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Journal of Chromatography A, 1042 (2004) 23–35
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Effect of temperature on the chromatographic retention of ionizable compounds I. Methanol–water mobile phases

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Received 9 January 2004; received in revised form 29 April 2004; accepted 29 April 2004

Abstract

The retention mechanism of acids and bases in reversed-phase liquid chromatography (RPLC) has been experimentally studied by examining the temperature dependence of retention, with emphasis on the role of the buffer ionization equilibria in the retention and selectivity. Retention factors of several ionizable compounds in a typical octadecylsilica column and using buffers dissolved in 50% (w/w) methanol as eluents at three temperatures in the range of 25–50 °C were measured. Two pairs of buffer solutions were prepared by a close adjusting of their pH at 25 °C; differences in their ionization enthalpies determined a different degree of ionization when temperature was raised and, as a consequence, a different shift in the eluent pH. Predictive equations of retention that take into account the temperature effect on both the transfer and the ionization processes are proposed. This study demonstrates the significant role that the selected buffer would have in retention and selectivity in RPLC at temperatures higher than 25 °C, particularly for co-eluted solutes.

Keywords: Temperature effects; Mobile phase composition; Retention behavior; Acid–base equilibria; Ionizable solutes

1. Introduction

Useful method development strategies for optimizing selectivity of ionizable solutes are based on manipulating the experimental conditions, such as stationary phase type, mobile phase pH and composition, and much less often temperature. The analyst is often faced with the separation of mixtures with a variable behavior of their components, which makes good resolution sometimes extremely difficult, and therefore, it is always useful to handle as many variables as possible for such complex mixtures. The range of permissible mobile phase pH and temperature for preserving the integrity of the typical HPLC supports have been the main impediment for using high temperature and pH for optimization strategies of ionizable solutes. However, nowadays chemically more stable supports are available.

The influence of pH on retention and selectivity is now well established. The pioneering studies of Horváth et al. provided a model for explaining retention of acids and bases as a function of pH under a single reversed-phase retention mechanism and under a more complex retention mechanism [1,2]. The retention of an ionizable analyte can vary strongly with the pH of the mobile phase, especially around the pK_a of the analyte. In this case the pH of the mobile phase needs to be tightly controlled.

The importance of using temperature as a tool to optimize chromatographic parameters such as retention, efficiency and selectivity, especially for large solutes, has become more widely appreciated [3]. It is well known that an increase in temperature produces: (1) increase in diffusion coefficient, (2) increase in sorption–desorption kinetics, and (3) reduction in eluent viscosity. The first two facts lead to a decrease in the mass transfer resistance at the common linear

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doi:10.1016/j.chroma.2004.04.073

velocities of the eluent, improving plate heights; the reduction in viscosity allows the increasing in flow-rates to speed the analysis [4–6].

The influence of temperature on retention and selectivity of ionizable solutes in reversed-phase conditions has been much less studied and it is still far from clear. Non-comprehensive examples are as follows. Horváth carried out studies of retention and selectivity of protonable solutes conducted in water as mobile phase [7]. This work demonstrated that the buffer nature is able to modify significantly the selectivity with temperature due to its enthalpy of ionization. Snyder et al. carried out a series of optimization studies as a function of mobile phase composition and temperature [8–11], which include a group of weak acids and bases as solutes [10]. Li published a theoretical work about the situations where the temperature changes the ionization status of protonable species and, as a consequence, changes the selectivity [12]. McCalley qualitatively investigated the effect of temperature on analysis of three basic solutes at two eluent pH on a typical reversed-phase liquid chromatography (RPLC) column [13]. He found that retention of nortryptyline at pH 7 and quinine at pH 7 and 5 increases with temperature over the range 20–60 °C. This is a non-expected behavior since retention under the single hydrophobic mechanism is exothermic and the enthalpic term dominates, so retention should decrease as temperature increases. Vervoort et al. estimated thermodynamic data of the retention of basic compounds with the aim of characterizing reversed-phase stationary phases [14]. They compared the transference enthalpies of amines at pH 3 and 7 in different ODS columns, and in all cases the enthalpy values were negative.

The aim of this study is to evaluate the effect of temperature on the selectivity of several ionizable compounds when elute with different buffers in methanol/water mobile phases having practically the same pH at 25 °C but quite different enthalpies of ionization. We have selected a pair of buffer solutions to control acidic mobile phase pH and a second pair controlling alkaline mobile phases pH to demonstrate the critical effect that a change on temperature can have over the selectivity as a function of the buffer nature used in a mobile phase.

The rigorous investigation of the chromatographic behavior of the studied compound requires information about dissociation constants as much of the buffer substances as of the different solutes studied at temperatures and solvent compositions. Since the published information is very scarce in a previous paper we have presented pK_a values of some common compounds at 50% (w/w) methanol/water mixtures in the temperature range of 20–50 °C determined by means of a potentiometric technique [15]. The selected compounds were representative of different chemical families; some of them are commonly used in the preparation of buffered mobile phases. In this work, we extend the pK_a measurements to other two weak acids used to prepare buffer solutions in the present chromatographic determinations.

2. Theoretical

2.1. Dependence of retention from mobile phase pH

The ionization equilibria for a weak analyte HA can be written as:

$$HA^z \rightleftarrows A^{(Z-1)} + H^+$$

$$\Delta G^\circ_{a(an)} = -RT \ln K_{a(an)}; \quad K_a = \left(\frac{[A]}{[HA]}\right) a_{II^+} \tag{1}$$

where the charge of the analyte's species have been suppressed and the activity coefficient was considered the unity for simplicity. $K_{a(an)}$ and $\Delta G^\circ_{a(an)}$ are the acidity constant and the standard Gibbs free energy, respectively. By assuming that the ionization reaction occurs only in mobile phase and that the rate of dissociation in mobile phase is very large (ion-pair concentration is negligible), the transfer of analyte A from the mobile to the stationary phase is given by:

$$HA^z_m \rightleftarrows HA^z_s \quad \Delta_t G^\circ_{HA} = -RT \ln K_{D(HA)};$$
$$K_{D(HA)} = \frac{[HA]_s}{[HA]_m} \tag{2a}$$

$$A^{z-1}_m \rightleftarrows A^{z-1}_s \quad \Delta_t G^\circ_A = -RT \ln K_{D(A)};$$
$$K_{D(A)} = \frac{[A]_s}{[A]_m} \tag{2b}$$

where $K_{D(i)}$ and $\Delta_t G^\circ_i$ represent the equilibrium constant and standard Gibbs free energy of transfer of i from the mobile to the stationary phase, respectively. The observed retention factors for an analyte (an), k, is the average retention of both the protonated (k_{HA}) and neutral forms (k_A) [2,16–20]:

$$k = \frac{k_{HA} + k_A(K_{a(an)}/a_{H^+})}{1 + (K_{a(an)}/a_{H^+})} = \frac{k_{HA} + k_A 10^{(pH - pK_{a(an)})}}{1 + 10^{(pH - pK_{a(an)})}} \tag{3}$$

where $k_{HA} = \varphi K_{D(HA)}$, $k_A = \varphi K_{D(A)}$ and φ is the phase ratio. Eq. (3) applies to cases where the interaction between a sorbent and a solute is exclusively controlled by their hydrophobicity. It is not applicable if more interactions of different origin are involved in the separation process. When the pH of the mobile phase is buffered by the pair HB/B, whose dissociation equilibrium is given by:

$$HB^z \rightleftarrows B^{z-1} + H^+$$

$$\Delta G^\circ_{a(buff)} = -RT \ln K_{a(buff)}; \quad K_{a(buff)} = \left(\frac{[B]}{[HB]}\right) a_{H^+} \tag{4}$$

where $K_{a(buff)}$ is the acidity constant of the buffer compound. Thus, a_{H^+} can be approximated by:

$$a_{II^+} = K_{a(buff)} \left(\frac{[HB]}{[B]}\right) = K_{a(buff)} \frac{(C_{HB} - a_{H^+} + a_{OH^-})}{(C_B + a_{H^+} - a_{OH^-})}$$
$$\cong K_{a(buff)} \left(\frac{C_{HB}}{C_B}\right) \tag{5}$$

C_{HB} and C_B are the analytical concentrations of acid and conjugated base. Considering that the buffer is concentrated enough and the pH of the mobile phase is far from 0 and from the pK_{ap} (K_{ap} is the autoprotolysis constant of the solvent) so the last approximation is valid. Substituting Eq. (5) in Eq. (3) results in:

$$k = \frac{k_{HA} + k_A w}{1 + w} \tag{6}$$

where

$$w = \left(\frac{K_{a(an)}}{K_{a(buff)}}\right)\left(\frac{C_B}{C_{HB}}\right) \tag{7}$$

It is important to highlight that pH and pK_a are referring to a unique standard state for all dissolved species, i.e., solutions of all analytes in a given aqueous organic solvent mixture (s) and a standard state referred to the solutes infinitely diluted in the same mixture: ${}^{s}_{s}$pH and ${}^{s}_{s}$pK_a according to the IUPAC recommendations for nomenclature [21,22].

