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Journal of Chromatography A, 1077 (2005) 159–169
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Effect of temperature on the chromatographic retention of ionizable compounds
II. Acetonitrile–water mobile phases

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Received 22 January 2005; received in revised form 20 April 2005; accepted 25 April 2005
Available online 13 May 2005

Abstract

The retentive behavior of weak acids and bases in reversed-phase liquid chromatography (RPLC) upon changes in column temperature has been theoretically and experimentally studied. The study focuses on examining the temperature dependence of the retention of various solutes at eluent pH close to their corresponding pK_a values, and on the indirect role exerted by the buffer ionization equilibria on retention and selectivity. Retention factors of several ionizable compounds in a typical octadecylsilica column and using buffer solutions dissolved in 30% (v/v) acetonitrile as eluent at five temperatures in the range from 25 to 50 °C were carefully measured. Six buffer solutions were prepared from judiciously chosen conjugated pairs of different chemical nature. Their pK_a values in this acetonitrile–water composition and within the range of 15–50 °C were determined potentiometrically. These compounds exhibit very different standard ionization enthalpies within this temperature range. Thus, whenever they are used to control mobile phase pH, the column temperature determines their final pH. Predictive equations of retention that take into account the temperature effect on both the transfer and the ionization processes are evaluated. This study demonstrates the significant role that the selected buffer would have on retention and selectivity in RPLC at temperatures higher than 25 °C, particularly for solutes that coelute.

Keywords: Temperature; Reversed-phase selectivity; Acid–base equilibria; Acetonitrile–water mixtures; Ionizable solutes

1. Introduction

Reversed-phase liquid chromatography (RPLC) continues being the most extensively used mode of liquid chromatography. Thus, the proposal of a single approach that would explain retention and selectivity in RPLC as a function of the most significant experimental variables would be highly appreciated. The basic aspects of RPLC retention have been extensively studied by several groups and a variety of different retention models, including hydrophobic, partition and adsorption models have been proposed to explain and predict retention and selectivity [1]. Today, the dependence of the retention on a single or combined experimental variables such as the solvent type and composition, mobile phase pH, chemical nature of the analyte and of the stationary phase is quite understandable [2]. However, the influence of temperature as a critical variable governing the retention mechanism has received much less attention [3–12].

Whenever the reversed-phase separation of weak acid–base electrolytes has been the target, mobile phase pH is usually the first trial. Models predicting that plots of ln k as a function of pH will be sigmoidal with an inflection point corresponding to the pK_a of the solute have been theoretical deduced and experimentally corroborated [13]. These models were subsequently extended to predict retention as a function of pH, ionic strength and solvent composition [14–17]. Rosés and Bosch [18] and Espinosa et al. [19] have critically demonstrated that these sigmoidal functions refer always to the solvent system used as mobile phase, not to the pK_a values

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doi:10.1016/j.chroma.2005.04.070

in water. All these studies were carried out under isothermal conditions.

Horváth and coworkers [20] were the pioneers in conducting studies that revealed the combined influence of temperature and pH on the retention of weak electrolytes. More than a decade elapsed before analysts again focused on the temperature effects over retention of ionogenic solutes [21–27]. This was probably due to the fear in the potential damage towards the silica-based packings upon heating their columns with mobile phases containing buffers. Nowadays, the availability of chemically and thermally more stable chromatographic supports should overcome this drawback and change our perspective. On the other hand, from the point of view of the complexity of real samples, the consideration of temperature as another leading variable allows to add another degree of freedom to optimize resolution of multiple peaks from a sample.

Our goal in this series of studies is to evaluate the combined effect of pH and temperature on the selectivity of ionizable compounds when they elute with different buffers in the usual RPLC mobile phases. In this particular study, we focus on buffers that have the same pH in 30% (v/v) acetonitrile–water and at 25 °C but quite different enthalpies of ionization.

Methanol–water and acetonitrile–water solvent mixtures are, by far, the most used mobile phases in liquid chromatography. In a previous work we have discussed a simple approach to predict the effect of temperature on the retention of ionogenic solutes as a function of the nature of the buffer in a mixture containing 50% (w/w) methanol [27]. In this paper, we shall extend these relationships to acetonitrile–water mobile phases. However, to test the truthfulness of the previously proposed approach we require information about dissociation constants (pK_a) of the buffer substances and of the solutes studied at different temperatures and acetonitrile–water compositions. Measurements of acid–base equilibria of ionizable compounds in acetonitrile–water mixtures are very scarce [28–32] and even more if we aimed at temperatures different from 25 °C. Therefore, potentiometric measurements were conducted to obtain pK_a values of those compounds used to prepare buffer solutions at 25% (w/w) acetonitrile–water mixtures in the temperature range of 15–50 °C. This solvent composition corresponds exactly to 30% (v/v) of the mixture at 25 °C.

2. Theory

2.1. Chromatographic retention of ionizable analytes

For a monoprotic analyte, HA, with an acid–base equilibrium ruled by an acidity constant $K_{a(an)}$, the retention factor will be a function of the mobile phase pH according to [13,14,16,18]

$$k = \frac{k_{HA} + k_A(K_{a(an)}/a_{H^+})}{1 + (K_{a(an)}/a_{H^+})} \quad (1)$$

In this equation, k_{HA} and k_A refer to the retention factors of the fully protonated and the deprotonated forms of the ionogenic compound, and a_{H^+} is the activity of the hydrogen ion in the mobile phase. The standard states for both K_a and pH are referred to the solutes infinitely diluted in the solvent mixture. By following the nomenclature recommended by IUPAC [33,34] for these quantities: ${}^{s}_{s}$pH and ${}^{s}_{s}$pK_a represent pH and pK_a, respectively.

Since a_{H^+}, is usually controlled by the relative concentrations of a conjugated pair, HB/B, Eq. (1) can be re-written as:

$$k = \frac{k_{HA} + k_A(K_{a(an)}/K_{a(buff)})(m_B/m_{HB})}{1 + (K_{a(an)}/K_{a(buff)})(m_B/m_{HB})} \quad (2)$$

where $K_{a(buff)}$ represents the buffer acidity constant and m_{HB} and m_B are the analytical molal concentrations of the acid and its conjugated base, respectively.

The thermodynamic energies of transfer $\Delta_t G^\circ_{HA}$ and $\Delta_t G^\circ_A$ along with the standard free energies of the ionization of the analyte, $\Delta G^\circ_{a(an)}$, and of the buffer, $\Delta G^\circ_{a(buff)}$, are implicit in Eq. (2):

$$k = \varphi \frac{e^{[-\Delta_t G^\circ_{HA}/RT]} + (m_B/m_{HB})\, e^{[-\Delta_t G^\circ_A/RT]}\, e^{[-(\Delta G^\circ_{a(an)} - \Delta G^\circ_{a(buff)})/RT]}}{1 + (m_B/m_{HB}) e^{[-(\Delta G^\circ_{a(an)} - \Delta G^\circ_{a(buff)})/RT]}} \quad (3)$$

where φ represents the chromatographic phase ratio.

Any change in temperature will change *all* the equilibrium phenomena: the transfers of solutes A and HA from the eluent to the stationary phase and also the ionization constants of the solute and of the buffer $K_{a(an)}$ and $K_{a(buff)}$ and, as a direct consequence, the mobile phase pH.

