and ionic forms of the different cinnamic and benzoic acid species are given in Table 3 for 10, 20 and 30% (v/v) MeCN. The variations in the pK_a values with

Table 3
Chromatographic capacity factors for phenolic species

Compound	Percentage of acetonitrile (v/v)					
	10%		20%		30%	
	k_{HA}	k_{A^-}	k_{HA}	k_{A^-}	k_{HA}	k_{A^-}
Gallic acid	0.99 (0.05)	0.00 (0.04)	0.54 (0.07)	0.01 (0.06)	0.36 (0.01)	0.03 (0.01)
Protocathechuic acid	2.28 (0.05)	0.02 (0.05)	0.93 (0.02)	0.03 (0.02)	0.59 (0.02)	0.05 (0.02)
p-Hydroxybenzoic acid	4.74 (0.14)	0.04 (0.12)	1.76 (0.04)	0.08 (0.04)	0.94 (0.05)	0.14 (0.06)
Vanillic acid	6.46 (0.15)	0.08 (0.13)	2.02 (0.05)	0.06 (0.05)	1.04 (0.02)	0.09 (0.02)
Caffeic acid	7.17 (0.15)	0.07 (0.15)	1.96 (0.03)	0.11 (0.04)	0.91 (0.03)	0.14 (0.03)
p-Coumaric acid	15.77 (0.22)	0.18 (0.21)	3.97 (0.06)	0.20 (0.06)	1.60 (0.05)	0.25 (0.06)
Benzoic acid	21.34 (0.37)	0.52 (0.24)	7.33 (0.17)	0.24 (0.13)	3.49 (0.22)	0.23 (0.14)
Ferulic acid	22.05 (0.54)	0.27 (0.49)	4.78 (0.07)	0.27 (0.07)	1.83 (0.04)	0.26 (0.04)
Sinapinic acid	23.08 (1.14)	0.12 (1.11)	4.60 (0.07)	0.27 (0.07)	1.64 (0.04)	0.24 (0.05)

Values in parentheses are the standard deviations.

percentage of MeCN are different for each compound, although, in general, the pK_a values increase as the MeCN content increases, as expected [38,39].

In order to optimize the proportion of organic modifier, the normalized E_T^N scale of solvent polarity proposed by Reichardt [40] was used:

$$\log k = C + eE_T^N \qquad (8)$$

The correlation between the experimental $\log k$ values of the solutes studied over the whole experimental range of MeCN contents and the E_T^N values of the different mobile phases are shown in Table 4. According to Eq. (8), logarithms of the retention factor correlate linearly ($r^2 > 0.99$) with the polarity of the mobile phase for the compounds studied. This linearity allows prediction of the elution behavior of polyphenolic compounds and hence optimization of the composition for the best separation from only two experimental measurements of the capacity factors for each analyte. In order to examine the accuracy of retention prediction by Eq. (8), the selectivity for adjacent solute pairs in some of the studied mixtures was calculated in the usual way, $\alpha = k_i/k_j$ ($k_i > k_j$). The selectivity between adjacent pairs of compounds in the chromatogram versus percentage of MeCN in the mobile phase is shown in Fig. 3. Points represent experimental selectivity values and lines represent the selectivity obtained using Eq. (8) in order to obtain k values. As can be observed from Fig. 3, there is good concordance of both selectivity values over the whole MeCN range for all compounds studied.
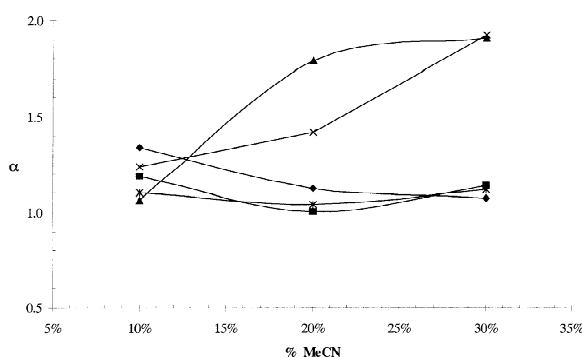
Achieving a good resolution between all of the