2.2. Effect of temperature

Taking into account that, the thermodynamics of ionization of analyte and buffer can be expressed respectively as:

$$\Delta G^{\circ}_{a(an)} = \Delta H^{\circ}_{a(an)} - T\,\Delta S^{\circ}_{a(an)} \tag{8a}$$

$$\Delta G^{\circ}_{a(buff)} = \Delta H_{a(buff)} - T\,\Delta S_{a(buff)} \tag{8b}$$

whereas the thermodynamics of transfer from mobile to stationary phase is given by,

$$\Delta_t G^{\circ}_{IIA} = \Delta_t H^{\circ}_{IIA} - T\,\Delta_t S^{\circ}_{IIA} \tag{8c}$$

$$\Delta_t G^{\circ}_{A} = \Delta_t H^{\circ}_{A} - T\,\Delta_t S^{\circ}_{A} \tag{8d}$$

where $\Delta H^{\circ}_{a(i)}$ and $\Delta S^{\circ}_{a(i)}$ represent standard enthalpy and entropy changes for the equilibrium under consideration, respectively. A change in temperature will change, not only the transfer of solute from mobile to stationary phase, but also $K_{a(an)}$, $K_{a(buff)}$ and its ratio, as well as a_{H^+} (or pH).

Replacing these thermodynamic expressions in the retention factor (Eq. (6)), applying logarithm and differentiating with respect to (1/T), the apparent enthalpy of the chromatographic process, $\Delta H^{\circ}_{app(an)}$ can be estimated (see Appendix A):

$$\Delta H^{\circ}_{app(an)} = -R\left(\frac{d\ln k}{d(1/T)}\right) = \left[\frac{\Delta_t H^{\circ}_{HA} + gw\,\Delta_t H^{\circ}_{A}}{1 + gw}\right] + \left[\frac{w(g-1)(\Delta H^{\circ}_{a(an)} - \Delta H^{\circ}_{a(buff)})}{(1+gw)(1+w)}\right] \tag{9}$$

where $g = k_A/k_{HA}$.

Eq. (9) clearly shows that the retention of the analyte at different temperatures will depend on the ionization enthalpies of both analyte and buffer, and a plot of ln k versus (1/T) would likely be non linear. Only if $\Delta H^{\circ}_{a(an)} = \Delta H^{\circ}_{a(buff)}$ (equal heat of ionization for buffer and analyte), results:

$$\Delta H^{\circ}_{app(an)} = \frac{\Delta_t H^{\circ}_{HA} + gw\,\Delta_t H^{\circ}_{A}}{1 + gw} \tag{9a}$$

the apparent enthalpy will depend only on the enthalpies of transference of HA and A and on the equilibrium constants of the ionization of analyte and buffer, in addition to the fraction of each buffer species present. When $\Delta H^{\circ}_{a(an)} \neq \Delta H^{\circ}_{a(buff)}$, the heat of ionization of the selected buffer would strongly affect the dependence of k with temperature. Eq. (9) clearly indicates that the apparent enthalpy evaluated from a typical van't Hoff plot can be a complex function of the operating conditions even in the simple case of RP-interactions.

The ratio between retention factors of deprotonated and protonated forms, g, is a quantity independent of pH. For a typical reversed-phase with a unique retention mechanism $0 < g < 1$ for neutral ($z = 0$) and $g > 1$ for cationic acids ($z = 1$) like protonated amines. Its dependence with temperature will be positive or negative depending on the magnitude of enthalpies of transfer for the protonated and the deprotonated species from mobile to stationary phase. In a RPLC system, where hydrophobic interactions dominates retention, it is expected that $\Delta_t H^{\circ}_{HA}$ is more negative than $\Delta_t H^{\circ}_{A}$ for neutral acids and less negative for protonated salts. If this assumption is correct, then g would increase for acids (and decrease for amines) as temperature increases.

On the other hand, as temperature increases, w values will decrease ($\Delta H^{\circ}_{a(an)} < \Delta H^{\circ}_{a(buff)}$) or increase ($\Delta H^{\circ}_{a(an)} > \Delta H^{\circ}_{a(buff)}$) as a function of the dissociation enthalpies of buffer and of analyte.

The two additional assumptions in Eqs. (8) and (9) are as follows. First, all $\Delta H^{\circ}_{a(i)}$ and $\Delta S^{\circ}_{a(i)}$ are independent of T (heat capacities are negligible), which is reasonable for a small range in temperatures. Second, activity coefficients of all species have been considered to be unity. In spite this is not true, the variation of the activity coefficients with temperature is negligible compared to the pK_a variation.

2.3. Significance

Several connotations can be inferred from the previous part. First, retention of the protonated form of the solute, k_{HA}, and the corresponding enthalpy of transfer, $\Delta_t H^{\circ}_{HA}$, can be measured by using a buffered mobile phase at pH $\ll$ p$K_{a(an)}$ through van't Hoff plots. Similarly, k_A and $\Delta_t H^{\circ}_{A}$ can be measured with an eluent of the same organic/water composition buffered at pH $\gg$ p$K_{a(an)}$. Ideally, these plots should be independent of the selected buffer. Second, two solutes chemically similar will present larger retention differences in the region of their pK_a's; and thus, the choice of temperature and buffer type (within this pH region) can determine the success of the separation. Chromatographers usually prefer to develop a method at a pH where the

dependence of retention on pH is small, i.e., far away from the pK_a of the analyte. However, this unrealistic situation does not apply to real separation problems where retention of many analytes change over a broad pH range, and, moreover, a separation may only be possible at a given pH value. Therefore, whenever the method exhibits a high level of pH sensitivity the premise is to control the mobile phase pH appropriately.

3. Experimental

3.1. Instrumentation

pH measurements were taken with a combined glass electrode, Ross Combination Electrode Orion 8102 SC, in a commercial pH-meter (Crison micropH 2002) with a precision of ±0.002 pH units. Buffer solutions were placed into a temperature-controlled bath and a thermometer calibrated at ±0.1 °C was used for temperature readings.

Chromatographic measurements were carried out with an apparatus consisting in an ISCO 2350 pump, a manual injector, and an UV Shimadzu SPD-10A detector connected to a data acquisition Class VP software (Shimadzu). The wavelength was set at 254 nm for detecting analytes and at 200 nm to detect the dead volume marker (KBr). In order to avoid strong interactions of basic solutes with the residual silanols in the silica surface, an RPLC column which is practically free of acidic silanol groups was chosen [23]. Thus, a 150 mm × 4.6 mm i.d. X-Terra MS-C18 column (Waters) was kept in a temperature controlled thermostatic bath. A 20 cm stainless steel capillary tube immersed in the bath allowed the preheating of the incoming mobile phase.

3.2. Chemicals

The solvents used were methanol HPLC-grade (99.9%, Merck) and water purified by a Milli-Q plus system (Millipore). Buffers were prepared from the reagents p.a. grade or better: phosphoric acid (Merck, 85%), potassium dihydrogen phosphate (Merck, >99.5%), disodium hydrogen phosphate (Merck, >99%), 2-amino-2-(hydroxymethyl)-1,3-propanediol (from now *tris*) (Fluka, >99.8%), hydrochloric acid (Merck, 25% in water), 1-aminobutane (Aldrich, 99.5%), glacial acetic acid (Merck), sodium acetate (Carlo Erba, >99%), piperazine (Fluka, >99%) 2,6-dichlorophenol (Fluka), 2,4-dichlorophenol (Merck), benzyldimethylamine (Merck), benzylamine (Fluka >99%), benzoic acid (Merck, p.a.), 2-methylbenzoic acid (Aldrich, >99%), 2,4-dinitrophenol (Doesder), 2,6-dinitrophenol (Fluka, >98%), 2-aminotoluene (C. Erba), 4-aminotoluene (Scharlau), benzimidazole (Fluka), papaverine hydrochloride (Fluka, >98%), trazodone (Sigma), *N*,*N*-dimethylaniline (Merck), pyridine (Merck), and quinoline (Fluka) were used as solutes.

