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Retention of ionisable compounds on high-performance liquid chromatography XV. Estimation of the pH variation of aqueous buffers with the change of the acetonitrile fraction of the mobile phase

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Abstract

The most commonly used mobile phases in reversed-phase high-performance liquid chromatography (RP-HPLC) are hydro-organic mixtures of an aqueous buffer and an organic modifier. The addition of this organic solvent to buffered aqueous solutions involves a variation of the buffer properties (pH and buffer capacity). In this paper, the pH variation is studied for acetic acid–acetate, phosphoric acid–dihydrogenphosphate–hydrogenphosphate, citric acid–dihydrogencitrate–citrate, and ammonium–ammonia buffers. The proposed equations allow pH estimation of acetonitrile–water buffered mobile phases up to 60% (v/v) of organic modifier and initial aqueous buffer concentrations between 0.001 and 0.1 mol L^{-1}, from the initial aqueous pH. The estimated pH variation of the mobile phase and the pK_a variation of the analytes allow us to predict the degree of ionisation of the analytes and from this and analyte hydrophobicities, to interpret the relative retention and separation of analyte mixtures.

Keywords: Mobile phase composition; Acetonitrile–water mixtures; pH; Buffers; Chromatographic retention

1. Introduction

Careful pH control and measurement of the mobile phase is essential for a reproducible and successful chromatographic analysis of ionisable analytes. There are three different pH scales commonly used in pH measurement of reversed-phase high-performance liquid chromatography (RP-HPLC) mobile phases. The IUPAC recommends to measure pH in the mobile phase, after mixing aqueous buffer and organic modifier. The pH electrode system can be calibrated with aqueous buffers and thus the pH readings provide directly the ${}^{s}_{w}$pH values of the mobile phase, i.e. the pH value in the mobile phase solvent (s) relative to water (w) as standard state solvent [1]. Alternatively, the pH electrode system can be calibrated with buffers prepared in the water organic solvent mixture used as mobile phase, and the pH readings provide ${}^{s}_{s}$pH values, i.e. the pH value in the mobile phase solvent (s) relative to the same solvent (s) as standard state solvent [1]. The two IUPAC pH scales can be easily related by means of the δ parameter [2–4]:

$$ {}^{s}_{w}\mathrm{pH} = {}^{s}_{s}\mathrm{pH} + \delta \quad (1) $$

The δ parameter includes the primary medium effect and the difference between the liquid-junction potentials of the electrode system in the mobile phase and water. The primary medium effect depends only on the solvent at which pH is measured (mobile phase solvent composition), but the liquid-

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Table 1
Properties of relevant interest for pH measurements in acetonitrile–water mixtures at 25 °C [4]

MeCN (%, v/v)	x_{MeCN}	A	a_0B	${}^{s}_{s}pK_{ap}$	δ
0	0.000	0.528	1.52	14.00	0.00
10	0.040	0.566	1.55	14.24	−0.01
20	0.079	0.604	1.59	14.47	−0.03
30	0.130	0.655	1.63	14.74	−0.04
40	0.186	0.712	1.68	15.08	−0.14
50	0.260	0.791	1.74	15.48	−0.22
60	0.339	0.877	1.80	15.90	−0.46

x_{MeCN}: molar fraction of acetonitrile in the mixture; A and a_0B: Debye–Hückel equation parameters; ${}^{s}_{s}pK_{ap}$: autoprotolysis constant of the solvent mixture; δ: interconversion parameter between ${}^{s}_{s}$pH and ${}^{s}_{w}$pH scales.

junction potential depends also on the particular electrode system, pH standards, and sample used. Therefore, general interlaboratory conversion between both pH scales is only possible if the different electrode systems are designed to have a negligible residual liquid-junction potential, i.e. if the junction potential of the electrode system in the measured mobile phase is close to the junction potential in the calibration solution in water [4].

The ${}^{s}_{w}$pH scale is specially suitable for its simplicity of measurement, because it does not require pH standards for each hydro-organic composition. Table 1 reports δ values obtained in this lab for the electrode system described in the experimental part and for some acetonitrile–water mixtures, as well as other parameters of interest for pH estimation in these mobile phases. Nevertheless, the most common pH scale used in chromatography is the aqueous pH scale (${}^{w}_{w}$pH) [1], which is obtained when the electrode system is calibrated with aqueous buffers and the pH measured in the RP-HPLC aqueous buffer before mixing it with the organic modifier. The relationship between ${}^{w}_{w}$pH value and ${}^{s}_{w}$pH or ${}^{s}_{s}$pH is buffer dependent [5–7] and it has been pointed out that adjusting the pH in the aqueous buffer may lead to significant differences in RP-HPLC retention when the same organic modifier is added to aqueous buffers of the same pH value, but prepared from different buffer components [5]. For instance, buffered solutions prepared from anionic and neutral acids increase their pH value when acetonitrile is added, whereas cationic acids show the reverse trend [5]. The pK_a variation of analytes follows a similar tendency: the same analyte in two aqueous buffers of the same pH, but prepared from different acids and bases, may show a different degree of ionisation, and thus different chromatographic retention, when acetonitrile is added to prepare the mobile phase [5].

In this paper, the variation of the aqueous pH of common chromatographic buffers upon addition of acetonitrile is studied for different initial buffer concentration and pH. Several chromatographic examples, in both isochratic and gradient elution, are presented to illustrate how the variation of buffer pH changes ionisation of acid–base analytes and thus chromatographic retention.

2. Experimental

2.1. Apparatus

Potentiometric measurements were taken with a Ross combination electrode Orion 8102 (glass electrode and a reference electrode with a 3.0 mol L^{-1} KCl solution in water as salt bridge) in a Crison MicropH 2002 potentiometer with a precision of ±0.1 mV. All the solutions were thermostated externally at 25 ± 0.1 °C. The retention data were measured on a 15 cm × 4.6 mm i.d. XTerra MS C_{18} 5-μm (Waters) column with a flow rate of 1 mL min^{-1} in isochratic mode. A Shimadzu (Kyoto, Japan) HPLC system consisting of two LC-10ADvp dual reciprocating plunger solvent delivery modules, a SIL-10ADvp autoinjector fixed to 10 μL, a SPD-10AVvp ultra-violet visible spectrophotometric detector set at 254 nm, a CTO-10ASvp column oven at 25 ± 0.1 °C and a SCL-10Avp system controller was employed.

2.2. Chemicals

Acetonitrile was RP-HPLC gradient grade from Merck and water purified by the Milli-Q plus system from Millipore. The studied buffers were prepared from acetic acid (Merck, glacial, for analysis), sodium acetate (Carlo Erba, 99%), phosphoric acid (Merck, 85%, for analysis), potassium dihydrogenphosphate (Merck, for analysis), sodium hydrogenphosphate (Merck, for analysis), citric acid (Fluka, for analysis), potassium dihydrogencitrate (Fluka, >99%), sodium citrate (Merck, for analysis), ammonia (Merck, 25%, for analysis) and ammonium chloride (Merck, for analysis), using hydrochloric acid (Merck, 25%, for analysis) and potassium hydroxide (Panreac, for analysis) to adjust the pH to the wanted value. The chromatographied compounds were 2-nitrophenol (Fluka, >99%), 3-bromophenol (Schuchardt, 90%), 2,4,6-trimethylpyridine (Merck, 96%) and *N*,*N*-dimethylbenzylamine (Merck–Schuchardt, for synthesis).