Fig. 3. Plot of selectivity, α, vs. percentage of MeCN for pairs of studied polyphenolic acids: vanillic acid/*p*-hydroxybenzoic acid ($\blacklozenge$); caffeic acid/vanillic acid ($\blacksquare$); benzoic acid/*p*-coumaric acid ($\blacktriangle$); sinapinic acid/benzoic acid ($\times$); ferulic acid/sinapinic acid (*). Experimental conditions: mixtures containing acetonitrile–water at different percentages, containing 0.1% (v/v) formic acid, and pH adjusted with NaOH.

analytes of interest is the main goal of chromatographic separation. In terms of fundamental chromatographic parameters, the resolution, R_s, between two adjacent peaks is given by:

$$R_s = (1/4)\sqrt{N}\ \underset{\text{Efficiency}}{}\ [(\alpha - 1)/\alpha]\ \underset{\text{Selectivity}}{}\ [k/(1+k)] \qquad (9)$$

where N is the number of theoretical plates. Although the selectivity term is generally regarded as the most important in LC, full attention must be given to all of the terms in Eq. (9). Fig. 4 shows the variation of R_s for adjacent solute pairs for each

Table 4
Relationships between experimental $\log k$ values for the studied polyphenolic acids versus the E_T^N value of the mobile phase for 10, 20 and 30% (v/v) acetonitrile containing 0.1% formic acid at pH 4

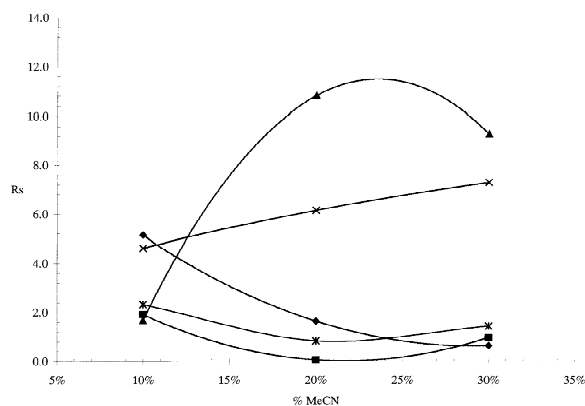| Compound	Equation	r^2
Gallic acid	$\log k = -3.44 + 3.45E_T^N$	0.999
Protocathechuic acid	$\log k = -5.14 + 5.67E_T^N$	0.979
p-Hydroxybenzoic acid	$\log k = -6.75 + 5.85E_T^N$	0.993
Vanillic acid	$\log k = -6.88 + 7.97E_T^N$	0.991
Caffeic acid	$\log k = -8.07 + 9.29E_T^N$	0.992
p-Coumaric acid	$\log k = -8.62 + 10.24E_T^N$	0.996
Benzoic acid	$\log k = -5.87 + 7.39E_T^N$	0.999
Ferulic acid	$\log k = -9.66 + 11.50E_T^N$	0.995
Sinapinic acid	$\log k = -9.74 + 11.55E_T^N$	0.998

Fig. 4. Plot of resolution, R_s, vs. percentage of acetonitrile in the mobile phase for the pairs of studied polyphenolic acids. Symbols and experimental conditions as in Fig. 3.

percentage of MeCN in the mobile phase. Only the resolution between solutes for which the separation is not suitable has been considered in order to simplify the graphical representation. Solid lines indicate the resolution values obtained from two retention measurements using Eqs. (8) and (9) and symbols represent experimental values.

Figs. 3 and 4 show that good chromatographic separation of the polyphenolics studied can be expected in a reasonable time when the MeCN content of the mobile phase is 10–13% (v/v). With a lower proportion of organic modifier, long retention times are obtained and, at higher proportions of MeCN, the R_s values indicate that some phenolic compounds are not well resolved (Fig. 4). We chose as the optimal composition 12% (v/v) MeCN in the mobile phase due to the appropriate selectivity, resolution and retention time.