The effect of temperature on all these equilibrium processes can be explicitly taken into account by splitting the corresponding standard free energies into the enthalpic and the entropic terms as:

$$k = \varphi \left\{ \frac{e^{[-\Delta_t H^\circ_{HA}/RT]}\, e^{[\Delta_t S^\circ_{HA}/R]} + (m_B/m_{HB})\, e^{[-(\Delta_t H^\circ_A + \Delta H^\circ_{a(an)} - \Delta H^\circ_{a(buff)})/RT]}\, e^{[(\Delta_t S^\circ_A + \Delta S^\circ_{a(an)} - \Delta S^\circ_{a(buff)})/R]}}{1 + (m_B/m_{HB})\, e^{[-(\Delta H^\circ_{a(an)} - \Delta H^\circ_{a(buff)})/RT]}\, e^{[(\Delta S^\circ_{a(an)} - \Delta S^\circ_{a(buff)})/R]}} \right\} \quad (4)$$

Within a small range of temperature, enthalpies of each process can be considered constant, thus applying the logarithm and differentiating with respect to (1/T), the apparent enthalpy of the chromatographic process, $\Delta H^\circ_{app(an)}$ can be estimated [20,22,27]:

$$\Delta H^\circ_{app(an)} = -R\left(\frac{d\ln k}{d(1/T)}\right) = \left[\frac{\Delta_t H^\circ_{HA} + gw\Delta_t H^\circ_A}{(1+gw)}\right] + \left[\frac{w(g-1)(\Delta H^\circ_{a(an)} - \Delta H^\circ_{a(buff)})}{(1+gw)(1+w)}\right] \quad (5)$$

where $g = k_A/k_{HA}$, the ratio between retention factors of both forms of the solute, and $w = [(m_B/m_{HB})(K_{a(an)}/K_{a(buff)})]$. The g ratio is independent of the eluent pH; for a typical reversed-phase, $0 < g < 1$ for neutral weak acids, and $g > 1$ for cationic acids such as protonated amines. Expression (5) allows us to predict the trend in the retentive behavior of an ionogenic solute in a mobile phase system containing a buffer B upon changes in column temperature. The changes in temperature will shift the eluent pH according to the sign and absolute value of $\Delta H^\circ_{a(buff)}$. At once, $\Delta H^\circ_{a(an)}$ will dictate the own shift in the analyte acid–base equilibrium due to the change in temperature, only. As a consequence of these two combined effects, the relative ratio between HA and A at the new temperature will determine the new retention factor. The first term in Eq. (5) reflects a weighted average between the standard enthalpies of transfer of both HA and A from the mobile to the stationary phase and considering that a unique partition retention mechanism takes place their values are usually negative. The second term in the equation can be either negative or positive depending on both ionization enthalpies and on the $(g - 1)$ coefficient. In other words, the ionization enthalpies of the selected buffer would strongly affect the dependence of k on temperature.

3. Experimental

3.1. Instrumentation

A combined glass electrode, Ross Combination Electrode Orion 8102 SC, in a commercial pH-meter (Crison micropH 2002) was used for ${}^{s}_{w}$pH measurements of those buffer solutions prepared for determining ionization constants (pK_a). The precision was estimated to be within ±0.01 pH units. Aqueous standards and buffer solutions were placed into a temperature-controlled bath and a thermometer calibrated at ±0.1 °C was used for temperature readings.

The HPLC operations were carried out in a Shimadzu LC-10A instrument, equipped with helium degasser, LC-10AD pump, Sil-10A autoinjector, SPD-M10A diode array detector and computer-based Class-VP Chemstation. A wavelength maximum at 254 nm was chosen for detecting analytes and at 200 nm for the dead volume marker (KBr).

A 150 mm × 4.6 mm I.D. X-Terra® MS-C18 column (Waters) was used for all the measurements. This silicon organic-inorganic hybrid material exhibits no silanol activity as demonstrated by the lack of retention of the cation Li^+ within the pH range 3–11 [35,36]. The column, along with a 20 cm stainless steel capillary tube for preheating the incoming mobile phase, was immersed in a temperature controlled thermostatic bath. Temperature was taken with a thermometer calibrated at ±0.1 °C.

pH measurements of mobile phase solutions were conducted with a Schott Blueline combined glass electrode, connected to a 702 SM Titrino pH-meter (Metrohm) with a precision of ±0.01 pH units.

3.2. Chemicals

The solvents used were acetonitrile HPLC-grade (99.9%, Mallinckrodt) and water purified by a Milli-Q® system (Simplicity 185, Millipore). Buffers were prepared from the reagents p.a. grade or better: phosphoric acid (Merck, 85%), potassium dihydrogen phosphate (Merck p.a. >99.5%), disodium hydrogen phosphate (Merck, >99%), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) (Baker z.a. >99.5%), hydrochloric acid (Merck, 25% in water), 1-aminobutane (Aldrich, >99.5%), glacial acetic acid (Merck p.a., 99–100%), sodium acetate anhydrous (Merck, >99%), piperazine (Fluka, >99%). Solutes (reagent grade or better) were dissolved in 30% (v/v) acetonitrile–water mixtures.

3.3. pK_a measurements

Acidic constants of the compounds used to prepare the chromatographic buffer solutions were measured in 25% (w/w) acetonitrile–water over the temperature range of 15–50 °C. At least five solutions containing different ratios between each component of the conjugate pair were prepared; the total molality was approximately 0.05 molal. ${}^{s}_{w}$pH(T) was carefully measured after thermal equilibrium of these solutions and of the aqueous standards. The experimental ${}^{s}_{w}$pH values have been converted into the ${}^{s}_{s}$pH by subtraction of the δ-value, being $\delta = -0.06$ the obtained value for the used acetonitrile–water mixture at 25 °C. Because of the lack of δ values at different temperatures we assumed the constancy of this value with temperature based on previous results obtained in methanol–water mixtures. Mixtures which contain from 10 to 50% methanol which exhibited a δ-shift of 0.04 pH units by raising temperature from 20 to 50 °C [37]. The corresponding ${}^{s}_{s}$p$K_a(T)$ were computed by introduction of corrections for non-idealities as follows:

$$ {}^{s}_{s}\mathrm{p}K_a(T) = {}^{s}_{s}\mathrm{pH}(T) - \log\left(\frac{m_X}{m_{HX}}\right) - \log\left(\frac{\gamma_X(T)}{\gamma_{HX}(T)}\right) \quad (6) $$

where m_i is the molality of species i in solvent s at the equilibrium, and $\gamma_i(T)$ refers to the activity coefficients of i in solvent mixture at each temperature. Activity coefficients of uncharged compounds were considered to be unity and the molal activity coefficients of ionic species were calculated from the ionic strength (I) of the solution by using the Debye–Hückel equation:

$$ -\log\gamma_i = \frac{z_i^2 A\sqrt{I}}{1 + a_0 B\sqrt{I}} \quad (7) $$

where z is the charge of the i ion, A and B are solvent- and temperature-dependent parameters, which can be estimated from the densities and dielectric constants of the medium at each temperature. The product a_0B at each temperature was estimated by following the Bates–Guggenheim convention [34,38,39]:

$$ (a_0 B)_T = 1.5\sqrt{[({}^{w}\varepsilon^{s}\rho)/({}^{s}\varepsilon^{w}\rho)]_T} \quad (8) $$

Table 1
Macroscopic properties of 30% (v/v) or 25.03% (w/w) acetonitrile–water mixtures at several temperatures

Temperature (°C)	Density, ρ (kg dm^{-3})	Dielectric constant, ε	A	a_0B
15	0.9510	71.49	0.6018	1.574
20	0.9485	69.70	0.6085	1.572
25	0.9454	67.95	0.6152	1.570
30	0.9418	66.24	0.6222	1.567
35	0.9379	64.58	0.6294	1.564
40	0.9341	62.96	0.6370	1.562
45	0.9305	61.38	0.6450	1.560
50	0.9273	59.84	0.6534	1.558

${}^{w}\varepsilon$, ${}^{w}\rho$, ${}^{s}\varepsilon$ and ${}^{s}\rho$ denote the dielectric constants and the densities of water and of the acetonitrile–water solvent mixture at the given temperature, respectively. The A parameter can be computed from [40]:

$$A = \frac{1.8246 \times 10^6 \sqrt{{}^{s}\rho}}{({}^{s}\varepsilon T)^{3/2}} \quad (9)$$

A bibliographic search revealed very scarce data about these physical properties within a wide temperature range [41–46]. From this data set the required values were interpolated or extrapolated and are reported in Table 1.