2.3. Procedure

The required aqueous acid and base concentrations for the selected pH is calculated before the preparation of the buffer, considering total buffer aqueous concentrations of 0.001, 0.01 and 0.1 mol L^{-1}. The pH is finally slightly adjusted by addition of small amounts of concentrated solutions of potassium hydroxide. Acetonitrile–water buffers were prepared by addition of acetonitrile to the aqueous buffers. In all instances, the electrode system was calibrated using the usual aqueous standard reference buffers of potassium hydrogenphthalate (${}^{w}_{w}$pH 4.01 at 25 °C) and potassium dihydrogenphosphate–disodium hydrogenphosphate (${}^{w}_{w}$pH 7.00 at 25 °C). All pH readings were done in the ${}^{s}_{w}$pH scale, i.e. after mixing aqueous buffer with acetonitrile.

Chromatographic data were obtained isochratically and in a fast gradient mode (0.00 → 2.50 min: 10 → 100% MeCN;

2.50 → 3.00 min: 100%; 3.00 → 3.20 min: 100 → 10%; 3.20 → 4.00 min: 10%).

3. Results and discussion

3.1. Model development

Previous work [5] shows that ${}^{s}_{w}\mathrm{pH}$ variation of buffers at the initial aqueous ${}^{w}_{w}\mathrm{pH}$ with the addition of acetonitrile (φ_{MeCN} on volume fraction of acetonitrile in the mixture) can be approximately fitted to a linear equation:

$$ {}^{s}_{w}\mathrm{pH} - {}^{w}_{w}\mathrm{pH} = m_{\mathrm{pH}}\varphi_{\mathrm{MeCN}} \quad (2) $$

with a m_{pH} value that depends on the particular buffer used and initial ${}^{w}_{w}\mathrm{pH}$ of the buffer. m_{pH} is the proportionality coefficient between pH and mobile phase solvent composition changes. The pH variation is caused by the variation of the pK_a values of buffer components when the solvent composition changes. The variation of the pK_a values of the studied acids (buffer components) is presented in Table 2, and some examples of pH variation with the volume fraction of acetonitrile added depending on the initial ${}^{w}_{w}\mathrm{pH}$ values are presented in Fig. 1.

Table 2
${}^{s}_{s}\mathrm{p}K_a$ values of the acids studied as buffer components in acetonitrile–water mixtures [5]

Buffer	${}^{s}_{s}\mathrm{p}K_a$ (%, v/v) of acetonitrile						
	0	10	20	30	40	50	60
Acetic acid	4.74	4.94	5.17	5.44	5.76	6.15	6.62
Phosphoric acid	2.21	2.39	2.62	2.80	3.11	3.42	3.75
Dihydrogenphosphate	7.23	7.40	7.60	7.82	8.08	8.38	8.73
Citric acid	3.16	3.31	3.49	3.68	3.90	4.16	4.45
Dihydrogencitrate	4.79	4.95	5.14	5.35	5.60	5.91	6.28
Hydrogencitrate	6.42	6.62	6.85	7.11	7.40	7.74	8.13
Ammonium	9.29	9.27	9.21	9.17	9.19	9.21	9.34

Espinosa et al. [5] proposed the following equation to describe the variation of the slope (m_{pH}) of Eq. (1) with the initial aqueous ${}^{w}_{w}\mathrm{pH}$ of the buffer:

$$ m_{\mathrm{pH}} = \frac{a_0 + \sum_{i=1}^{n} a_i 10^{s(i\mathrm{pH}-b_i)} + a_{n+1}10^{s[(n+1)\mathrm{pH}-b_{n+1}]}}{1 + \sum_{i=1}^{n} 10^{s(i\mathrm{pH}-b_i)} + 10^{s[(n+1)\mathrm{pH}-b_{n+1}]}} \quad (3) $$

The a_0 term in the numerator and the 1 value in the denominator predominate over the other terms at low pH values, when the solution is buffered by strong acids. Then, for strong acids, a_0 parameter is taken equal to zero. The $n+1$ term predom-

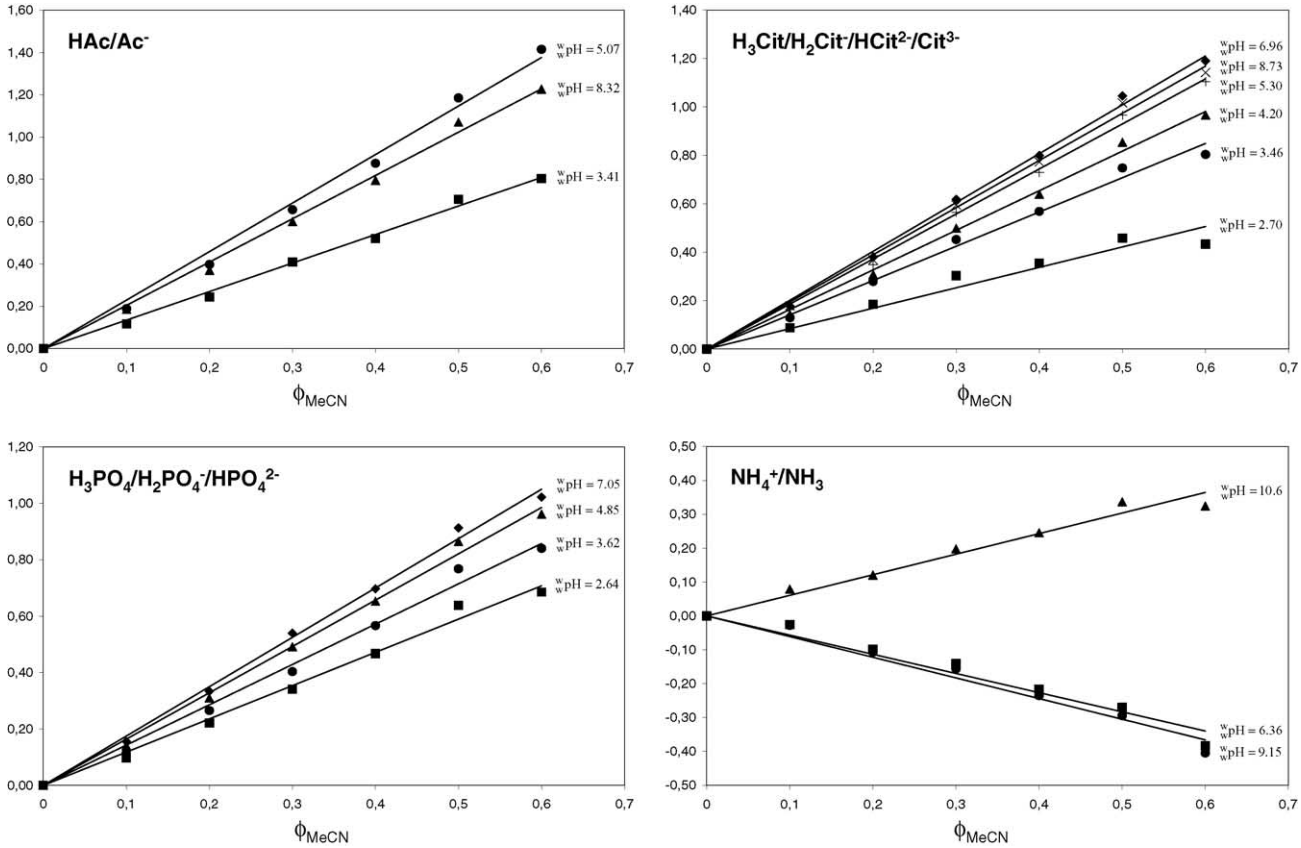

Fig. 1. Variation of the pH values (${}^{s}_{w}\mathrm{pH} - {}^{w}_{w}\mathrm{pH}$) of several studied solutions depending on the acetonitrile fraction added to aqueous buffer 0.01 M and initial aqueous ${}^{w}_{w}\mathrm{pH}$.

inates at very basic pH values (buffers with strong bases), and a_{n+1} of strong bases has an estimated value of 1.81 [5]. The intermediate terms prevail in the pH zones close to the acid–base conjugate equilibria of the buffered system, represented by their n pK_a values. The meaning of these terms will be discussed later.