To optimize the pH of the mobile phase, we can use Eq. (6), a linearized form of Eq. (5). When the pK_a values of the compounds are known, a plot of $k(1 + Ka/(a_{H^+}\gamma))$ vs. $Ka/(a_{H^+}\gamma)$ suggests the possibility of making predictions of k from just two experimental measurements of k for each compound at two different pH values. Fig. 5 shows the corresponding plots for the polyphenolic compounds studied. These relationships also permit the k_{HA} and k_A values to be obtain from the intercept and slope of Eq. 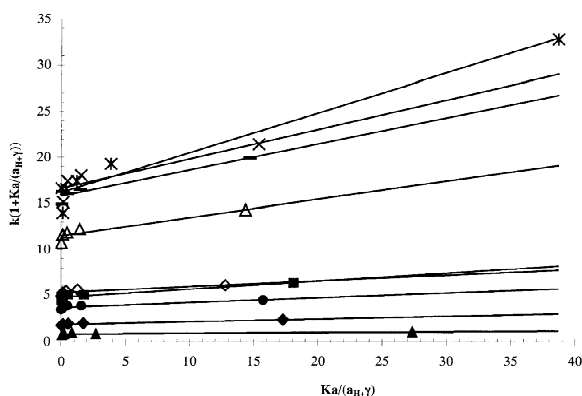(6) (Table 3). To ensure good predictions, the estimated k value should be within the range covered by the two experimental data points. In order to examine the accuracy of the retention predictions obtained using Eq. (6), data measured at pH 3.0 and 7.0 in MeCN–water mixtures as mobile phase were considered. From just two measurements per compound, the k values of the compounds considered at all different pH values were calculated taking into account the pK_1 values. Thus, selectivities were obtained for adjacent solute pairs in the usual way and are plotted vs. selected pH values in Fig. 6. Solid lines indicate α values obtained from two measurements per compound using linearized Eq. (6) and the symbols are experimental α values. The concordance between both sets of values indicates that only two experimental measurements per compound are sufficient to predict accurately the effect of pH and ionic strength on the chromatographic behavior of the compounds.

Fig. 7 shows the variation of R_s for the solute pairs, obtained from k values estimated from the linearized Eq. (6), with the pH of the MeCN–water mobile phase; symbols are experimental R_s values. Figs. 6 and 7 show that good chromatographic separation can be obtained for the polyphenolics studied with MeCN–water mixtures containing 12% (v/v) MeCN when the pH of the mobile phase is
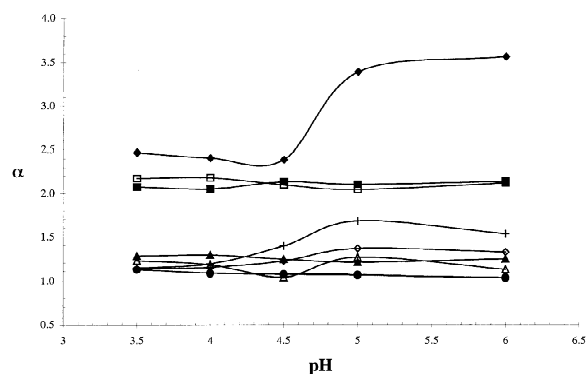

Fig. 5. Plot of $k[Ka/(a_{H^+}\gamma)]$ vs. $Ka/(a_{H^+}\gamma)$ for the studied polyphenolic acids. Mobile phase: 12% (v/v) MeCN–water, containing 0.1% formic acid, pH adjusted with NaOH. Symbols as in Fig. 2.

Fig. 6. Plot of selectivity, α, vs. pH of the mobile phase for pairs of the studied polyphenolic acids: protocathechuic acid/gallic acid (♦); p-hydroxybenzoic acid/protocathechuic acid (■); vanillic acid/p-hydroxybenzoic acid (▲); caffeic acid/vanillic acid (◊); p-coumaric acid/caffeic acid (□); benzoic acid/p-coumaric acid (△); ferulic acid/benzoic acid (+); sinapinic acid/ferulic acid (●); Experimental conditions as in Fig. 4.
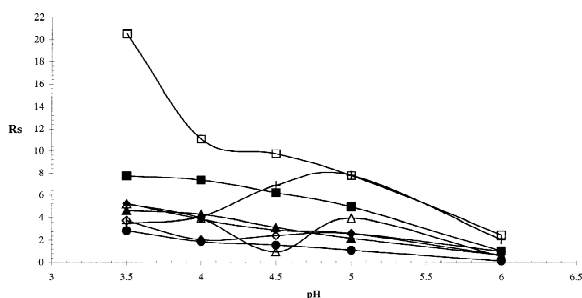
Fig. 7. Plot of resolution, R_s, vs. pH of the mobile phase for pairs of the studied polyphenolic acids: Symbols as in Fig. 6. Experimental conditions as in Fig. 4.