3.3. pK_a measurements

The first ionization constants of phosphoric acid and of piperazinium in 50% (w/w) methanol/water mixtures were measured over the temperature range of 15–50 °C. Five solutions approximately 0.05 molal, and containing different ratios between each component of the conjugate pair were prepared. After thermal equilibrium of these solutions and of the aqueous standards, the corresponding ${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{pH}(T)$ were carefully measured. ${}^{\mathrm{s}}_{\mathrm{s}}\mathrm{pH}(T)$ was calculated by subtraction of the corresponding δ-parameter at each temperature. δ-value at 15 °C was estimated in 0.15 pH units by lineal extrapolation from experimental values obtained in the range of 20–50 °C [15]. Finally, ${}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_{\mathrm{a}}(T)$ were computed by introduction of correction for non-idealities as follows:

$$ {}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_{\mathrm{a}}(T) = {}^{\mathrm{s}}_{\mathrm{s}}\mathrm{pH}(T) - \log\left(\frac{m_{\mathrm{X}}}{m_{\mathrm{HX}}}\right) - \log\left(\frac{{}^{\mathrm{s}}_{\mathrm{s}}\gamma_{\mathrm{X}}(T)}{{}^{\mathrm{s}}_{\mathrm{s}}\gamma_{\mathrm{HX}}(T)}\right) \quad (11) $$

where m_i are the molalities of species i in solvent s at the equilibrium, and ${}^{\mathrm{s}}_{\mathrm{s}}\gamma_i(T)$ refers to the activity coefficients of i in solvent mixture and referred to the standard state in the same solvent at each temperature. Since ionic strengths were kept below 0.1 m, activity coefficients were estimated from the Debye–Hückel equation:

$$ -\log {}^{\mathrm{s}}_{\mathrm{s}}\gamma_i = \frac{z_i^2 A\sqrt{I}}{1 + a_0 B\sqrt{I}} \quad (12) $$

where z is the charge of the i ion, A and B are two, solvent- and temperature-dependent parameters. The product a_0B at each temperature was estimated by following the Bates–Guggenheim convention [22,24,25]:

$$ ({}^{\mathrm{s}}a_0{}^{\mathrm{s}}B)_T = 1.5\sqrt{\left[\frac{({}^{\mathrm{w}}\varepsilon^{\mathrm{s}}\rho)}{({}^{\mathrm{s}}\varepsilon^{\mathrm{w}}\rho)}\right]_T} \quad (13) $$

${}^{\mathrm{w}}\varepsilon$, ${}^{\mathrm{w}}\rho$, ${}^{\mathrm{s}}\varepsilon$ and ${}^{\mathrm{s}}\rho$ denote the dielectric constants and the densities of water and of the methanol/water solvent mixture at the given temperature, respectively. These physical properties were taken from Refs. [26,27]. The A parameter has been computed from [28]:

$$ A = \frac{1.8246 \times 10^6\sqrt{{}^{\mathrm{s}}\rho}}{({}^{\mathrm{s}}\varepsilon T)^{3/2}} \quad (14) $$

Ionic strength calculations, which require the knowledge of hydrogen and of lyate ion concentrations in the solvent mixture, were calculated by an iterative scheme that provided both I and ${}^{\mathrm{s}}_{\mathrm{s}}\gamma$ values. Activity coefficient of uncharged molecules can be considered as unity in comparison with those of ions.

3.4. Chromatography

In all the cases, buffer solutions were prepared in the molal scale, and at the fixed methanol compositions of 50% (w/w). Buffers for chromatographic experiments were prepared by mixing: H_3PO_4 and KH_2PO_4, KH_2PO_4 and

Table 1
Buffer solutions prepared in 50% (w/w) methanol/water

Buffer solutions		Concentrations (mmolal)	Ionic strength (mmolal)	${}^{s}_{s}pH_{exp}$ [a]	${}^{s}_{s}pH(T)$ [b]			ΔH°_{a}(kJ mol^{-1})
					25 °C	37 °C	50 °C	
B1	H_3PO_4/KH_2PO_4	6.43/2.50	2.5	3.04	3.00	3.01	3.05	−4.4
B2	Piperazine:HCl/piperazine	16.52/12.01	20	5.04	4.99	4.75	4.52	35.8
B3	Acetic acid/Sodium Acetate	8.03/2.00	2	5.05	5.02	5.03	5.06	−0.21[c]
B4	KH_2PO_4/Na_2HPO_4	7.70/5.94	19	8.13	8.03	8.02	8.01	1.9
B5	Acetic acid/sodium acetate	5.02/5.01	5	5.64	5.60	5.62	5.64	−0.21
B6	*Tris*:HCl/*tris*	5.02/9.10	5	8.09	8.13	7.81	7.53	47.8
B7	KH_2PO_4/Na_2HPO_4	5.65/4.35	19	8.10	8.03	8.02	8.01	1.9
B8	1-Aminobutane:HCl/1-aminobutane	2.54/7.46	2.5	10.30	10.40	9.97	9.61	58.4

[a] ${}^{s}_{s}pH$ (25 °C) calculated by subtraction of δ-values from experimental ${}^{s}_{w}pH$ (25 °C).
[b] ${}^{s}_{s}pH(T)$ calculated from the corresponding ${}^{s}_{s}pK_a$ values obtained from either this work or Refs. [15,37–39].
[c] From Ref. [40].

Na_2HPO_4 or acetic acid and sodium acetate solutions or by mixing hydrochloric acid solution with *tris*, 1-aminobutane or piperazine solutions and ending with the addition of the organic modifier (methanol). When neccesary after the addition of the methanol the pH was adjusted with small volumes of concentrated hydrochloric acid or with a solution of one the species of the buffer prepared in methanol 50% (w/w) to keep the solvent composition. Concentrations, ionic strengths, the corresponding ${}^{s}_{s}pH$ at three temperatures and enthalpies of ionization in 50% (w/w) methanol/water mixture are reported in Table 1. The column was stabilized at each temperature for at least 1 h (about 40 column volumes). Eluent flow-rate was 1 mL/min and injection volume was 5 μL. Hold-up time was measured with potassium bromide (Merck for spectroscopy). Solute retention times at each temperature are the average of at least three determinations. Their precision, as measured by the relative standard deviation, was better than 1%. Since the extracolumn contributions could be significant at the highest temperatures studied and for the less retained solutes, retention factors k_i were computed from:

$$k_i = \frac{t_i - t_0}{t_0 - t_{ex}} \quad (15)$$

where t_i is the retention time measured at the peak maximum, t_0 the elution time of the marker and t_{ex} the time spent by the marker between the injector and detector connected without the column in phase.

4. Results and discussion

4.1. Dissociation constants of phosphoric acid and of piperazine

Dissociation constants of several compounds in pure water at different temperatures have been compiled by Christensen et al. [29] and by Palm [30]. However, a very scarce information about dissociation constants in methanol/water mixtures at higher temperatures is available in the literature. Although the trend in dissociation with temperature is expected to be similar to that for water, careful measurements of ${}^{s}_{s}pK_a(T)$ values for compounds commonly used to prepare buffer solutions are required for predictive purposes. In this work, the first dissociation constant of phosphoric acid and of piperazine in 50% (w/w) methanol/water mixtures at several temperatures over the range of 15–50 °C have been determined.

Eq. (11) was used to estimate the ${}^{s}_{s}pK_a$ values from pH measurements. In that equation, m_i refers to the molality of i once the equilibrium was reached, i.e., $m_X = m^{\circ}_X + m_H - m_S$, and $m_{HX} = m^{\circ}_X - m_H + m_S$, where m°_i are the analytical molal concentrations and m_H and m_S, molality of protons and of solvent lyate anions, respectively. For piperazine buffer solutions, analytical molalities are higher than both m_H and m_S and thus, these quantities can be approximated to $m_X \cong m^{\circ}_X$ and $m_{HX} \cong m^{\circ}_{HX}$ without significant difference. However, for phosphate buffer solutions only m_S can be neglected, and m_H must be considered ($= {}^{s}_{s}a_{H^+}/{}^{s}_{s}\gamma$).

The results are reported in Table 2 along with phosphoric ${}^{s}_{s}pK_a$ values at 25 °C published in the literature. Stan-

Table 2
${}^{s}_{s}pK_a$ values of phosphoric acid and piperazine in 50% methanol/water at several temperatures

Temperature (°C)	Phosphoric acid		Piperazine:HCl	
	Exp.	Lit.	Exp.	Lit.
15	3.33 (±0.01)		4.79 (±0.03)	
20	3.35 (±0.01)		4.68 (±0.03)	
25	3.37 (±0.01)	3.33[a] 3.435[b]	4.58 (±0.03)	
30	3.37 (±0.01)		4.47 (±0.03)	
35	3.37 (±0.01)		4.36 (±0.03)	
40	3.39 (±0.01)		4.27 (±0.03)	
45	3.41 (±0.01)		4.18 (±0.03)	
50	3.42 (±0.01)		4.09 (±0.03)	
${}^{s}_{s}\Delta H^{\circ}_{a}$ kJ mol^{-1}	−4.56 (±0.01)		35.8 (±0.3)	
${}^{w}_{w}\Delta H^{\circ c}_{a}$ (kJ mol^{-1})		−7.9		29.8

[a] From Ref. [41].
[b] Calculated from Ref. [42].
[c] From Ref. [43].

dard deviations of piperazine pK_a were systematically higher than standard deviations of pK_a of phosphoric acid. The last rows in Table 2 gathered the dissociation enthalpies estimated from a linear dependence of pK_a with the reciprocal of temperature. The standard errors associated to the linear estimations were 0.007 and 0.03 for phosphoric and for piperazine, respectively. A more sophisticated approach, which takes into account the dependence of $\Delta H^{\circ}_{a(i)}$ with temperature for estimation of these ionization enthalpies, did not improve the fits [31].