The studied range of acetonitrile–water mixtures goes up to 60% (v/v) of organic modifier. In this high water content medium homoconjugation and ionic pair formation can be neglected, and the involved acid–base equilibria are quite similar to the ones in aqueous solutions.

The ${}^{s}_{s}$pH values of several series of buffers were calculated at 0, 10, 20, 30, 40, 50 and 60% (v/v) of acetonitrile taking into account the dilution coefficient, the molar activity coefficient (by means of the Debye–Hückel equation), and the ${}^{s}_{s}$pK_a of each buffer component at the corresponding hydro-organic composition (Tables 1 and 2). The dielectric constants of the studied solvent mixtures are higher than 40 [4] and, thus, ion pairing should be insignificant in them [8] and was not considered in pH calculation. The autoprotolysis constant of each solvent composition was also considered in the calculations (Table 1). This calculation has been carried out for thirteen different aqueous buffer concentrations: 0.001, 0.003, 0.005, 0.007, 0.01, 0.02, 0.03, 0.04, 0.05, 0.0625, 0.075, 0.0875 and 0.1 mol L^{-1}. Then, the ${}^{s}_{s}$pH calculated values were converted to the ${}^{s}_{w}$pH scale by means of the δ values (Table 1 and Eq. (1)). For each initial aqueous ${}^{w}_{w}$pH and the subsequent acetonitrile additions, the m_{pH} value was calculated.

The m_{pH} values for the studied buffers and concentrations were plotted against their corresponding initial aqueous ${}^{w}_{w}$pH value, and fitted to Eq. (3). Fig. 2 shows three of the most representative studied concentrations (0.001, 0.01 and 0.1 mol L^{-1}) for several buffered systems.

Table 3 shows the fitted s, a_i and b_i parameters corresponding to the studied buffered systems (acetic acid–acetate, citric acid–dihydrogencitrate–hydrogencitrate–citrate, phosphoric–dihydrogenphosphate–hydrogenphosphate, ammonium–ammonia) at three different representative concentrations.

In the acetic acid system, the a_0 parameter corresponds to the estimated value of a strong acid ($a_0 \approx 0$), a_1 is referred to the m_{pH} maximum value of acetic acid/acetate solutions, a_2 is the supposed value for a strong base ($a_2 \approx 1.81$), b_1 corresponds to the ${}^{w}_{w}$pH value of the inflection point of the upward curve (only acetic acid solutions) and $b_2 - b_1$ corresponds to the ${}^{w}_{w}$pH value of the inflection point of the downward curve (only acetate solutions). s is a fitting parameter related to the sharpness of the transitions between the different a_i values (Table 4).

Due to the high number of polynomial variables (s, a_1, a_2, a_3, b_1, b_2, b_3 and b_4; $a_0 \approx 0.00$ and $a_4 \approx 1.81$) in the citric acid buffered system, b_4 has been fixed before the iteration process to reach a better adjustment. This parameter can be easily known because $b_4 - b_3$ agree with the ${}^{w}_{w}$pH value corresponding to solutions with only citrate. When hydrogencitrate is the

Table 3
Parameters of Eq. (2) for the variation of the ${}^{s}_{w}$pH values of the buffers with the addition of acetonitrile, at three representative initial aqueous buffer concentration

Parameter	Acetic acid			Citric acid			Phosphoric acid			Ammonia		
	0.001 mol L^{-1}	0.01 mol L^{-1}	0.1 mol L^{-1}	0.001 mol L^{-1}	0.01 mol L^{-1}	0.1 mol L^{-1}	0.001 mol L^{-1}	0.01 mol L^{-1}	0.1 mol L^{-1}	0.001 mol L^{-1}	0.01 mol L^{-1}	0.1 mol L^{-1}
s	3.06	3.07	3.48	1.87	1.94	2.40	2.60	1.80	2.26	3.19	3.22	3.60
a_0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
a_1	2.27	2.29	2.27	1.09	1.42	1.45	0.64	1.44	1.77	−0.58	−0.60	−0.59
a_2	1.81	1.81	1.81	1.71	1.70	1.61	1.77	1.76	1.67	1.81	1.81	1.81
a_3	–	–	–	2.09	2.02	1.80	1.81	1.81	1.81	–	–	–
a_4	–	–	–	1.81	1.81	1.81	–	–	–	–	–	–
b_1	3.89	3.36	2.85	3.23	2.61	2.06	3.07	2.27	1.65	6.23	5.73	5.31
b_2	11.73	11.68	11.59	7.33	6.54	5.74	8.20	7.04	6.18	16.35	16.38	16.47
b_3	–	–	–	12.83	11.83	10.61	17.26	16.51	15.98	–	–	–
b_4	–	–	–	21.44	20.78	19.96	–	–	–	–	–	–
$b_2 - b_1$	7.84	8.32	8.74	4.10	3.93	3.68	5.13	4.77	4.53	10.12	10.65	11.16
$b_3 - b_2$	–	–	–	5.50	5.29	4.87	9.06	9.47	9.80	–	–	–
$b_4 - b_3$	–	–	–	8.61	8.95	9.35	–	–	–	–	–	–
N	45	45	45	112	118	122	54	60	81	45	45	45
S.D.	0.021	0.017	0.014	0.003	0.004	0.004	0.008	0.014	0.021	0.025	0.020	0.018
r^2	0.998	0.998	0.998	1.000	1.000	1.000	1.000	0.997	0.995	0.998	0.998	0.998