3.4. Chromatography

Mobile phase solutions were pre-mixed at a fixed acetonitrile composition of 30% (v/v). This solvent content corresponds exactly to 25.03% (w/w) of acetonitrile in water at 25 °C. Buffer solutions were prepared at this solvent composition in the molal scale, thus independent of temperature, by mixing the corresponding reagents. Concentrations, ionic strengths, the measured ${}^{s}_{w}$pH at room temperature and that corrected at the other experimental temperatures are reported in Table 2.

The chromatographic column was kept at the corresponding temperature for at least 1 h before injection. The eluent flow-rate was 1 mL/min and the injection volume was 5 μL. The hold-up time was measured with potassium bromide or uracil. Solute retention times at each temperature are the average of three determinations. Their precision, as measured by the relative standard deviation, was better than 0.6%. Since the extracolumn contributions could be non-negligible for the less retained solutes, retention factors k_i were calculated from

$$k_i = \frac{t_i - t_0}{t_0 - t_{ex}} \quad (10)$$

where t_i is the retention time measured at the peak maximum, t_0 is the elution time of the void volume marker and t_{ex} is the time spent by the marker between the injector and detector connected without column. Since this early eluted peak is highly tailed, the extracolumn and also the hold-up times were taken at an acquisition sampling rate of 0.24 s, and they were computed from calculation of the first statistical moment. All results are the average of at least triplicate injections.

4. Results and discussion

4.1. Dissociation constants

The ${}^{s}_{s}pK_a(T)$ values of those organic and inorganic weak acids, which were used to prepare buffer solutions in 30% (v/v) acetonitrile in water, were potentiometrically measured over the range of temperature from 15 to 50 °C. Eq. (6) was used to calculate the ${}^{s}_{s}pK_a$ values from pH measurements. In that equation, m_i refers to the molality of i once the equilibrium is reached, i.e., $m_X = m^{\circ}_X + m_{H^+} - m_{S^-}$, and $m_{HX} = m^{\circ}_{HX} - m_{H^+} + m_{S^-}$, where m°_i are the analytical molal concentrations and m_{H^+} and m_{S^-}, the molality of protons and of solvent lyate anions, respectively. For all but phosphoric acid solutions, analytical molalities are significantly higher than both m_{H^+} and m_{S^-} and thus, the substitution $m_X \cong m^{\circ}_X$ and $m_{HX} \cong m^{\circ}_{HX}$ is a valid approach. For ${}^{s}_{s}pK_{a1}$ of phosphoric acid only m_S was neglected, and m_{H^+} was considered as $m_{H^+} = 10^{-{}^{s}_{s}pH}/\gamma$.

The results of ${}^{s}_{s}pK_a$ and the corresponding standard deviations are presented in Table 3. Unfortunately, very few data in acetonitrile–water mixtures have been reported in the literature, and most of them are data measured at 25 °C. We have included those data into the table; all of them compare favorably with the values reported here.

Standard enthalpies of ionization are presented at the bottom of Table 3. We also included $\Delta^{w}_{w}H^{\circ}_a$ compiled from the literature. Within the temperature range of 35 °C the com-

Table 2
Buffer solutions prepared in 25% (w/w) acetonitrile–water

Buffer solutions		Concentrations (mmolal)	Ionic strength (mmolal)	${}^{s}_{w}pH^{25C}_{exp}$	${}^{s}_{w}pH(T)$[a]			
					31.2 °C	37.0 °C	43.8 °C	50.0 °C
B1	H_3PO_4–KH_2PO_4	18.95, 5.98	9.8	2.42	2.44	2.45	2.47	2.49
B2	Acetic acid–sodium acetate	16.42, 7.68	7.7	4.95	4.95	4.95	4.95	4.95
B3	Piperazine–HCl	25, 40.05	55	4.95	4.83	4.71	4.59	4.47
B4	KH_2PO_4–Na_2HPO_4	11.1, 13.9	53	7.84	7.83	7.82	7.82	7.81
B5	*Tris*–HCl	25, 17.3	17.3	7.85	7.69	7.53	7.37	7.21
B6	Butylamine–HCl	39, 14.5	14.5	10.71	10.50	10.29	10.08	9.87

[a] ${}^{s}_{w}pH(T)$ calculated from the corresponding ${}^{s}_{s}pK_a$ values measured in this work.

Table 3
$^{s}_{s}pK_a(T)$ values of weak acids used as buffer in 25% (w/w) acetonitrile–water mixtures at several temperatures

Temperature (°C)	H_3PO_4		Acetic acid	Piperazine–2HCl	Tris–HCl	Butylamine–HCl
	pK_{a1}	pK_{a2}				
15	2.75 (±0.01)[a]	7.88 (±0.01)	5.45 (±0.01)	5.42 (±0.01)	8.25 (±0.01)	10.70 (±0.02)
20	2.77 (±0.01)	7.88 (±0.01)	5.45 (±0.01)	5.31 (±0.01)	8.10 (±0.01)	10.51 (±0.01)
25	2.79 (±0.01)	7.86 (±0.01)	5.45 (±0.01)	5.20 (±0.01)	7.96 (±0.01)	10.32 (±0.02)
	2.76[b]	7.78[b]	5.40[b]			10.41[c]
30	2.81 (±0.01)	7.85 (±0.01)	5.45 (±0.01)	5.10 (±0.01)	7.83 (±0.01)	10.14 (±0.02)
35	2.83 (±0.01)	7.84 (±0.01)	5.45 (±0.01)	4.99 (±0.01)	7.69 (±0.01)	9.97 (±0.02)
40	2.85 (±0.01)	7.83 (±0.01)	5.45 (±0.01)	4.89 (±0.01)	7.56 (±0.01)	9.80 (±0.01)
45	2.86 (±0.01)	7.83 (±0.01)	5.45 (±0.01)	4.79 (±0.01)	7.44 (±0.01)	9.64 (±0.01)
50	2.87 (±0.01)	7.83 (±0.01)	5.45 (±0.01)	4.69 (±0.01)	7.33 (±0.01)	9.47 (±0.01)
$^{s}_{s}\Delta H^{\circ}_{a}$ (kJ mol^{-1})	−6.3 (±0.2)	3.05 (±0.17)	−0.34 (±0.06)	37.2 (±0.3)	47.0 (±0.1)	62.3 (±0.1)
$^{w}_{w}\Delta H^{\circ}_{a}$ (kJ mol^{-1})[d]	−7.9	4.1	−0.4	29.8	47.6	58

[a] Values in parentheses are standard deviations.
[b] pK_a values taken from the literature [28,55].
[c] Estimated from equations given in [32].
[d] From refs. [56,57].

pounds behave with a typical van't Hoff dependence. From these enthalpy values, it is clear that the mobile phase pH shift will be very dependent of the buffer type solution. Whereas weak acid pK_a's are almost independent of solution temperature, amine salts become stronger acids in 30% (v/v) acetonitrile–water as the temperature is raised from 15 to 50 °C.