4.2. Chromatographic results

We first noted that system dead time, estimated from the elution time of potassium bromide, was dependent on both buffer chemical nature and temperature. The flow-rate through the warm column will be higher than flow-rate measured at room temperature. Thus, the decrease in hold-up time as temperature increase is in agreement with the decrease in fluid density [32]. On the other hand, the dependence of different hold-up time markers with the buffer solutions (even at identical pH) and methanol composition had been previously reported [33,34]. It has been found a rough relationship between this time and the medium ionic strength. In this study, with very few buffer solutions, this relationship does not strictly apply. Thus, for consistent results, hold-up times in each buffer and temperature were measured 8–12 times and these t_0 values were averaged with those obtained in the other buffers under identical column temperature.

Retention factors of a number of solutes eluted from a reversed-phase octadecylsilica column with buffer solutions prepared in 50% (w/w) methanol at three temperatures: 25, 37 and 50 °C were determined. Four mobile phase solutions were prepared: one at the lower pH value, B1 ($H_3PO_4/H_2PO_4^-$, ${}^{s}_{s}pH = 3.04$); a pair of buffers at the same pH at room temperature, B2 (piperazine + HCl ${}^{s}_{s}pH = 5.04$) and B3 (acetic/acetate, ${}^{s}_{s}pH = 5.05$); and one last buffer for the higher pH, B4 ($H_2PO_4^-/HPO_4^=$, ${}^{s}_{s}pH = 8.13$). Solutes were carefully chosen to have ionization constants close to 5, thus they will be completely protonated at the pH of B1, totally deprotonated at the pH of B4 and partially dissociated at the pH of B2 and B3. The solutes along with their dissociation constants in 50% (w/w) methanol–water mixture and at 25 °C are listed in Table 3.

Retention factors of these analytes eluted with buffer B3 were depicted as a function of the reciprocal of temperature in Fig. 1, splitted in plots A and B. There is a co-elution of analytes at 25 °C (*o*-toluic acid/papaverine in plot A, and benzoic acid/*p*-toluidine and 2,4-dinitrophenol/benzimidazole/pyridine in plot B). Selectivity of these analytes change when temperature is raised from 25 to 50 °C. In Fig. 2, we have superimposed the chromatograms of four of these analytes (*o*-toluic acid, papaverine, quinoline and trazodone) obtained at 25 °C in one plot and at 50 °C on the other. Baseline resolution of the four analytes at 50 °C is feasible due to a larger decrease in retention of *o*-toluic acid and of quinoline as temperature is raised as compared to the other two solutes.

The replacement of acetic/acetate buffer (B3) by piperazine + HCl as buffer (B2) led to completely different elution profiles. The buffer was prepared with identical composition in methanol and at very similar ${}^{s}_{s}pH$ at 25 °C. Retention factors ($\ln k$) are shown as a function of ($1/T$)

Table 3
List of solutes and ${}^{s}_{s}pK_a$ 25 °C values. Apparent enthalpies of transfer of solutes from different buffers in the mobile phase to a C18 column[a]

Solute	${}^{s}_{s}pK_a$ (25 °C)	$-\Delta H_{app}$ (kJ mol^{-1})[b]			
		B1	B2	B3	B4
Benzoic acid	5.43[c]	15.2 (±0.3)	4.3 (±1.5)	14.2 (±1.0)	15.5 (±3.0)
2-Methylbenzoic acid	5.32[d]	16.5 (±0.1)	5.4 (±1.8)	14.8 (±1.7)	14.7 (±0.8)
4-Aminotoluene	4.54[e]	8.4 (±4.1)	11.0 (±0.8)	3.6 (±3.1)	10.4 (±0.3)
Trazodone	6.33[f]	20.7 (±1.9)	18.1 (±0.2)	0.3 (±0.1)	16.1 (±0.7)
Benzimidazole	4.95[g]	35.8 (±5.6)	15.0 (±0.2)	3.9 (±1.5)	13.8 (±0.5)
Papaverine	5.62[g]	18.4 (±0.1)	18.8 (±0.8)	0.1 (±2.1)	14.9 (±0.4)
2-Aminotoluene	3.91[e]	−5.4 (±2.8)	10.1 (±0.1)	6.1 (±1.0)	10.0 (±1.1)
N,*N*-Dimethylaniline	4.28[c]	−13.3 (±1.5)	14.0 (±0.1)	7.9 (±1.1)	13.5 (±0.4)
Pyridine	4.08[c]	14.8 (±12.8)	11.4 (±1.9)	2.9 (±2.8)	9.2 (±0.2)
Quinoline	3.64[e]	1.5 (±0.4)	14.2 (±0.1)	11.2 (±0.9)	12.4 (±0.4)
2,4-Dinitrophenol	4.51[e]	16.6 (±0.7)	8.7 (±1.0)	24.9 (±2.7)	19.8 (±1.6)
2,6-Dinitrophenol	4.18[h]	14.3 (±0.9)	11.7 (±0.3)	24.2 (±6.7)	18.7 (±2.9)

[a] Eluent: 50% (w/w) methanol–buffer solutions. For buffer compositions see Table 1.
[b] Computed from the van't Hoff plots.
[c] From Ref. [15].
[d] From Ref. [30].
[e] Estimated from [44].
[f] From Ref. [45].
[g] Unpublished results.
[h] From Ref. [40].

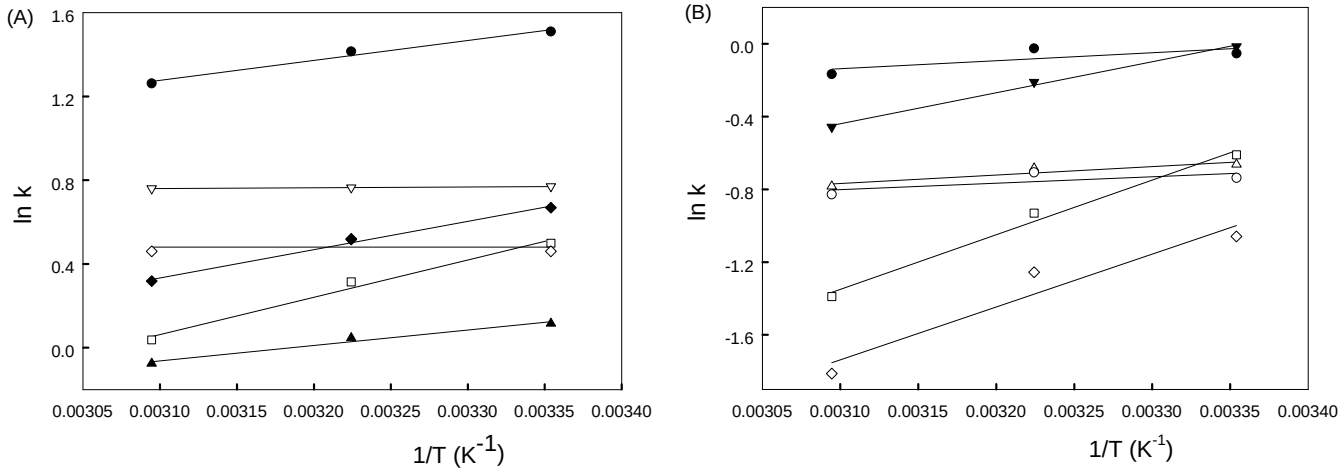

Fig. 1. van't Hoff plots of acidic and basic analytes eluted from a MS X-Terra C18 (15 cm × 0.46 cm i.d.). Acetic/acetate buffer $^{s}_{s}$pH (25 °C) = 5.05. (A) (□) 2-methylbenzoic acid; (▽) trazodone; (◇) papaverine; (▲) 2-aminotoluene; (●) *N*,*N*-dimethylaniline; (◆) quinoline. (B): (○) benzimidazole; (△) pyridine; (□) 2,4-dinitrophenol; (◇) 2,6-dinitrophenol; (▼) benzoic acid; (●) 4-aminotoluene.