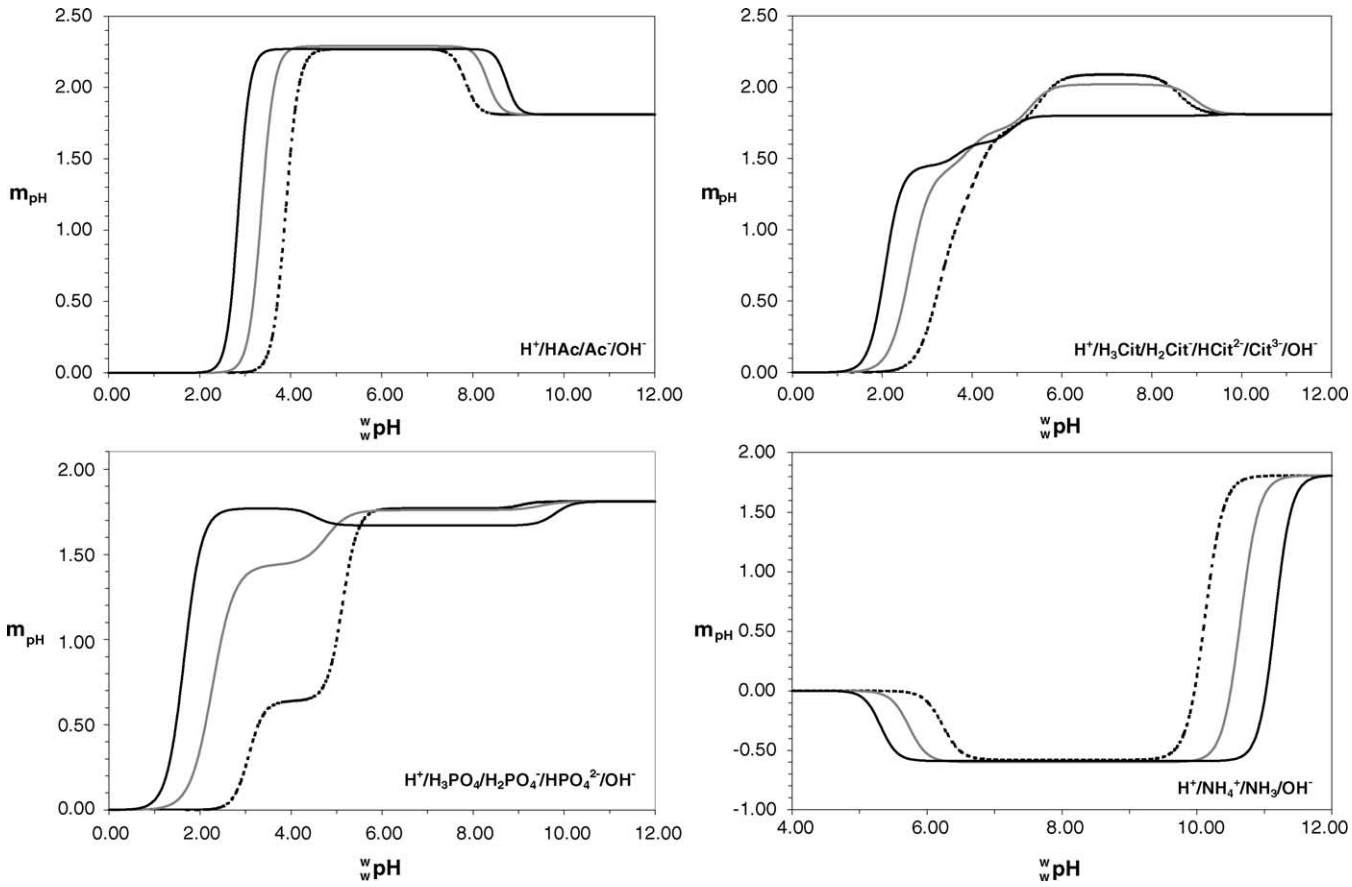

Fig. 2. Variation of the slope (m_{pH}) of Eq. (1) vs. volume fraction of acetonitrile with the initial aqueous pH of the buffer ($^{w}_{w}$pH). Dashed lines represent buffer aqueous concentrations at 0.001 mol L^{-1}, grey continuous lines 0.01 mol L^{-1} and black continuous lines 0.1 mol L^{-1}.

only species present in the buffered system, the $^{w}_{w}$pH value corresponds to $b_3 - b_2$. Analogously, $b_2 - b_1$ corresponds to dihydrogencitrate and b_1 to citric acid. On the other hand, a_1 refers to m_{pH} slope of citric acid–dihydrogencitrate solutions, a_2 to dihydrogencitrate–hydrogencitrate and a_3 to hydrogencitrate–citrate (Table 5).

In the calculation of the pH involved in the phosphoric acid buffer system, we have only been able to consider the contribution of the phosphoric acid, dihydrogenphosphate and hydrogenphosphate because of the absence of literature $^{s}_{s}pK_{a_3}$ values in acetonitrile–water mixtures, and phosphate insolubility when the fraction of organic modifier is high.

Table 4
Linear variation of the s, a_i and b_i parameters for the acetic acid–acetate buffer system depending on the aqueous buffer concentration (c_T)

Parameter	Acetic acid–acetate		
	Equation	N	S.D.
s	$0.20 \log c_T + 3.56$	13	0.085
a_0	0.00	–	–
a_1	2.28	13	0.007
a_2	1.81	–	–
b_1	$-0.52 \log c_T + 2.33$	13	0.005
$b_2 - b_1$	$0.45 \log c_T + 9.20$	13	0.012

To get a better polynomial fit in the iteration process, as we considered before for the citric acid system, parameters b_3 and b_2 were fixed. We are able to calculate these parameters considering that $b_3 - b_2$ corresponds to the $^{w}_{w}$pH value when the only species of the buffer system is the dihydrogenphosphate, $b_2 - b_1$ to the hydrogenphosphate and b_1 to phosphoric acid. Table 6 and Fig. 2 show that for aqueous concentrations of the buffer above 0.05 mol L^{-1}, the a_1 value is higher than

Table 5
Linear variation of the s, a_i and b_i parameters for the citric acid–dihydrogencitrate–hydrogencitrate–citrate buffer system depending on the aqueous buffer concentration (c_T)

Parameter	Citric acid–dihydrogencitrate–hydrogencitrate–citrate		
	Equation	N	S.D.
s	$0.29 \log c_T + 2.59$	13	0.067
a_0	0.00	–	–
a_1	$0.14 \log c_T + 1.63$	13	0.057
a_2	$-0.06 \log c_T + 1.56$	13	0.015
a_3	$-0.16 \log c_T + 1.67$	13	0.027
a_4	1.81	–	–
b_1	$-0.58 \log c_T + 1.47$	13	0.015
$b_2 - b_1$	$-0.21 \log c_T + 3.47$	13	0.014
$b_3 - b_2$	$-0.34 \log c_T + 4.58$	13	0.054
$b_4 - b_3$	$0.38 \log c_T + 9.72$	13	0.030

Table 6
Linear variation of the s, a_i and b_i parameters for the phosphoric acid–dihydrogenphosphate–hydrogenphosphate buffer system depending on the aqueous buffer concentration (c_T)

Parameter	Phosphoric acid–dihydrogenphosphate–hydrogenphosphate		
	Equation	N	S.D.
s	$-0.04 \log c_T + 1.99$	13	0.243
a_0	0.00	–	–
a_1	$0.53 \log c_T + 2.40$	13	0.086
a_2	$-0.06 \log c_T + 1.63$	13	0.015
a_3	1.81	–	–
b_1	$-0.69 \log c_T + 0.93$	13	0.036
$b_2 - b_1$	$-0.29 \log c_T + 4.22$	13	0.022
$b_3 - b_2$	$0.36 \log c_T + 10.18$	13	0.014

a_2. This fact could be attributed to the impossibility of considering the contribution of the phosphate species to the buffer system.

Analogous considerations of the acetic acid system can be made for ammonia system, except for the negative m_{pH} values corresponding to ammonium–ammonia solutions (Table 7).