4.2. Chromatographic results

Several methods for the measurement of the holdup time have been proposed [47–49]; but they often lead to different t_0 values. When working with a typical reversed phase stationary phase, the most reasonable method seems to be the use of an ionic solute, such as $NaNO_3$ or KBr, which are not partitioned into the stationary phase. For the most basic mobile phase (B6) it was not possible to detect bromide ions and in this case a very polar compound often chosen as void volume marker, uracil, was used. Potassium bromide was used for the rest of the mobile phases and the expected constancy of the hold-up time between different buffer conditions was not observed: the obtained values vary depending not only on temperature but also on the buffer type. Similar behavior has been discussed previously [15,50]. In view of these discrepancies, the average of the t_0 values measured with KBr in different buffer solutions at each temperature was taken. Thus, dead time were 1.436 (SD = 0.034), 1.432 (SD = 0.031), 1.427 (SD = 0.027), 1.423 (SD = 0.026) and 1.417 (SD = 0.021) at 25, 31.5, 37, 42.5 and 50 °C, respectively. These values are consistent with a column porosity of 0.61, in good agreement with the column porosity estimated by Gritti and Guiochon [51].

Chromatographic retention of a group of ionogenic analytes in an octadecylsilica reversed-phase column and using several buffer solutions in a solvent mixture containing 30% (v/v) acetonitrile and at five temperatures was measured. Solutes were chosen according to their acidic dissociation constants: a group of them have pK_a values close to the pH of the buffered mobile phases B2 and B3 at 25 °C (pH ∼ 5). In these buffer mobile phase solutions, these solutes will be partially ionized and, therefore, retention factors should reflect this ionization status: *k* values are expected to be in between those at a pH larger and smaller than pH 5. A second group of solutes have pK_a values around pH 8, the pH of buffer solutions B4 and B5. The solutes studied along with their pK_a values are given in Table 4. The chromatographic data measured in all buffer solutions are given in Table 5. Retention factor values of solutes measured with mobile phases B2 and B3 at 25 °C are slightly smaller when using piperazine buffer (B3) than acetic buffer (B2), even using exactly the same temperature, solvent composition and pH (25 °C) in both cases. A possible explanation of this observation is the more than seven-fold

Table 4
Acid–base dissociation constants of the solutes in pure water and in 30% (v/v) acetonitrile–water mixtures at 25 °C

Solute	Abbreviation	$^{w}_{w}pK_a$ (25 °C)[a]	$^{s}_{s}pK_a$ (25 °C)[b]
Benzoic acid	S1	4.21	5.05
2-Methylbenzoic acid	S2	3.91	4.64
3-Methylbenzoic acid	S3	4.21–4.24	4.98
3-Bromobenzoic acid	S4	3.80–3.82	4.56
Cinnamic acid	S5	4.41	5.19
4-Amino-2-hydroxybenzoic acid	S6	3.66	
4-Methylaniline	S7	5.08	4.73
N-Ethylaniline	S8	5.12	4.77
4-Ethoxyaniline	S9	5.24	4.81
Benzimidazole	S10	5.48	
2,4,6-Trimethylpyridine	S11	7.25	6.75
2-Nitrophenol	S12	7.24–7.23	7.71
4-Nitrophenol	S13	7.15	7.81
2,6-Dinitrophenol	S14	3.69–3.71	3.72
4-Aminopyridine	S15	9.12	
Codeine phosphate	S16	8.21	7.91

[a] From refs. [57,58].
[b] From refs. [29,30,32,59].

Table 5
Retention factors of solutes in six buffer solutions at five temperatures

Temperature (°C)	Solute															
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16
Buffer B1																
25	2.28	4.11	4.55	7.42	4.81	0.84	0.16	0.19	0.24	0.07	3.43	6.49	3.21	5.66	–	–
31.2	2.13	3.74	4.19	6.68	4.36	0.75	0.17	0.21	0.24	0.09	3.06	5.79	3.05	4.90	–	–
37	1.98	3.48	3.86	6.09	4.00	0.68	0.17	0.22	0.24	0.09	2.69	5.26	2.72	4.32	–	–
43	1.78	3.11	3.46	5.33	3.53	0.61	0.18	0.23	0.22	0.07	2.35	4.63	2.35	3.70	–	–
50	1.65	2.85	3.15	4.82	3.22	0.55	0.16	0.24	0.21	0.06	2.10	4.17	2.09	3.26	–	–
Buffer B2																
25	1.13	1.87	2.51	2.03	2.85	0.48	2.36	6.98	1.48	0.52	–	6.15	3.31	0.91	0.13	0.40
31.2	1.03	1.71	2.25	1.78	2.55	0.46	2.35	7.03	1.52	0.50	–	5.51	2.90	0.79	0.10	0.35
37	1.00	1.68	2.24	1.76	2.52	0.45	2.43	7.32	1.61	0.53	–	5.36	2.79	0.77	0.11	0.36
43	0.89	1.50	1.80	1.55	2.21	0.42	2.36	7.04	1.61	0.51	–	4.73	2.39	0.67	0.10	0.32
50	0.79	1.31	1.71	1.31	1.87	0.38	2.20	6.59	1.57	0.48	–	4.09	2.06	0.55	0.07	0.29
Buffer B3																
25	1.01	1.68	2.27	1.82	2.66	0.47	2.28	6.81	1.39	0.48	–	6.29	3.41	0.84	0.07	0.29
31.2	1.08	1.78	2.34	1.92	2.65	0.44	2.10	6.15	1.30	0.42	–	5.58	2.97	0.82	0.06	0.30
37	1.12	1.86	2.38	2.04	2.59	0.41	1.94	5.62	1.23	0.39	–	5.04	2.64	0.82	0.07	0.30
43	1.15	1.90	2.37	2.13	2.56	0.38	1.79	5.08	1.14	0.36	–	4.49	2.29	0.82	0.08	0.29
50	1.15	1.92	2.34	2.22	2.47	0.35	1.65	4.62	1.10	0.33	–	4.04	2.03	–	0.07	0.28
Buffer B4																
25	0.13	0.12	0.20	0.37	0.30	0.54	3.43	11.17	2.45	0.86	4.31	3.38	1.55	0.62	0.08	1.17
31.2	0.12	0.12	0.20	0.35	0.29	0.49	3.14	10.20	2.30	0.80	4.21	2.81	1.26	0.57	0.08	1.31
37	0.12	0.12	0.20	0.34	0.27	0.46	2.93	9.42	2.18	0.75	4.11	2.39	1.05	0.51	0.09	1.46
43	0.11	0.11	0.19	0.31	0.26	0.42	2.72	8.60	2.06	0.69	3.98	1.99	0.84	0.48	0.09	1.60
50	0.12	0.12	0.19	0.31	0.25	0.40	2.53	7.88	1.94	0.66	3.84	1.70	0.70	0.44	0.11	1.74
Buffer B5																
25	–	0.05	0.12	0.31	0.19	0.51	3.33	10.75	2.38	0.85	4.40	3.00	1.29	0.42	0.18	1.53
31.2	–	0.07	0.12	0.30	0.19	0.49	3.05	9.77	2.24	0.79	4.11	3.00	1.29	0.40	0.17	1.43
37	–	0.07	0.14	0.29	0.20	0.45	2.85	9.05	2.13	0.75	3.88	2.98	1.29	0.38	0.16	1.38
43	–	0.08	0.14	0.28	0.21	0.43	2.64	8.26	1.96	0.70	3.63	2.92	1.27	0.36	0.15	1.28
50	–	0.08	0.15	0.27	0.22	0.41	2.47	7.59	1.91	0.66	3.43	2.83	1.24	0.33	0.15	1.22
Buffer B6																
25	0.08	0.09	0.16	0.33	0.24	0.32	3.20	10.75	2.27	0.78	–	0.62	0.25	0.93	0.30	2.30
31.2	0.08	0.08	0.16	0.31	0.23	0.33	2.97	9.84	2.16	0.74	–	0.57	0.24	0.90	0.29	2.33
37	0.07	0.08	0.15	0.30	0.22	0.34	2.75	9.03	2.05	0.70	–	0.51	0.22	0.87	0.28	2.35
43	0.06	0.08	0.14	0.28	0.20	0.34	2.55	8.15	1.93	0.66	–	0.46	0.21	0.85	0.27	2.37
50	0.06	0.07	0.13	0.26	0.19	0.34	2.38	7.44	1.83	0.63	–	0.41	0.20	0.84	0.27	2.38