in Fig. 3. At 25 °C, a slight difference in retention factors of most solutes between both buffer solutions can be noted. These differences would be attributed to the 10-times increase in the ionic strength of buffer B2 as compared with buffer B3. In this simplified model, retention was considered only due to partition of the ionizable analytes between mobile and stationary phases, and thus dependent on methanol composition, pH and temperature. However, the true retention of these compounds would certainly be affected by multiple and simultaneous processes such as ion-pair formation with ions present in the eluent or hydrophobic interactions induced by the ionic strength of the medium. In this particular case, silanophylic interactions with the surface should be minimized due to the election

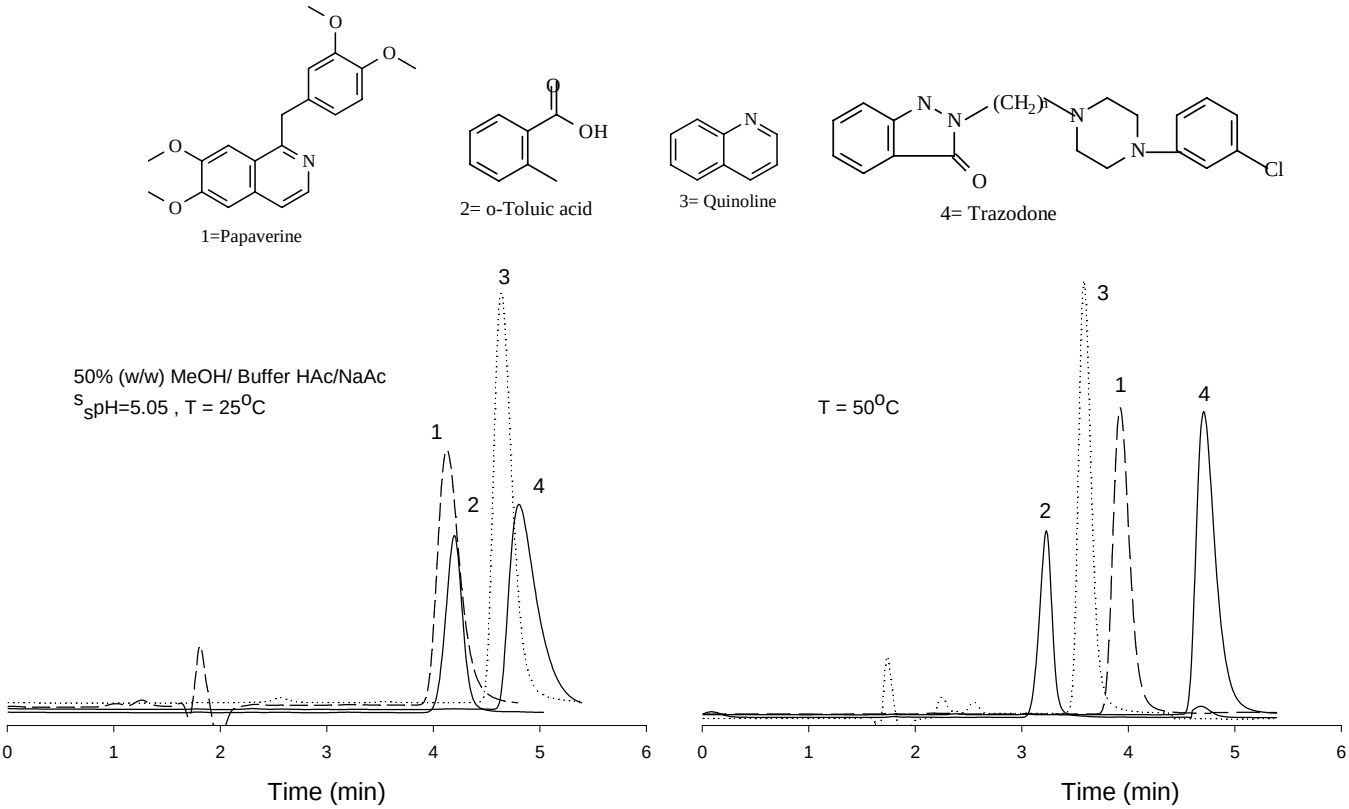

Fig. 2. Influence of temperature on retention and selectivity. Chromatograms of analytes eluted at 25 and 50 °C. Acetic/acetate buffer $^{s}_{s}$pH (25 °C) = 5.05. Analytes: papaverine, 2-methylbenzoic acid, quinoline and trazodone. Other conditions as in Fig. 1.

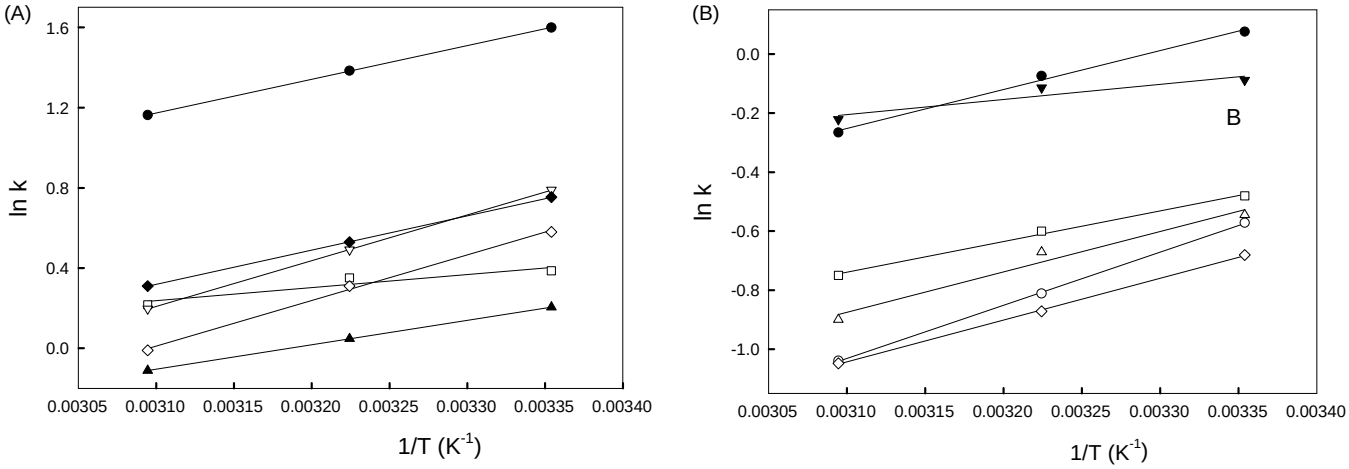

Fig. 3. van't Hoff plots of acidic and basic analytes. Piperazine + HCl buffer $^{s}_{s}$pH (25 °C) = 5.04. (A) (□) 2-methylbenzoic acid; (▽) trazodone; (◇) papaverine; (▲) 2-aminotoluene; (●) *N*,*N*-dimethylaniline; (◆) quinoline. (B): (○) benzimidazole; (△) pyridine; (□) 2,4-dinitrophenol; (◇) 2,6-dinitrophenol; (▼) benzoic acid; (●) 4-aminotoluene. Other conditions as in Fig. 1.

of the reversed-phase column. In spite of these differences in ionic strength, the effect of increasing the temperature was markedly different in both buffer systems. The comparison of Figs. 1 and 3 indicates several changes in the critical pairs depending on the buffer mobile phase. Chromatograms of the same four solutes, which where baseline separated at 50 °C in acetic buffer (Fig. 2), are shown in Fig. 4. A significant decrease in retention of the three basic analytes (papaverine, quinoline and trazodone) at 50 °C led to spoil resolution. *o*-Toluic was much less influenced by temperature.