A linear tendency is observed in the graphical representation of the parameters s, a_i and b_i value against the logarithm of the aqueous concentration of the buffer ($\log c_T$). For each buffer system, the results of the linear regression are shown in Tables 4–7. We have chosen the logarithmic linear regression because the solution pH is normally directly related to the present species concentration logarithm. Furthermore, it has been confirmed that this kind of approximation is better than the direct fitting to the concentration values. Although for all buffers the worse linear relationship corresponds to the polynomial adjustment parameter s, the fitting of all equations is quite good. A second degree equation has been considered to fit the s parameters as a function of concentration logarithm, but the results obtained in pH estimation are not significantly different from the ones estimated by means of the linear regression.

Quantitative measurement of buffer ability to keep pH can be expressed in terms of buffer capacity (β) of buffered solutions, which can be calculated by means of the following differential equation [2,3]:

$$\beta = \frac{dc_b}{d(pH)} \quad (4)$$

Table 7
Linear variation of the s, a_i and b_i parameters for the ammonium–ammonia buffer system depending on the aqueous buffer concentration (c_T)

Parameter	Ammonium–ammonia		
	Equation	N	S.D.
s	$0.20 \log c_T + 3.71$	13	0.086
a_0	0.00	–	–
a_1	−0.60	13	0.007
a_2	1.81	–	–
b_1	$-0.45 \log c_T + 4.84$	13	0.014
$b_2 - b_1$	$0.52 \log c_T + 11.67$	13	0.005

i.e. in rough terms, the strong base amount (expressed in equivalents) required to produce a one pH unit change in the buffer solution. Buffer capacity can be calculated by means of the algorithms used to determine the pH of the solution, calculating the pH change produced by a small change of the base concentration (e.g. 0.1%). For a weak acid/weak base, maximum buffer capacity of a protolyte occurs when the acid species concentration is equal to the concentration of conjugate base.

3.2. Experimental evaluation of the model

In order to calculate the accuracy of the model in the estimation of the pH variation of buffer with the variation of the mobile phase composition, several buffers at different composition, concentration and initial aqueous pH have been prepared and their pH values measured. To calculate the pH variation, we determine first the parameters (s, a_i and b_i) as a function of the aqueous buffer concentration (Tables 4–7). Then, when these values are fixed, the m_{pH} value can be estimated through Eq. (2) for each ${}^{w}_{w}$pH value. Finally, through the estimated value of m_{pH}, we can estimate the ${}^{s}_{w}$pH value corresponding to any acetonitrile–water mixture up to 60% (v/v) (Eq. (1)), and compare it with the experimental value.

Fig. 3 represents graphically the estimated ${}^{s}_{w}$pH values against the experimental ${}^{s}_{w}$pH values for all studied buffers. There is a good agreement between these measured pH values and the expected straight line of unitary slope and null origin ordinate. This figure also shows the variation of the buffer capacity as a function of ${}^{s}_{w}$pH values for different acetonitrile–water compositions.

In the acetic acid–acetate buffer, the highest dispersion is observed for basic ${}^{s}_{w}$pH (>7.5), perhaps because of its low buffer capacity in this pH range. As pointed earlier, maximum buffer capacity (also shown in the plot for $c = 0.01$ mol L^{-1}) occurs when pH value equals the pK_a value, and the ${}^{w}_{w}$pK_a of this buffer equals to 4.74.

The correspondence between estimated and measured ${}^{s}_{w}$pH values in the citric acid buffer system is really good for all series of ${}^{w}_{w}$pH up to 8. Above this pH value, when the buffer capacity of this system decreases, the potentiometric measured values become slightly lower than the estimated ones. This tendency becomes more marked with the increase of the acetonitrile fraction in the hydro-organic buffer mixture.

In the phosphoric acid buffer system, the estimated ${}^{s}_{w}$pH values are consistent with the experimental ones in most cases, only observing a certain variation at ${}^{s}_{w}$pH above 9, since we are not able to take into account the contribution of the phosphate species.

In any case, positive deviations observed at basic pH values can be attributed to the CO_2 absorption by the solution.

There is a satisfactory correspondence between estimated and measured ${}^{s}_{w}$pH values in the ammonium–ammonia buffer except for a little deviation on high organic fraction mixture, possibly due to the volatility of the ammonia. Moreover, we