4–4.5. At lower pH values, the retention times would be too long for practical purposes. Fig. 8 shows a chromatogram of the separation of a mixture of standards of the studied compounds in a mobile phase of MeCN–water with 12% (v/v) organic modifier at pH 4.25. A good separation is obtained with an analysis time of 30 min.

In order to validate the method we applied the optimized method to the separation of the polyphenolic compounds present in a strawberry sample. In these fruits, the distribution, as well as the content, of phenolic compounds are basically composed of hydroxybenzoic acids and hydroxycinnamic acids accompanied by flavonoids and anthocyanidines. The compounds most frequently present in this kind of sample are hydroxybenzoic acids such as gallic, *p*-hydroxybenzoic, protocathechuic and vanillic acids, and cinnamic acids such as *p*-coumaric, ferulic and caffeic acid [2,29]. A comparison of the two hydrolysis methods described above was made but, with the first method, only *p*-coumaric acid was obtained, perhaps due to degradation of the compounds at high temperature. Therefore, the second hydrolysis method was applied in this work. From the chromatogram obtained from the juice and pulp of strawberries, we detected gallic acid, protocathechuic acid, *p*-hydroxybenzoic acid, vanillic acid and *p*-coumaric acid. Chromatograms were monitored at 280 nm for standards and samples and the maximum absorption at 320 nm was also checked for cinnamic acids. All the strawberry samples injected were fresh and stored in a deep freeze in the dark. In the chromatogram of strawberry juice (Fig. 9a), *p*-hydroxybenzoic acid and *p*-coumaric acid can clearly be seen in amounts greater than for the other compounds. The chromatogram of strawberry pulp (Fig. 9b) was the same as for strawberry juice, but exhibited smaller amounts of the compounds compared to the juice. Gallic acid,
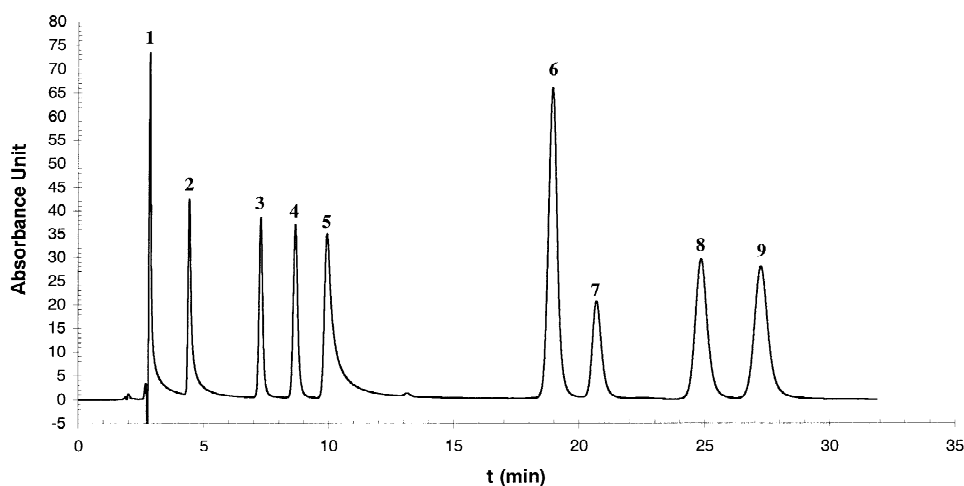