In order to put these observations in a quantitative context, we intend to test the validity of the model depicted in the Introduction. The apparent enthalpies for the chromatographic process can be experimentally estimated from the slopes of these plots (see Appendix A). Although the number of data-points is undoubtedly insufficient for obtaining rigorous thermodynamic information, with the purpose of studying the tendencies, enthalpies were estimated from the consideration of linearity in the van't Hoff plots. The results are presented in Table 3. Enthalpies of transfer for the protonated and the deprotonated solutes from the mobile to the stationary phase, $\Delta_t H^{\circ}_{HA}$ and $\Delta_t H^{\circ}_{A}$, respectively, can be estimated from the van't Hoff plots measured with mobile phases regulated at pH lower and higher than the solute pK_a. Thus, solutes were eluted from the column with a mobile phase composed of 50% methanol/phosphoric buffer $^{s}_{s}$pH = 3.04. Solutes were protonated at this pH, i.e., acids were neutral and basic solutes were positively charged. Finally, a buffer controlling $^{s}_{s}$pH = 8.13 (B4) in 50% (w/w)

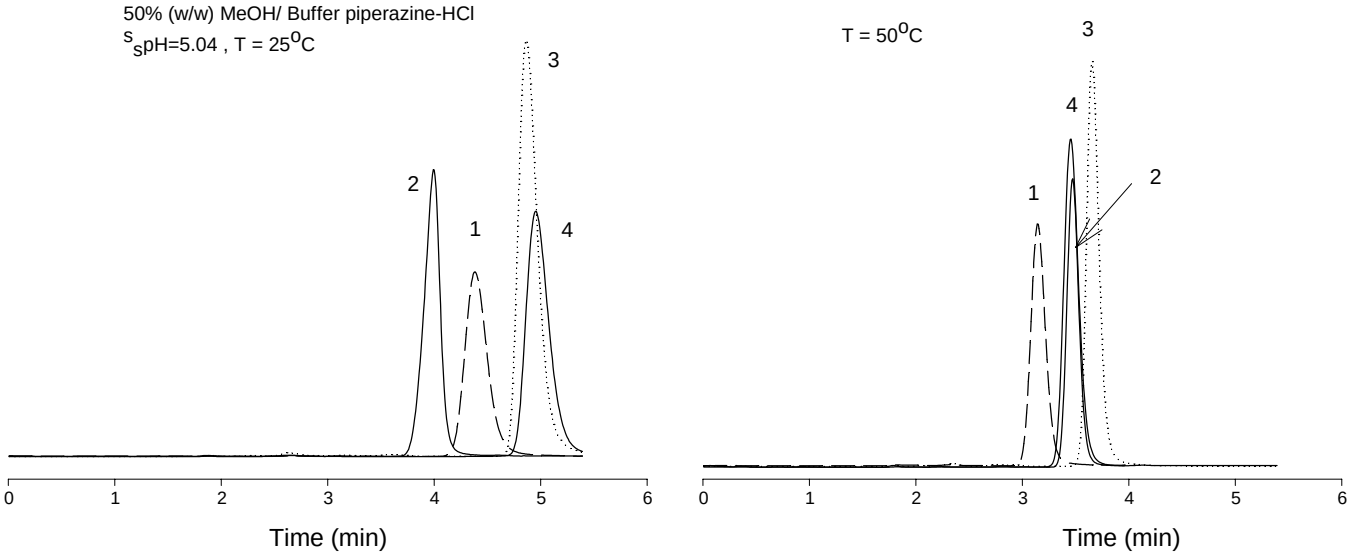

Fig. 4. Influence of temperature on retention and selectivity. Chromatograms of analytes eluted at 25 and 50 °C. Piperazine + HCl buffer $^{s}_{s}$pH (25 °C) = 5.04. Analytes: papaverine, 2-methylbenzoic acid, quinoline and trazodone. Other conditions and solute references as in Fig. 2.

Table 4
Comparison of apparent enthalpies calculated from Eq. (9) with experimental values

Solute	k_{HA}[a]	k_A[b]	ΔH_a (kJ mol^{-1})	Buffer B2 $^{s}_{s}$pH = 5.04					Buffer B3 $^{s}_{s}$pH = 5.05				
				w[c]	1st[d]	2nd[d]	ΔH_{app}		w[d]	1st[c]	2nd[c]	ΔH_{app}	
							Calc.	Exp.				Calc.	Exp.
Benzoic acid	1.563	0.224	0.6[e]	0.198	−15.3	4.8	−10.4	−4.3	0.417	−15.3	−0.2	−15.5	−14.2
2-Methylbenzoic acid	2.646	0.337	−5.86[f]	0.255	−16.5	7.1	−9.3	−5.4	0.537	−16.4	1.6	−14.8	−14.8
Pyridine	0.03	0.692	20.1[e]	4.423	−8.9	−2.7	−11.7	−11.4	9.336	−9.1	1.9	−7.2	−2.9
N,*N*-Dimethylaniline	0.296	5.896	28.3[e]	2.791	−13.0	−1.9	−14.9	−14.0	5.891	−13.3	3.9	−9.4	−7.9
Quinoline	0.339	2.245	22.4[f]	12.182	−12.2	−0.9	−13.0	−14.2	25.715	−12.3	0.7	−11.6	−11.2
2,4-Dinitrophenol	1.896	0.524	11.0[f]	1.643	−17.6	7.7	−10.0	−8.7	3.469	−18.2	−3.2	−21.4	−24.9
2,6-Dinitrophenol	1.269	0.482	7.61[f]	3.513	−16.8	5.8	−11.0	−11.7	7.416	−17.6	−1.1	−18.7	−24.2

[a] Retention factors. Mobile phase: 50% (w/w) methanol/buffer $^{s}_{s}$pH = 3.04 (buffer B1, Table 1).
[b] Retention factors. Mobile phase: 50% (w/w) methanol/buffer $^{s}_{s}$pH = 8.13 (buffer B4, Table 1).
[c] See Eq. (9). $w = (C_B/C_{HB})(K_{a(an)}/K_{a(buff)})$.
[d] Refers to first and second term on the right-hand of Eq. (9).
[e] From Ref. [15].
[f] Enthalpy of ionization of solutes in water.

methanol was used. In this last case, solutes were eluted under the unprotonated form: negatively charged anions and neutral amines. The results were gathered in Table 3.

In the mobile phase buffered with the conjugated pair acetic/acetate (B3) the pH remained almost constant as temperature increased from 25 to 50 °C due to an almost zero heat of ionization of acetic acid (see Table 1). The decrease in retention factor of *o*-toluic acid in buffer B3 has to be attributed mainly to the enthalpies of transfer of both protonated and unprotonated species as is shown in the first term on the right-hand of Eq. (9). Quinoline, an aromatic amine with $^{s}_{s}pK_a = 3.64$, is present under the basic form at the pH of the eluent B3 and, although it has a large positive heat of ionization, the second term on Eq. (9) has little effect on the total apparent enthalpy (see Table 4). On the other hand both, papaverine and trazodone, have strong negative enthalpies of transfer for the charged and neutral species. Thus, their almost null apparent enthalpies for the chromatographic retention in acetic buffer are the result of a positive compensation due to the ionization enthalpies of these two amines (second term of Eq. (9)). In Table 4, apparent enthalpies of a few solutes, whose heats of ionization were available, are compared with the data obtained from experimental regression of $\ln k$ versus $1/T$. The results clearly confirm the qualitative explanation given in the precedent paragraph for *o*-toluic and quinoline.

In buffer solution B2, the pH of the eluent strongly depends on column temperature; heat of ionization of piperazine measured in 50% methanol is 35.8 kJ mol^{-1}. This temperature-induced shift in the eluent pH in addition to the significant pK_a change of solutes with temperature will *differentially* affect retention of weak acids and bases. Acidic solutes, such as *o*-toluic acid, shall partially compensate the negative first term (enthalpies of transfer) with a positive second term due to ionization of the buffer (shall be $(g - 1) < 1$). As a consequence, their apparent enthalpies will be smaller in this buffer system (see benzoic and toluic acids and dinitrophenols in Tables 3 and 4). For amines, like trazodone, papaverine and quinoline in Fig. 4, the relative dissociation enthalpies between solute and buffer compensates and, as a consequence, the incidence of this second term will be rather small, as it is clear in the results gathered in Table 3.

A similar temperature study was extended to buffers controlling alkaline pH. Phosphate buffer solution (B7) and *tris* + HCl solution (B6) in methanol/water solvent mixture were prepared so that their final $^{s}_{s}$pH at 25 °C were close. The following four solutes: 2,4-dichlorophenol, 2,6-dichlorophenol, benzylamine (BZA) and *N*,*N*-dimethylbenzylamine (DMBA) were run in both buffer systems at three temperatures. The results of retention factors are presented as $\ln k$ versus $1/T$ in Fig. 5A and B. Retention factors of BZA and DMBA increase in buffer phosphate as temperature is increased, however, both amines displayed a positive slope (decreased retention as temperature increases) in a mobile phase constituted by buffer *tris* + HCl in methanol/water. It is evident that the critical separation between 2,6-dichlorophenol and DMBA at 25 °C improves significantly at 50 °C only if buffer phosphate is chosen to control the pH of the eluent. The chromatograms of these two analytes in buffer phosphate at 25 and 50 °C are superimposed in Fig. 6; the elution profiles of the same analytes in buffer *tris* + HCl are shown in Fig. 7. In this case, because of the stronger dependence of retention with temperature for DMBA, the change in temperature within the range studied here modifies the elution order but does not have any beneficial effect on resolution. The slopes, computed as apparent enthalpies of the retentive mechanism, are presented in Table 5. In the table we also included the enthalpies of transfer for protonated (positively charged amines and neutral phenols) and unprotonated (basic amines and negatively charged phenols) analytes. These values were computed from the slopes of the corresponding van't Hoff plots obtained from retention factors in mobile phases consisting in acetic/acetate buffer (B5, $^{s}_{s}$pH = 5.60) and 1-aminobutane + HCl buffer (B8, $^{s}_{s}$pH = 10.30).