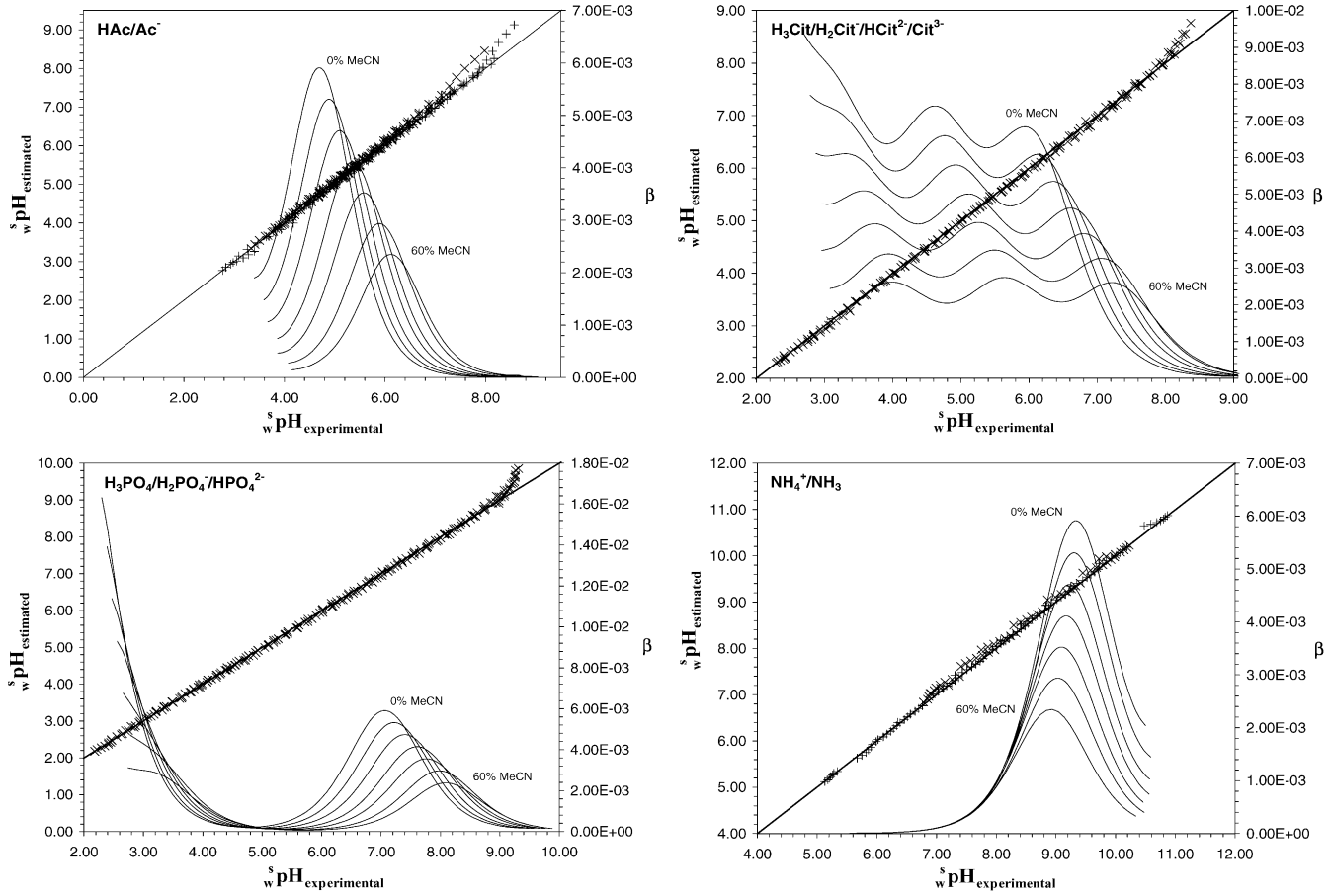

Fig. 3. Estimated $^{s}_{w}$pH values vs. experimental $^{s}_{w}$pH values plot. Straight line of unitary slope and null origin ordinate is also given. Buffer capacity variation is also shown for 0, 10, 20, 30, 40, 50 and 60% (v/v) acetonitrile–water compositions and an initial buffer concentration of 0.01 mol L^{-1}. Symbols for initial aqueous buffer concentration: (*) 0.001 mol L^{-1}, (×) 0.01 mol L^{-1} and (+) 0.1 mol L^{-1}.

must take into account that $^{s}_{w}pK_a$ and $^{s}_{w}$pH variation with acetonitrile fraction in BH^+–BH buffers is less close to linearity than HA–A^-, HA^-–A^{2-}, HA^{2-}–A^{3-} buffers.

Regarding buffer capacity, a decrease is observed when the acetonitrile fraction in the hydro-organic mixture increases, due to the decrease of the buffer concentration on increasing the volume of the solution. The addition of acetonitrile produces a shift of the maximum of buffer capacity towards higher $^{s}_{w}$pH values for neutral or anionic acid buffers (HAc–Ac^-, H_3Cit–H_2Cit^-, H_2Cit^-–$HCit^{2-}$, $HCit^{2-}$–Cit^{3-}, H_3PO_4–$H_2PO_4^-$, $H_2PO_4^-$–HPO_4^{2-}), but towards lower $^{s}_{w}$pH value for the cationic acid buffer (NH_4^+–NH_3). It is noteworthy the broad low buffered zone between the first and the second p*K*a of the phosphoric system, around $^{w}_{w}$pH 5, and the wide range of good buffer capacity of the citric acid system up to $^{w}_{w}$pH 7.

3.3. Estimation of the degree of ionisation and chromatographic retention

The retention of acid–base analytes in reversed-phase high-performance liquid chromatography depends on their hydrophobicity and ionisation degree [6,7,9–17]. Whereas the hydrophobicity of a substance is a non-modifiable property inherent to the own nature of the analyte, the degree of ionisation depends on both analyte dissociation constant and mobile phase pH. For a particular analyte, it can be tuned by an appropriate election of the buffer. As a general rule and for analytes of similar hydrophobicity, and since the neutral form is the most retained by the stationary phase, the higher the degree of ionisation, the lower the retention.

The ionisation degree (α) (or association degree, $1-\alpha$) of an ionisable analyte (HA^z/A^{z-1}) depends on its dissociation constant (K_a) and mobile phase pH through the Eqs. (5) and (6) [5]:

$$\alpha_A = \frac{[A^{z-1}]}{[HA^z]+[A^{z-1}]} = \frac{1}{1+10^{pK_a-pH}} \quad (5)$$

$$\alpha_{HA} = \frac{[HA^z]}{[HA^z]+[A^{z-1}]} = \frac{1}{1+10^{pH-pK_a}} \quad (6)$$

where α_A is the ionisation degree of a neutral acid ($z=0$) and α_{HA} corresponds to the ionisation degree of a neutral base ($z=1$). Strictly, pH and pK_a are referred to $^{s}_{s}$pH and $^{s}_{s}pK_a$, but

we can use here the corresponding ${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{pH}$ and ${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_\mathrm{a}$, because of ${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{pH} - {}^{\mathrm{s}}_{\mathrm{s}}\mathrm{pH} = {}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_\mathrm{a} - {}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_\mathrm{a} = \delta$ and ${}^{\mathrm{s}}_{\mathrm{s}}\mathrm{pH} - {}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_\mathrm{a} = {}^{\mathrm{s}}_{\mathrm{w}}\mathrm{pH} - {}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_\mathrm{a}$. Variation of mobile phase composition changes analyte dissociation constant (K_a) and mobile phase pH, and thus ionisation degree.

If the altogether pH variation of the hydro-organic mobile phase and the analyte pK_a change follow linear models such as those proposed in Eq. (1), the difference between these two values can be expressed in terms of [5]:

$$ {}^{\mathrm{s}}_{\mathrm{w}}\mathrm{pH} - {}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_\mathrm{a} = {}^{\mathrm{w}}_{\mathrm{w}}\mathrm{pH} - {}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_\mathrm{a} + (m_\mathrm{pH} - m_{\mathrm{p}K_\mathrm{a}})\varphi_\mathrm{MeCN} \quad (7) $$

This equation, together with Eqs. (5) and (6), shows that in an acetonitrile–water mobile phase the variation of an analyte ionisation degree on increasing the organic modifier fraction depends on the difference between the corresponding m_pH values of the buffer and $m_{\mathrm{p}K}$ of the analyte. If $m_\mathrm{pH} = m_{\mathrm{p}K}$, then there is no variation of the degree of ionisation with the change of the mobile phase composition. But this is not usually the case.

On one hand, the m_pH value can be estimated for all of the studied buffers in this paper in aqueous concentrations comprised between 0.001 and 0.1 mol L^{-1} by means of Eq. (3) and the parameters detailed in Tables 4–7. On the other hand, pK_a variations follow a linear relation with the acetonitrile fraction, analogous to Eq. (2):

$$ {}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_\mathrm{a} - {}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_\mathrm{a} = m_{\mathrm{p}K}\varphi_\mathrm{MeCN} \quad (8) $$

Literature [18] provides equations to estimate the acetonitrile–water pK_a values of several substances corresponding to one of these large families: pyridines, amines, carboxylic aromatic acids, carboxylic aliphatic acids and phenols. For each compound family and solvent composition, linear relations between ${}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_\mathrm{a}$ and ${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_\mathrm{a}$ were established:

$$ {}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_\mathrm{a} = a_\mathrm{s}\,{}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_\mathrm{a} + b_\mathrm{s} \quad (9) $$

The a_s and b_s sets of values obtained for each family were related to solvent composition through polynomials:

$$ a_\mathrm{s} = \frac{1 + a_{\mathrm{s}1}\varphi_\mathrm{MeCN} + a_{\mathrm{s}2}\varphi^2_\mathrm{MeCN}}{1 + a_{\mathrm{s}3}\varphi_\mathrm{MeCN} + a_{\mathrm{s}4}\varphi^2_\mathrm{MeCN}} \quad (10) $$

$$ b_\mathrm{s} = \frac{1 + b_{\mathrm{s}1}\varphi_\mathrm{MeCN} + b_{\mathrm{s}2}\varphi^2_\mathrm{MeCN}}{1 + b_{\mathrm{s}3}\varphi_\mathrm{MeCN} + b_{\mathrm{s}4}\varphi^2_\mathrm{MeCN}} \quad (11) $$

where $a_{\mathrm{s}1}$, $a_{\mathrm{s}2}$, $a_{\mathrm{s}3}$, $a_{\mathrm{s}4}$, $b_{\mathrm{s}1}$, $b_{\mathrm{s}2}$, $b_{\mathrm{s}3}$ and $b_{\mathrm{s}4}$ were fitting parameters constant for all acids of the same family at any acetonitrile–water composition up to 60% (v/v) of acetonitrile (100% for pyridines).