difference in ionic strength between both buffer solutions (see Table 2). Under this hypothesis, a higher ionic strength leads to an increase in the ratio ionized/neutral form of any analyte at the equilibrium, independently if this change increases or decreases the dissociation. An increase of the ionized/neutral ratio implies a decrease in retention time in reversed phase systems. By using Debye–Hückel equation, we can roughly estimate that the ionic strength difference (0.055 molal versus 0.008 molal) implies a reduction of about 15% in the activity coefficient of the ionic species, and therefore, the ratio ionized/neutral form would be similarly affected. Even though, analyzing the retention factor values of the solutes at the equilibrium when using the basic pair of mobile phases B4 and B5, the behavior is not completely explained by the previous hypothesis.

In Fig. 1, we show the superposed chromatograms of five solutes (3-bromobenzoic acid, 2-methylbenzoic acid, cinnamic acid, 4-methylaniline and 4-ethoxyaniline). They were eluted from an octadecylsilica column with a mobile phase containing acetic-acetate buffer (${}^{s}_{w}\mathrm{pH} = 4.95$) in 30% (v/v) acetonitrile and at three temperatures (25, 37 and 50 °C). As can be observed, it is feasible to partially resolve these analytes at 25 °C, although resolution between 3-bromobenzoic and 2-methylbenzoic acid is lower than one. In this case, an increase in column temperature from 25 to 37 °C leads to a lost of resolution due to a strong decrease in retention of 3-bromobenzoic and of 2-methylbenzoic acid whereas ethoxyaniline has practically the same elution time at both temperatures. Similarly, retention of the other carboxylic acid, cinnamic acid, exhibits a strong dependence with temperature, and it is not separated from 4-methylaniline. A further increase in temperature from 37 to 50 °C leads to coelution of 3-bromobenzoic and 2-methylbenzoic acid which now elute before ethoxyaniline.

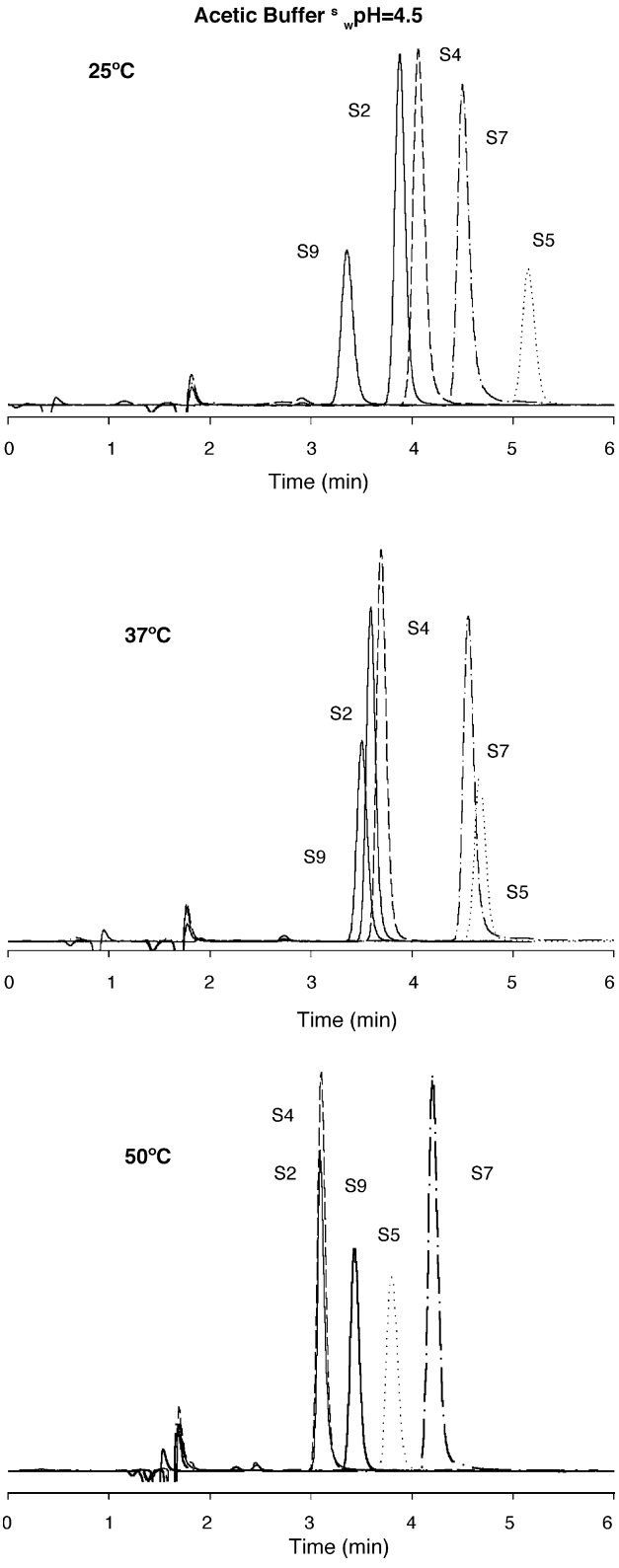

Fig. 1. Influence of temperature on retention and selectivity. Chromatograms of analytes eluted at 25, 37 and 50 °C. Column: MS X-Terra C18 (150 mm × 4.6 mm I.D.). Acetic/acetate buffer $^{s}_{w}$pH (25 °C) = 4.95 in 30% (v/v) acetonitrile–water mixture, flow rate = 1 mL/min. Injection volume: 5 μL. Solute concentrations: 0.1 mg/mL. See Table 4 for identification of the analytes.

The behavior of the same five analytes running in the same column but using piperazine–piperazine dihydrochloride buffer at $^{s}_{w}$pH = 4.95 in acetonitrile 30% (v/v) are shown in Fig. 2. The buffer solution was prepared by matching the pH at 25 °C with the one of mobile phase B2. Under this buffer condition, resolution is feasible at both temperatures: 25 and 50 °C, although the elution order is completely different. Thus, cinnamic acid, 4-methylaniline and 4-ethoxyaniline behave as usually expected, i.e., retention factors decrease as temperature is increased showing an apparent negative enthalpy of transfer of solute from the eluent to the stationary phase. On the other hand, the solutes 3-bromobenzoic acid and 2-methylbenzoic acid present an anomalous behavior: the retention increase with temperature.