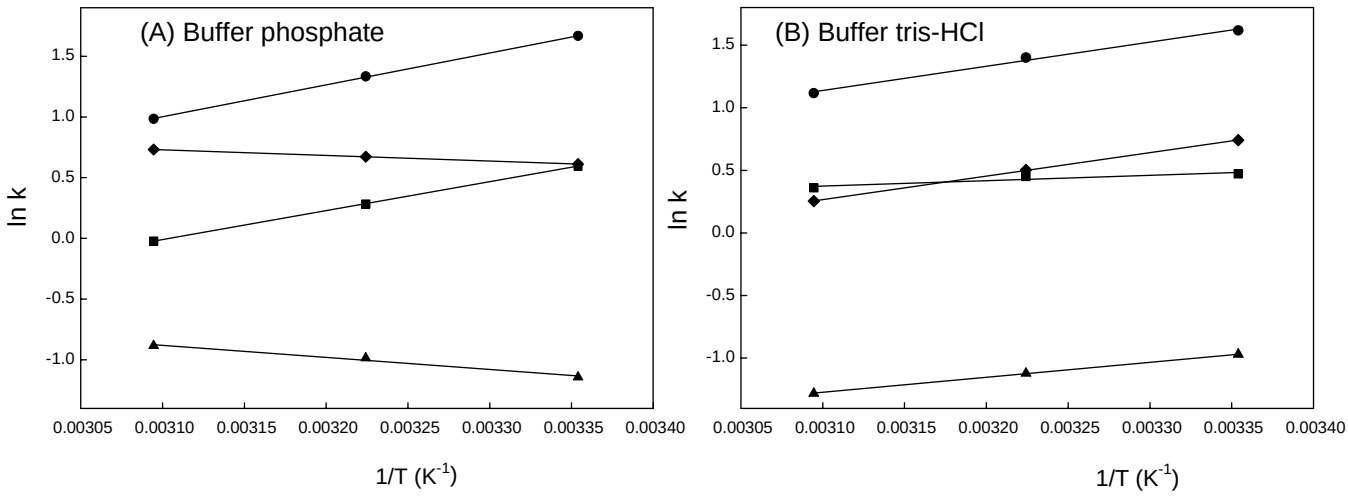

Fig. 5. van't Hoff plots of acidic and basic analytes. Phosphate buffer ${}^{\S}_{S}$pH (25 °C) = 8.10 (A), and *tris* + HCl buffer ${}^{\S}_{S}$pH (25 °C) = 8.09 (B). Analytes: (●) 2,4-dichlorophenol; (■) 2,6-dichlorophenol; (▲) benzylamine (◆) benzyldimethylamine. Other conditions as in Fig. 1.

Ionization enthalpies in methanol/water for these four solutes are not available, but it is expected that they behave similar to other compounds of their respective chemical families. With this last hypothesis both, amines and phenols, should have large positive heats of ionization, i.e., a large increase in the ratio [B]/[HB] and [A]/[HA] for amines and phenols, respectively, due to the decrease in the pK_a as temperature increases. These positive enthalpies of ionization for solutes shall not be compensated in phosphate buffer ($\Delta H^{\circ}_{a(i)} = 1.9\,\mathrm{kJ\,mol^{-1}}$) and since g value will be larger than 1 for amines, the positive apparent enthalpies of both amines in phosphate buffer are attributed to a large positive second term of Eq. (9). On the other hand, as enthalpies of transfer are close to zero, retention increase upon increasing the temperature on this ODS column because reversed-phase interactions are dominant, and the neutral form interacts with the ODS phase much stronger than does the charged form.

Phenols, in addition to the increase in the concentration of deprotonated species relative to the neutral ones, have exothermic heats of transfers from mobile to the stationary phase and thus, as a whole, will be less retained as temperature increases.

Recently McCalley studied the influence of temperature on retention of benzene and of other three basic compounds from an ODS column by using acetonitrile-phosphate buffers of pH 3 and 7 as eluents [13]. The van't Hoff plots over the range of 20–60 °C were linear, and depending on the pH of the buffer and the nature of the analyte, positive and negative slopes were obtained. However, the buffers were prepared by measuring the pH *before* mixing with a given amount

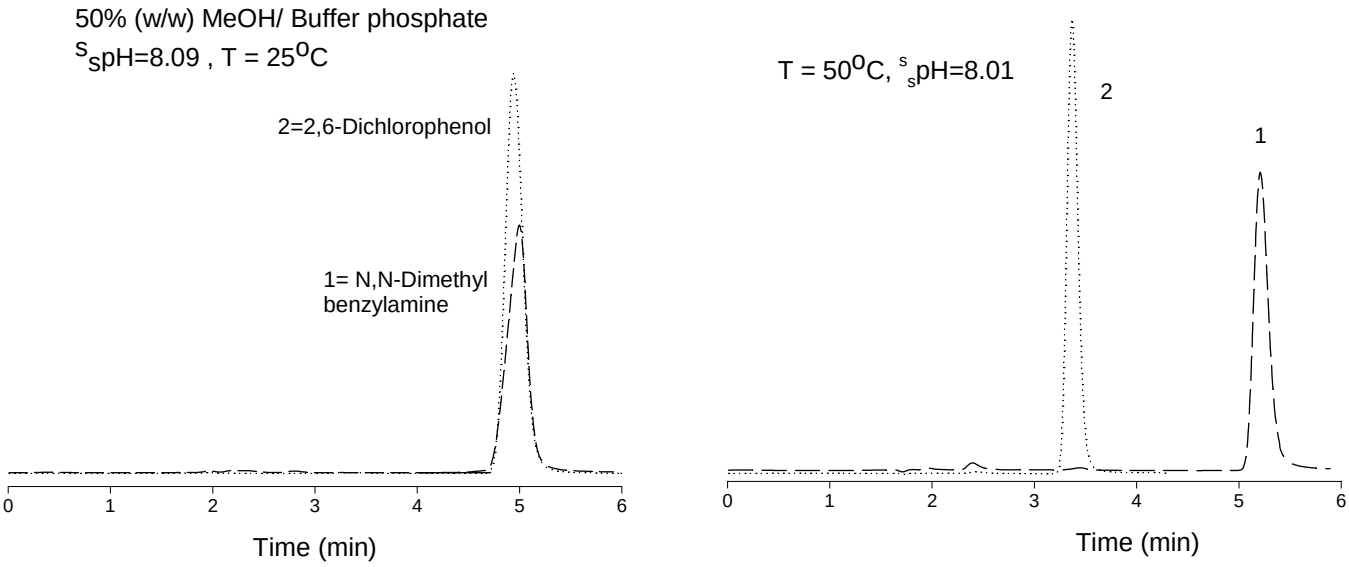

Fig. 6. Influence of temperature on retention and selectivity. Chromatograms of analytes eluted at 25 and 50 °C. Dihydrogenphosphate/hydrogenphosphate buffer ${}^{\S}_{S}$pH (25 °C) = 8.10. Analytes: papaverine, 2-methylbenzoic acid, quinoline and trazodone. Other conditions as in Fig. 1.