After checking the correspondence for several compounds between the experimental pK_a values and the estimated ones by means of these proposed equations, we observed a slight deviation in the case of carboxylic aromatic acids. Then, we repeated the calculations for this family of compounds, taking into account all the carboxylic aromatic acids considered before, except 1-naphtoic and 2-nitrobenzoic acid. The first one is a bicycled aromatic acid, whereas the rest are monocyclic aromatic acids, and the second one is the most acidic compound of the set, presenting an evident positive deviation in the linearity in relation to the others. Tables 8 and 9 summarize all $a_{\mathrm{s}i}$ and $b_{\mathrm{s}i}$ parameters for prediction of the slope (a_s) and the intercept (b_s) of the linear correlation between ${}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_\mathrm{a}$ (and ${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_\mathrm{a}$) values in acetonitrile–water and the ${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_\mathrm{a}$ in pure water.

Table 8
Parameters for prediction of the slope ($a_{\mathrm{s}i}$) of the linear correlations between ${}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_\mathrm{a}$ values in acetonitrile–water and the ${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_\mathrm{a}$ values in pure water

	$a_{\mathrm{s}1}$	$a_{\mathrm{s}2}$	$a_{\mathrm{s}3}$	$a_{\mathrm{s}4}$	S.D.	F
Aliphatic carboxylic acids	9.97	−8.59	8.83	−8.72	0.01	5464
Aromatic carboxylic acids	52.04	−10.93	49.33	−32.69	0.02	1695
Phenols	10.05	−10.04	7.97	−8.37	0.02	386
Amines	−0.73	−0.27	−0.87	−0.12	0.00	3476
Pyridines	−1.67	0.67	−1.66	0.67	0.03	38

Using the pH and pK_a estimation equations, the ionisation (α) or association ($1-\alpha$) degrees of different substances in any acetonitrile–water mobile phases can be easily calculated. A representative example is shown in Fig. 4, where the association degree (directly related to retention through hydrophobicity) of several substances are plotted as a function of the volume fraction of acetonitrile for two different buffered mobile phases of ${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{pH} = 8$. Also, the ${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_\mathrm{a}$ of all these analytes, namely 2-nitrophenol, 3-bromophenol, 2,4,6-trimetilpyridine and *N*,*N*-dimethylbenzylamine, is relatively close to 8 (7.24, 8.87, 7.49 and 8.91, respectively). Eq. (9) allows the computation of ${}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_\mathrm{a}$ values of analytes and from them, values of δ given in Table 1, and Eqs. (1) and (8), $m_{\mathrm{p}K}$ values are computed (2.46, 3.01, −2.25 and 1.48, respectively). The buffered solutions consisted of dihydrogenphosphate–hydrogenphosphate 0.01 mol L^{-1} and ammonium–ammonia 0.01 mol L^{-1}, and their estimated m_pH value (equations from Tables 4–7 and Eq. (2)) in relation to ${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{pH} = 8$ were 1.76 and −0.60, respectively.

Fig. 4 shows that an increase of the acetonitrile fraction in the hydro-organic mobile phase increases the association degree of analytes, although in a different degree that depends on the nature of the buffer used. In the case of dihydrogenphosphate–hydrogenphosphate (anionic acid), the association degrees of the phenols (neutral acids) slightly increase because of the higher variation of analyte pK_a in relation to buffer pH ($m_{\mathrm{p}K} > m_\mathrm{pH} > 0$). On the other hand, in the case of amines and pyridines (neutral bases) the variation in the association degree is larger, due to the reversed

Table 9
Parameters for prediction of the slope ($b_{\mathrm{s}i}$) of the linear correlations between ${}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_\mathrm{a}$ values in acetonitrile–water and the ${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_\mathrm{a}$ values in pure water

	$b_{\mathrm{s}1}$	$b_{\mathrm{s}2}$	$b_{\mathrm{s}3}$	$b_{\mathrm{s}4}$	S.D.	F
Aliphatic carboxylic acids	−0.68	9.94	8.45	−8.59	0.08	5152
Aromatic carboxylic acids	−5.32	8.99	22.56	−23.21	0.05	14456
Phenols	−5.33	9.95	0.19	−0.70	0.11	2406
Amines	−1.82	2.25	−1.75	0.90	0.05	1559
Pyridines	−1.78	1.89	−0.58	−0.40	0.10	1293

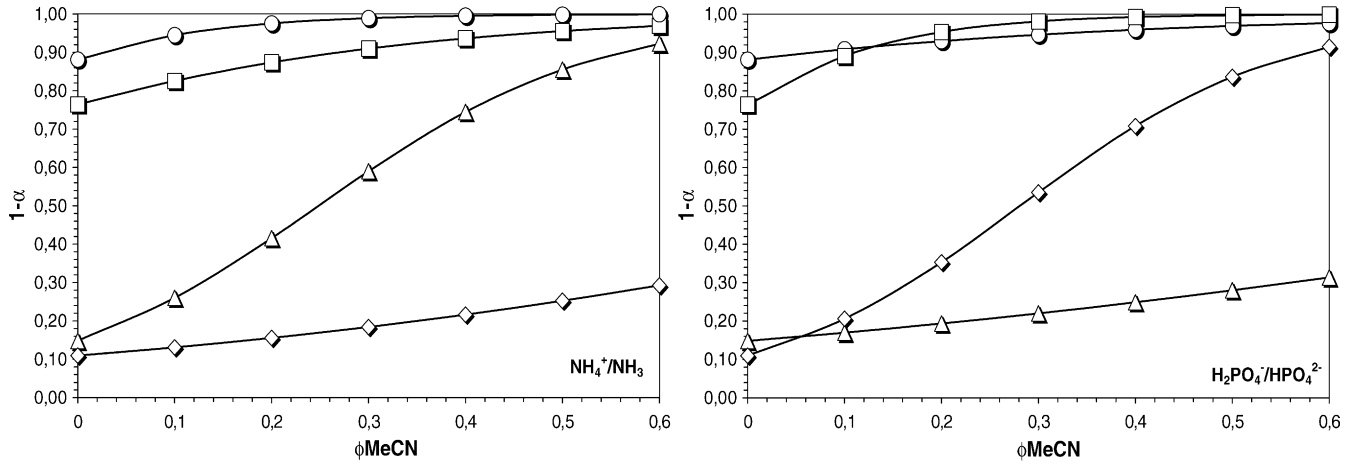

Fig. 4. Variation of the association degree of acid/base compounds with the addition of acetonitrile to NH_4^+–NH_3 and $H_2PO_4^-$–HPO_4^{2-} aqueous buffers of $^{w}_{w}$pH 8. Compounds: (◯) 3-bromophenol; (□) 2,4,6-trimethylpyridine; (△) 2-nitrophenol; (◊) *N*,*N*-dimethylbenzylamine.

trend of analyte pK_a variation in relation to buffer pH ($m_{pK} < 0 < m_{pH}$). The opposite phenomenon is observed in ammonium–ammonia (cationic acid) buffer, since $m_{pH} < 0$.