Several studies previously indicated that amines can be not "normally-behaved" in certain chromatographic systems [4,27,52–54]. Mao and Carr reported an increase in retention factors of seven antihistamines in an ODS column which was heated from 30 to 40 °C when using 40/60 (v/v) acetonitrile/phosphate buffer pH 7 as eluent mixture. Similarly, Kirkland reported negative van't Hoff slopes with a change in the slope around 50 °C in both a typical monomeric C18 and a bidentated silane stationary phases for trimipramine ($^{w}_{w}pK_a = 9$) in a (40/60) buffer phosphate ($^{w}_{w}$pH = 7)/acetonitrile eluent mixture. Buckenmaier et al. also observed the increase in retention of four amines from two C18 columns when eluted with phosphate buffer at $^{s}_{w}$pH = 7.8 within the temperature range of 30–60 °C. Since the increase in temperature leads to an amine pK_a shift towards lower values [20,37] and, concomitantly, the ratio between the neutral and the cationic form of the solute will be larger, all these experimental results can be fully rationalized. However, under our experimental conditions, the two amines 4-methylaniline and 4-ethoxyaniline behaved as expected whereas 3-bromobenzoic and 2-methylbenzoic acids exhibited a somewhat unexpected behavior.

A symmetrical study in the basic pH range was conducted by using two thermodynamically different buffer solutions. One was prepared from dihydrogen phosphate and hydrogen phosphate salts which were dissolved in acetonitrile at 30% (v/v) and pH was regulated at $^{s}_{w}$pH = 7.84. The other solution was prepared from *Tris* base, which was also dissolved in the same acetonitrile–water mixture, and pH was regulated by adding a hydrochloric acid solution prepared in the same solvent mixture.

We compare the elution times of two nitrophenols: 2-nitrophenol and 4-nitrophenol, and two amines: 2,4,6-trimethylpyridine and codeine at three temperatures in Fig. 3. The solutes were injected individually. The mobile phase for these chromatograms contained buffer phosphate $^{s}_{w}$pH = 7.85 in acetonitrile–water. With the exception of codeine, the increase in column temperature caused less retention. A selectivity crossover between codeine and 4-nitrophenol is evident at a temperature between 25 and 37 °C. We compared

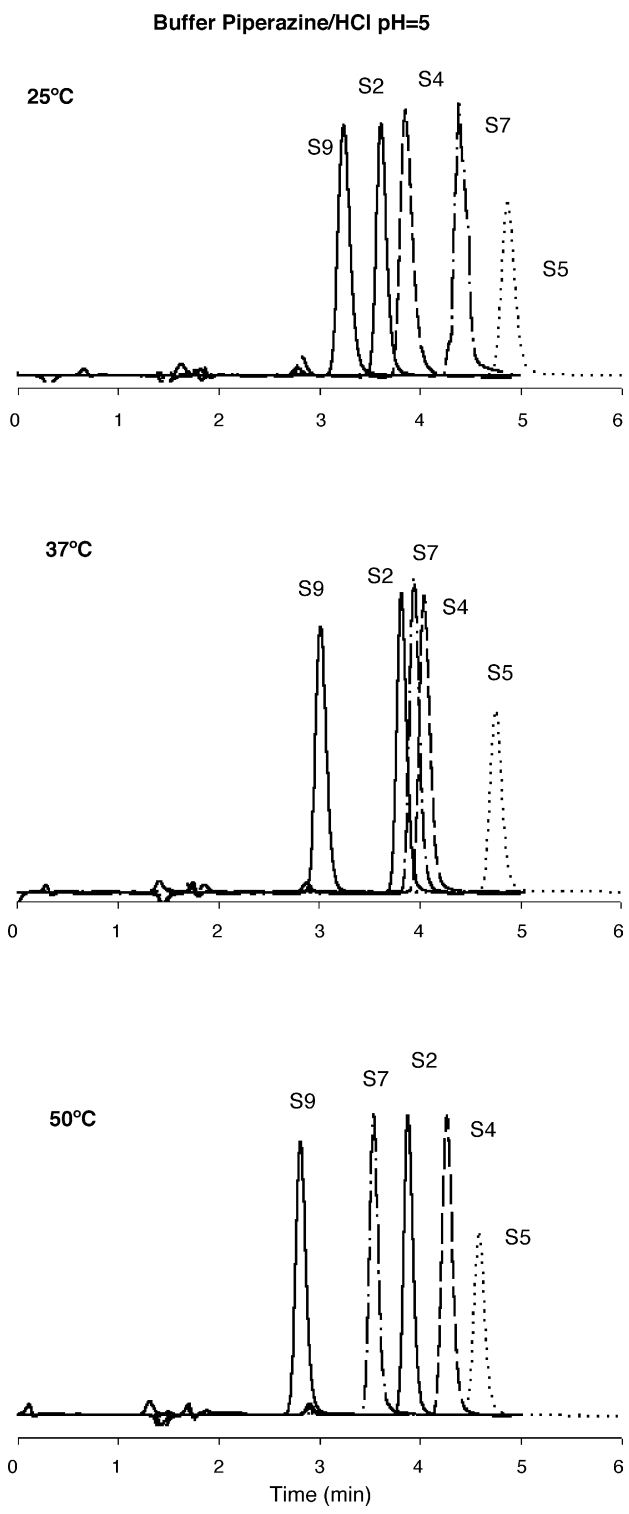

Fig. 2. Influence of temperature on retention and selectivity. Chromatograms of analytes eluted at 25, 37 and 50 °C. Piperazine–HCl buffer $^{s}_{w}$pH (25 °C) = 4.95 in 30% (v/v) acetonitrile–water mixture. Other conditions and solute references as Fig. 1.