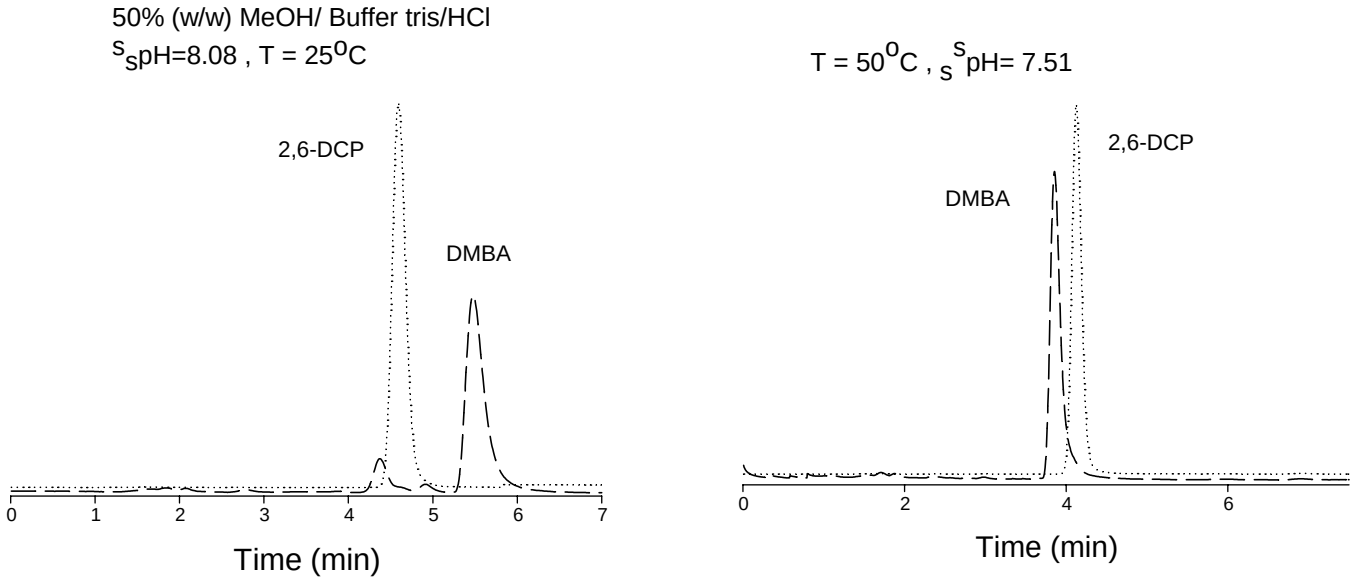

Fig. 7. Influence of temperature on retention and selectivity. Chromatograms of analytes eluted at 25 °C and at 50 °C. *Tris* + HCl buffer $^{s}_{s}$pH (25 °C) = 8.09. Analytes: 2,6-dichlorophenol (2,6-DCP) and benzyldimethylamine (DMBA). Other conditions as in Fig. 1.

Table 5
$^{s}_{s}pK_a$ (25 °C) values and apparent enthalpies of transfer for solutes on a C18 column and with different buffers in the mobile phase[a]

Solute	$^{s}_{s}pK_a$[b] (25 °C)	$-\Delta H_{app}$ (kJ mol^{-1})[c]			
		B5	B6	B7	B8
2,4-Dichlorophenol	8.60[d]	17.5 (±0.3)	16.0 (±1.2)	21.8 (±0.3)	11.0 (±1.2)
2,6-Dichlorophenol	7.68	14.1 (±0.2)	3.5 (±1.3)	19.8 (±0.1)	22.7 (±0.9)
Benzylamine	8.81[d]	−5.5 (±4.4)	10.0 (±0.2)	−8.3 (±1.0)	7.0 (±2.9)
Benzyldimethylamine	8.20	−1.6 (±2.3)	15.6 (±0.2)	−3.9 (±0.1)	10.0 (±1.4)

[a] Eluent: 50% (w/w) methanol-buffer solutions (see Table 1).
[b] From Ref. [30].
[c] Computed from the van't Hoff plots.
[d] $^{s}_{s}pK_a$ in 45% (w/w) methanol/water solvent mixture.

of acetonitrile, and actually an increase of about 0.7 pH units has been found for mixtures containing 40% acetonitrile [35]. Temperature has a small effect on the ionization of phosphoric acid and on dihydrogen phosphate in water and in methanol/water mixtures. Again, we assume a similar behavior in acetonitrile/water mixtures. On the other hand, amines (quinine, nortryptiline and pyridine) should have a large positive heat of ionization in water, and it is likely the same tendency in 35 or 40% (v/v) acetonitrile/water mixtures. As a consequence, the true mobile phase pH becomes close to that of pK_a of nortryptiline and of quinine when temperature is raised, and therefore, the apparent positive enthalpies found by McCalley are likely due to an increase in the ratio between [B]/[HB] at higher temperatures.

Mao and Carr [36], who also observed an increase in retention of antihistamines on a reversed-phase column as temperature increases, explained this behavior based on the change in the state of ionization of the basic solutes and the almost constant pH of the eluent controlled by a phosphate buffer. We agree about this condition which is necessary but is not enough to explain the negative slope of the van't Hoff plots. In addition, this *differential* ionization between solute and buffer must be dominant in comparison with the enthalpy of transfer of both ionic and neutral species from mobile to stationary phase.

5. Conclusions

This study provides background information about the influence of temperature in acid–base equilibria of ionizable important compounds in LC separations. The main remarks are as follows:

(1) Temperature plays a key role on dissociation constants of acidic and basic compounds in solvent mixtures and, thus, it affects differentially the retention and selectivity of weak electrolytes as a function of the chemical type of buffer used in mobile phase.
(2) A very significant effect of buffers on retention and selectivity will be observed when both protonated and unprotonated species are present (pH ≈ pK_a).

(3) Negative slopes in van't Hoff plots are expected when ionization of the buffer used to control the pH of the eluents have ionization enthalpies significantly different from those displayed by the solutes and, in addition, a small heat exchange occurs during the transfer of solute from mobile to stationary phase.

Further work will be faced towards obtaining useful thermodynamic data to demonstrate the role that temperature plays on dissociation constants of acidic and basic compounds in solvent mixtures. This information would be included in rational separation predictions.

Acknowledgements

We are thankful for financial support from MCYT of the spanish government, FEDER of E.U. (project BQU2001-2882) and catalan government (2001SRG00055). C.B.C. acknowledges to Fundación Antorchas and CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina) for her support.

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Appendix A

The apparent retention factor, Eq. (6), can be written as:

$$k = \varphi \left\{ \frac{e^{[-\Delta_t G^\circ_{HA}/RT]} + (C_B/C_{HB})\, e^{[-\Delta_t G^\circ_{A}/RT]}\, e^{[-(\Delta G^\circ_{a(an)} - \Delta G^\circ_{a(buff)})/RT]}}{1 + (C_B/C_{HB})\, e^{[-(\Delta G^\circ_{a(an)} - \Delta G^\circ_{a(buf\,f)})/RT]}} \right\} \tag{A.1}$$

where the phase ratio was introduced and thus, $k_i = \varphi K_{D(i)}$

By introducing the thermodynamic functions, Eq. (8), into Eq. (A.1):

$$k = \varphi \left\{ \frac{e^{[-\Delta_t H^\circ_{HA}/RT]}\, e^{[\Delta_t S^\circ_{HA}/R]} + r\, e^{[-(\Delta_t H^\circ_{A} + \Delta H^\circ_{a(an)} - \Delta H^\circ_{a(buff)})/RT]}\, e^{[(\Delta_t S^\circ_{A} + \Delta S^\circ_{a(an)} - \Delta S^\circ_{a(buff)})/R]}}{1 + r\, e^{[-(\Delta H^\circ_{a(an)} - \Delta H^\circ_{a(buff)})/RT]}\, e^{[(\Delta S^\circ_{a(an)} - \Delta S^\circ_{a(buff)})/R]}} \right\} \tag{A.2}$$

where $r = (C_B/C_{HB})$. By applying logarithm to Eq. (A.2) and differentiating respect to $(1/T)$:

$$\frac{d\ln k}{d(1/T)} = \frac{(-\Delta_t H^\circ_{HA}/R) K_{D(HA)} + r K_{D(A)} [-(\Delta_t H^\circ_{A} + \Delta H^\circ_{a(an)} - \Delta H^\circ_{a(HB)})/R] (K_{a(an)}/K_{a(buff)})}{K_{D(HA)} + r K_{D(A)} (K_{a(an)}/K_{a(buff)})} - \frac{r[-(\Delta H^\circ_{a(an)} - \Delta H^\circ_{a(buff)})/R](K_{a(an)}/K_{a(buff)})}{1 + r(K_{a(an)}/K_{a(buff)})} \tag{A.3}$$

The apparent enthalpy for the chromatographic process will be obtained from:

$$\Delta H^\circ_{app(an)} = -R \frac{d\ln k}{d(1/T)} = \frac{k_{HA} \Delta_t H^\circ_{HA} + k_A r(\Delta_t H^\circ_{A} + \Delta H^\circ_{a(buff)} - \Delta H^\circ_{a(buff)})(K_{a(an)}/K_{a(buff)})}{k_{HA} + k_A r(K_{a(an)}/K_{a(buff)})} - \frac{r[\Delta H^\circ_{a(an)} - \Delta H^\circ_{a(buff)}](K_{a(an)}/K_{a(buff)})}{1 + r(K_{a(an)}/K_{a(buff)})} \tag{A.4}$$

Making $w = r(K_{a(an)}/K_{a(buff)})$, $g = (k_A/k_{HA})$ and rearranging the equation becomes:

$$\Delta H^\circ_{app(an)} = \frac{\Delta_t H^\circ_{HA} + gw[\Delta_t H^\circ_{A} + (\Delta H^\circ_{a(an)} - \Delta H^\circ_{a(buff)})]}{1 + gw} - \frac{w(\Delta H^\circ_{a(an)} - \Delta H^\circ_{a(buff)})}{1 + w} \tag{A.5}$$

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