Fig. 8. Chromatogram of a standard mixture of polyphenolic compounds, with a mobile phase of MeCN–water (12:88, v/v) containing 0.1% (v/v) formic acid, with the pH adjusted to 4.25 with NaOH. Gallic acid (1), protocathechuic acid (2), *p*-hydroxybenzoic acid (3), vanillic acid (4), caffeic acid (5), *p*-coumaric acid (6), benzoic acid (7), ferulic acid (8), sinapinic acid (9).
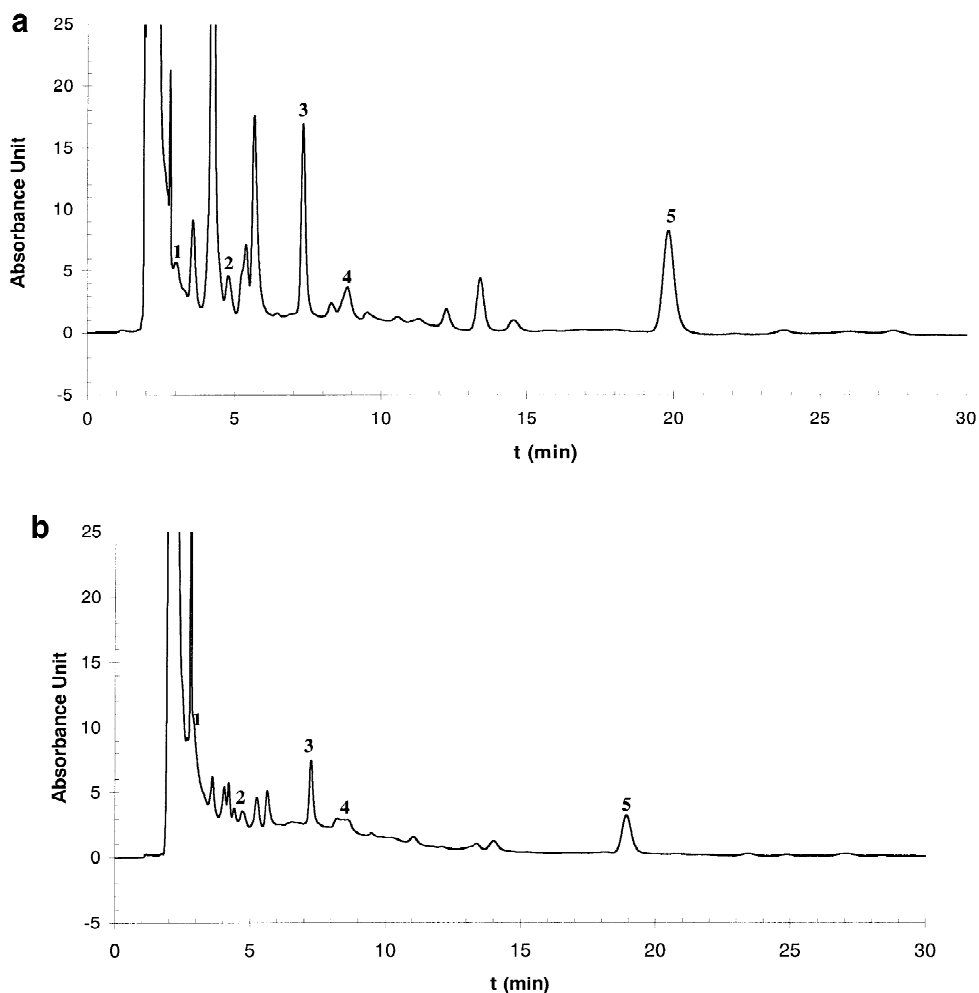
Fig. 9. (a) Chromatogram of strawberry juice, with a mobile phase of MeCN–water (12:88, v/v) containing 0.1% (v/v) formic acid, adjusted to pH 4.25 with NaOH. Gallic acid (1), protocathechuic acid (2), *p*-hydroxybenzoic acid (3), vanillic acid (4), *p*-coumaric acid (5). (b) Chromatogram of strawberry pulp. Gallic acid (1), protocathechuic acid (2), *p*-hydroxybenzoic acid (3), vanillic acid (4), *p*-coumaric acid (5). Experimental conditions as in (a).

protocathechuic acid and vanillic acid were also detected.

4. Conclusion

From this study it can be concluded that the proposed equation that relates the retention of ionizable compounds to the pH of the mobile phase may be of considerable use in the optimization of chromatographic methods. The obtained relationships can be combined with those that relate retention to the solvent composition (E_T^N values) of the mobile phase in order to establish a general model relating the elution behavior of compounds to the composition and pH of the mobile phase. The advantage of optimizing the standard polyphenolic separation is that, as we know the behavior of each compound over a wide range of conditions (organic phase and pH), we can predict the retention time for each percentage of organic phase and pH. As a consequence, when an interfering compound appears it is

possible to change the chromatographic conditions in order to avoid co-elution. For the series of polyphenolic compounds studied, the optimized mobile phase is composed of MeCN–water (12:88, v/v) and formic acid buffer adjusted to pH 4.25 with isocratic elution. The obtained retention factors can also be used for pK_a determination. The proposed method can be applied for the separation of the polyphenolic compounds present in strawberry samples.

Acknowledgements

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