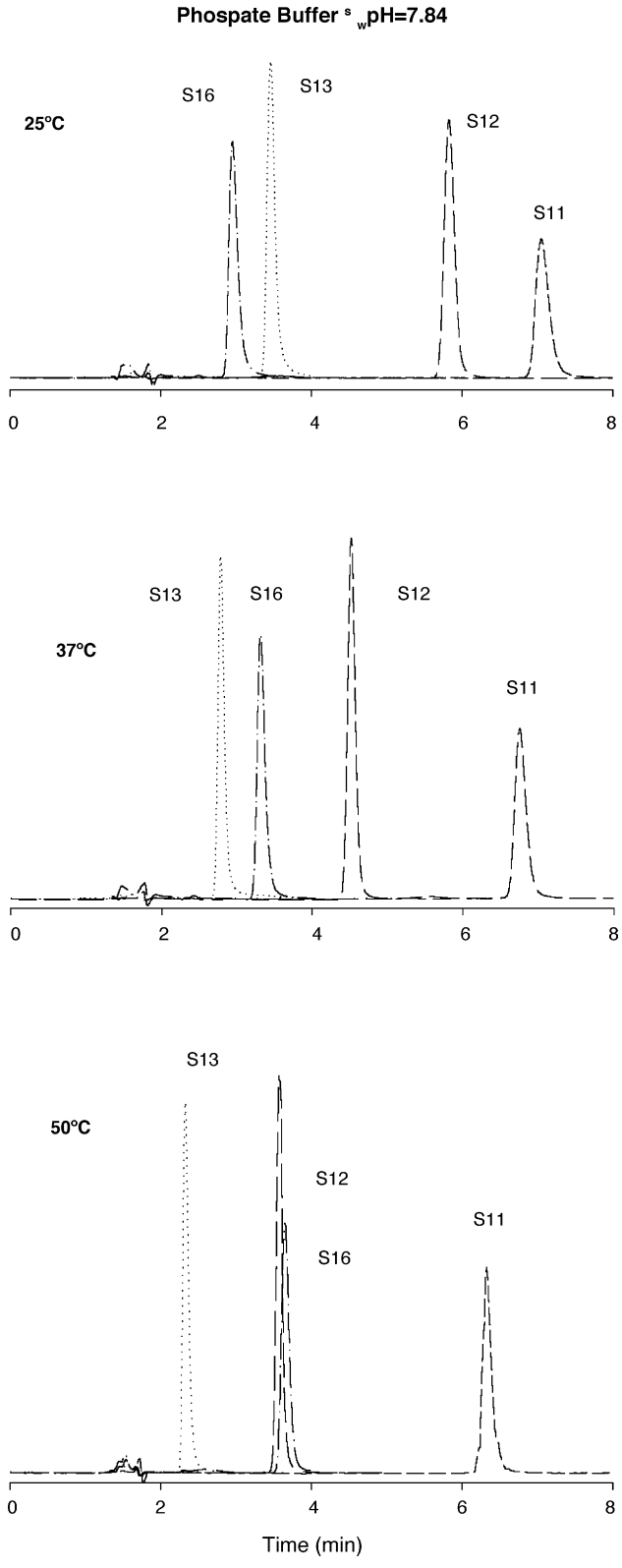

Fig. 3. Influence of temperature on retention and selectivity. Chromatograms of analytes eluted at 25, 37 and 50 °C. Dihydrogenphosphate/hydrogenphosphate buffer $^{s}_{w}$pH (25 °C) = 7.84. Analytes: see Table 4 for nomenclature. Other conditions as Fig. 1.

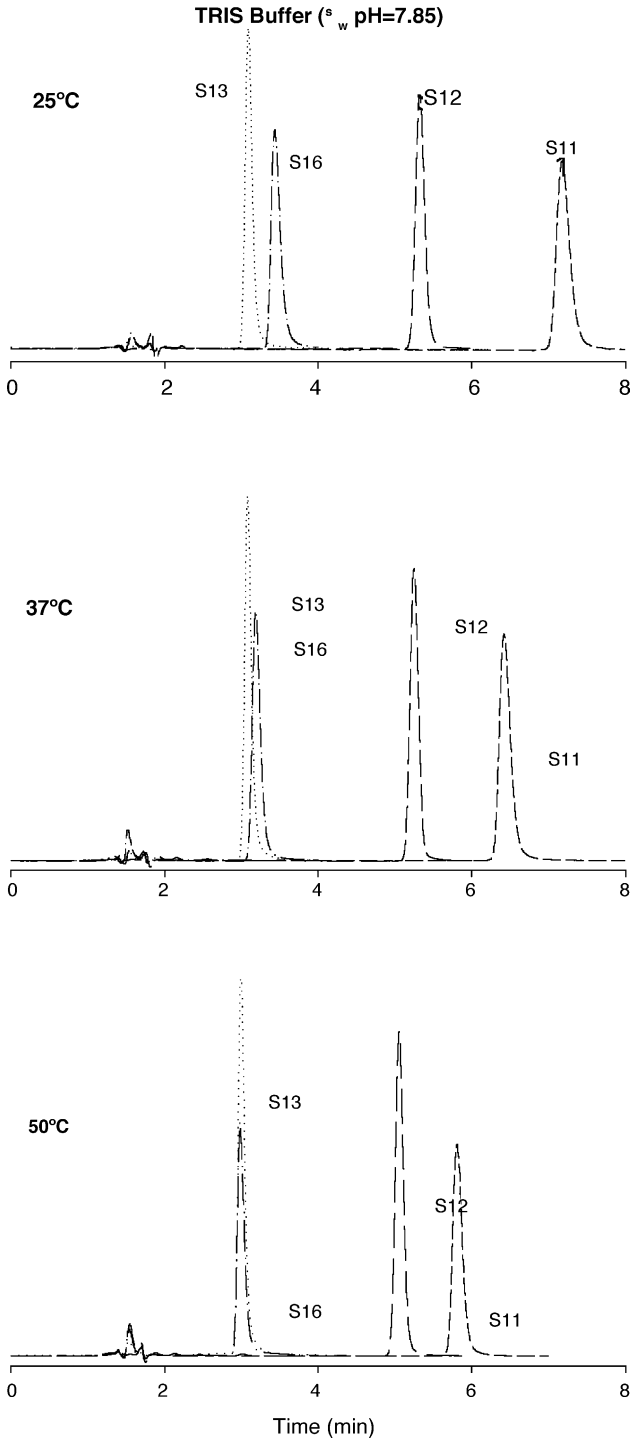

Fig. 4. Influence of temperature on retention and selectivity. Chromatograms of analytes eluted at 25, 37 and 50 °C. *Tris*–HCl buffer $^{s}_{w}$pH (25 °C) = 7.85. Other conditions as in Fig. 3.

these chromatograms with those obtained for these solutes from the same column but with a mobile phase containing a *Tris*–HCl buffer solution (Fig. 4). In this buffer solution the four solutes presented a decrease in retention factors as temperature is increased.

A more quantitative explanation of our results will be now attempted. From the slopes of the van't Hoff plots the apparent enthalpies of transfer of all solutes were estimated. Plots were linear within this temperature range, i.e., the experimental errors prevented us of searching for some non-linear behavior. These results and the standard deviation of each slope are presented in Table 6. As the model predicts, the enthalpy of transfer of a fully protonated and of deprotonated compound is independent of pH. This can be observed in Table 6 for solutes that were well-retained (subject to minimum errors in retention factors). Taking for comparison the three anilines and benzimidazole, whose pK_a are close to 5, and thus they will be as molecular bases in the three buffers B4–B6, their enthalpies of transfer are quite similar regardless of the buffer solution. On the other hand, similar enthalpies of transfer of 2- and 4-nitrophenol were measured in buffer solutions B1–B3, where the solutes are neutral acids.

The experimental "apparent" enthalpies of transfer (slopes of ln k with the reciprocal of temperature at the intermediate mobile phase pH) were compared with those predicted from the retention of the neat HA and A^- forms by applying Eq. (5). The results are gathered in Table 7; the agreement is quite good if we consider that in the calculations we used the solute ionization enthalpies measured in pure water. Other analytes could not be included since their ionization enthalpies were not available. A significant remark is the fact that the calculated enthalpies for the four benzoic acids in the piperazine buffer predict exactly the trends observed in the experimental chromatograms. Eq. (5) offers a simple explanation for the retention increase of this solute family, i.e., the high positive ionization enthalpy of piperazine implies

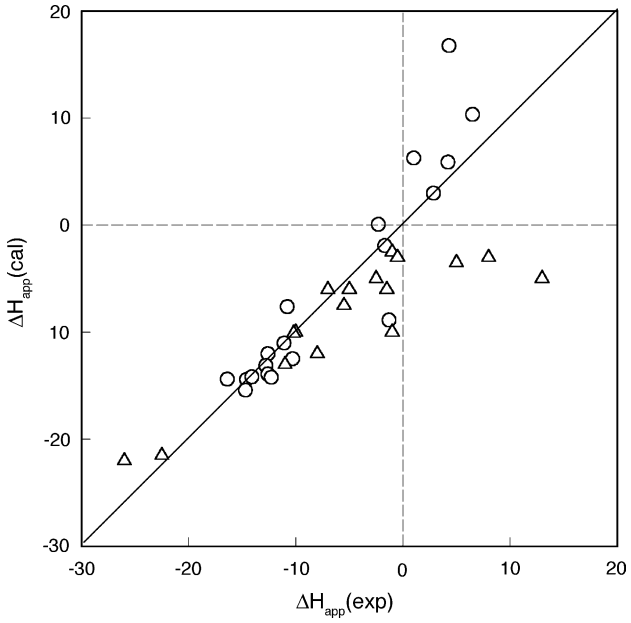

Fig. 5. Calculated versus experimental apparent enthalpies of transfer for solutes over all buffer conditions. Open circles correspond to mobile phases B2 and B3 and open triangles to mobile phases B4 and B5.

Table 6
Apparent enthalpies of transfer of solutes from the mobile phase to a C18 column[a]

Solute	ΔH_{app} (kJ mol^{-1})[b]					
	B1	B2	B3	B4	B5	B6
Benzoic acid	−10.6 (±0.5)	−11.1 (±1.3)	4.2 (±0.8)	−1.6 (±1.7)	–	−10.1(±1.7)
2-Methylbenzoic acid	−11.7 (±0.3)	−10.8 (±1.6)	4.3 (±0.8)	−2.2 (±1.0)	13.1(±1.1)	−7.0 (±1.1)
3-Methylbenzoic acid	−11.9 (±0.4)	−12.6 (±2)	1.0 (±0.7)	−2.9 (±0.3)	7.0 (±1.1)	−5.8 (±0.9)
3-Bromobenzoic acid	−13.9 (±0.4)	−12.8 (±1.9)	6.5 (±0.2)	−6.2 (±0.9)	−4.4 (±0.3)	−7.6 (±0.2)
Cinnamic acid	−13.0 (±0.3)	−12.6 (±2.0)	−2.3 (±0.4)	−6.6 (±0.4)	5.0 (±0.7)	−6.6 (±0.6)
4-Aminosalicylic acid	−13.8 (±0.1)	−6.9 (±1.2)	−9.3 (±0.2)	−9.6 (±0.3)	−6.9 (±0.4)	2.2 (±0.5)
4-Methylaniline	0.7 (±3)	−1.7 (±1.4)	−10.3 (±0.1)	−9.7 (±0.1)	−9.5 (±0.1)	−9.6 (±0.1)
N-Ethylaniline	6.2 (±0.9)	−1.4 (±1.6)	−12.3 (±0.1)	−11.2 (±0.1)	−11.2 (±0.1)	−11.9 (±0.2)
4-Ethoxyaniline	−4.5 (±1.6)	2.3 (±1.1)	−7.4 (±0.4)	−7.4 (±0.1)	−7.5 (±0.5)	−7.0 (±0.2)
Benzimidazole	−8.0 (±6)	−1.5 (±1.4)	−11.1 (±0.8)	−8.7 (±0.2)	−8.1 (±0.1)	−6.9 (±0.3)
2,4,6-Trimethylpyridine	−15.9 (±0.3)	–	–	−3.7 (±0.2)	−8.1 (±0.2)	–
2-Nitrophenol	−14.2 (±0.2)	−12.3 (±1.5)	−14.1 (±0.1)	−22.1 (±0.1)	−1.8 (±0.5)	−6.6 (±0.2)
4-Nitrophenol	−14.4 (±1.3)	−14.6 (±1.5)	−16.4 (±0.2)	−25.5 (±0.3)	−1.3 (±0.5)	−15.5 (±0.2)
2,6-Dinitrophenol	−17.7 (±0.2)	−14.7 (±2)	−1.3 (±0.5)	−10.8 (±0.6)	−7.5 (±0.5)	−13.3 (±0.4)
4-Aminopyridine	–	−16.2 (±5.4)	3.5 (±3.5)	9.3 (±1.3)	−6.6 (±0.9)	−4.0 (±0.3)
Codeine phosphate	–	−9.1 (±1.5)	−1.3 (±0.9)	12.6 (±0.4)	−7.4 (±0.2)	1.1 (±0.2)

[a] Eluent: 30% (v/v) acetonitrile-buffer solutions. For buffer compositions see Table 1.
[b] Computed from the van't Hoff plots.

Table 7
Comparison of apparent enthalpies calculated with Eq. (5) with experimental values

Solute	ΔH_a (kJ mol^{-1})[a]	Buffer B2 ($^{s}_{w}$pH = 4.95)				Buffer B3 ($^{s}_{w}$pH = 4.95)		
		First[d]	Second[d]	ΔH_{app}		Second[d]	ΔH_{app}	
				Calculated	Experimental		Calculated	Experimental
Benzoic acid	0.6	−10.58	−0.47	−11.0	−11.1	16.5	5.9	4.2
2-Methylbenzoic acid	−5.86	−11.41	3.80	−7.6	−10.8	28.2	16.8	4.3
3-Methylbenzoic acid	0.29	−11.62	−0.33	−12.0	−12.6	17.9	6.3	1.0
3-Bromobenzoic acid	−0.25	−13.03	−0.06	−13.1	−12.8	23.5	10.4	6.5
Cinnamic acid	2.51	−12.74	−1.19	−13.9	−12.6	12.9	0.1	−2.3
2-Nitrophenol	19.04	−14.20	−0.04	−14.2	−12.3	0.03	−14.2	−14.1
4-Nitrophenol	19.45	−14.40	−0.04	−14.4	−14.6	0.03	−14.4	−16.4
2,6-Dinitrophenol	7.61	−14.16	−1.25	−15.4	−14.7	5.47	−8.9	−1.3
4-Methylaniline	27.2[b]	−9.39	7.44	−1.9	−1.7	−3.11	−12.5	−10.3
4-Ethoxyaniline	33.47[c]	−6.88	9.48	2.6	2.3	−1.17	−8.0	−7.4

[a] Enthalpy of ionization of solutes in water, taken from [57].
[b] Ionization enthalpy of aniline.
[c] Ionization enthalpy of methoxyaniline.
[d] Refers to first and second term on the right-hand of Eq. (5).

a strong decrease in eluent pH with temperature raising. On the other hand, the acid base equilibrium of these analytes in 30% acetonitrile is barely affected by temperature. Both facts lead to a highly positive second term in Eq. (5), which is not completely compensated by the negative term, which is not sensitive to the nature of the buffer. Calculated versus experimental data were plotted in Fig. 5. In this plot the values shown are indicated with circles when using the acidic mobile phases B2 and B3, and with triangles when using basic mobile phases B4 and B5. Predictions for a few systems are far from the experimental values; these experimental slopes correspond to data measured from very small retention factors which are prone to the largest experimental uncertainties. Despite these errors, the trends between estimated and experimental apparent enthalpies are correct in most of the cases.

5. Conclusions

From a pair of buffer solutions controlling acidic mobile phase pH and a second pair controlling alkaline mobile phases pH, we demonstrated the critical effect that a change in temperature would have on the retention and selectivity of weak acids and bases as a function of the buffer nature used in the mobile phase. A quantitative expression for predicting the change in retention factors of these solutes as a function of buffer type was satisfactorily tested. The expression indicates that when the enthalpies of ionization are similar for both the buffer and the solute, or if the pK_a of the solute is far from that of the buffer, then no special effects would be expected. However, if the eluent pH is close to the pK_a of the analytes and if their respective ionization enthalpies differ significantly, an unexpected behavior of the

analytes upon change in column temperature is highly probable.

Acknowledgements

We are thankful for financial support from the Spanish Government (Project CTQ2004-00633/BQU) and the Catalan Government (2001SGR00055), and C.B.C. acknowledge Fundación Antorchas (Argentina), IFS (Sweden) and CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina) for their support.

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