The chromatographic retention of an analyte strongly depends on its ionisation (or association) degree, in addition to its hydrophobicity. The higher the hydrophobicity and association degree, the higher the retention time. The proposed method enables the association degree of a substance to be calculated in each studied aqueous buffer and acetonitrile content. In relation to the hydrophobicity of compound, it can be expressed by several parameters, although the octanol–water partition coefficient ($\log P_{o/w}$) is the most widely used.

As an example, the measured chromatographic retention times of several compounds with two different pH buffers at 20, 30, 40, 50 and 60% (v/v) of acetonitrile are plotted in Fig. 5. Significant differences in retention times are observed for acetonitrile fractions lower than 40%, since in higher fractions all compounds elute very fast, almost at the same time. At 20%, we can relate the retention times of the analytes with similar hydrophobicity ($\log P_{o/w}$ is 1.79, 1.88, 1.98 for 2-nitrophenol, 2,4,6-trimethylpyridine and *N*,*N*-dimethylbenzylamine, respectively) to their ionisation degree: the higher the compound ionisation, the lower the retention time. The retention of 2-nitrophenol in the dihydrogenphosphate–hydrogenphosphate buffer is lower than that of *N*,*N*-dimethylbenzylamine, whereas in the case of the ammonium–ammonia buffer the reversed behaviour is observed. This behaviour is explained because of the different ionisation trends of these compounds with the addition of acetonitrile to both aqueous buffers. On the other hand, although 3-bromophenol and 2,4,6-trimethylpyridine have similar ionisation degrees in both buffers, the phenol has a much higher retention times than the pyridine, because of its higher hydrophobicity ($\log P_{o/w} = 2.63$). These considerations can be extended to gradient elution, since when a gradient is applied the separation depends mainly on the different retention of analytes at the lowest fractions of organic

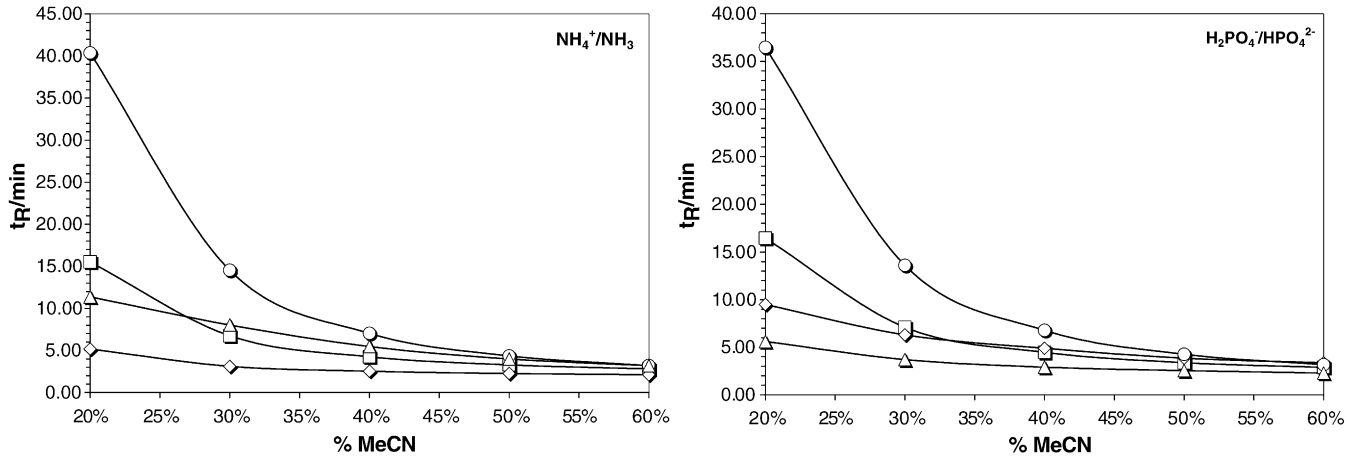

Fig. 5. Retention times of individual ionisable compounds at 20, 30, 40, 50 and 60% (v/v) of acetonitrile prepared from $H_2PO_4^-$–HPO_4^{2-} and NH_4^+–NH_3 aqueous buffers of $^{w}_{w}$pH 8. Symbols as in Fig. 4.

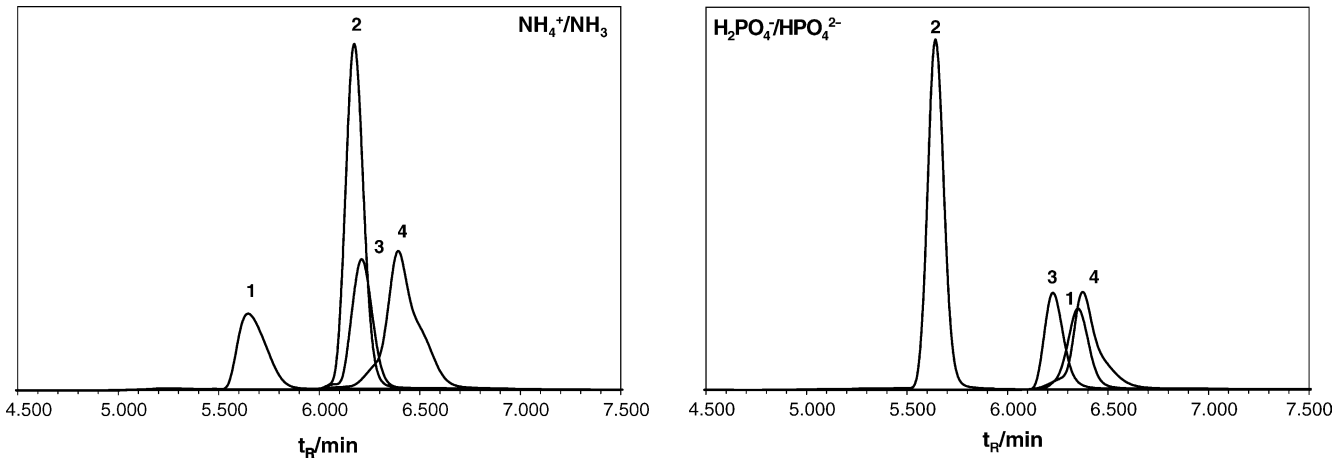

Fig. 6. Chromatograms for individual ionisable compounds, corresponding to the elution of their mixture, in a fast gradient prepared from $H_2PO_4^-$–HPO_4^{2-} and NH_4^+–NH_3 aqueous buffers of ${}^{w}_{w}$pH 8. Compounds: (1) *N*,*N*-dimethylbenzylamine; (2) 2-nitrophenol; (3) 2,4,6-trimethylpyridine; (4) 3-bromophenol.

modifier, when differences on analytes partition between the hydrophobic stationary phase and the hydro-organic mobile phase are more pronounced.

Fig. 6 shows the retention times of the four compounds mentioned above obtained in a fast gradient mode in ${}^{w}_{w}\text{pH} = 8$ dihydrogenphosphate–hydrogenphosphate and ammonium–ammonia buffered mobile phases. The elution order of the analytes corresponds to the expected one considering the compounds hydrophobicity and their ionisation degrees in each acetonitrile–water buffered system. Thus, 2-nitrophenol is the first eluted analyte in the anionic phosphate buffer, whereas in the cationic ammonia buffer the first one is *N*,*N*-dimethylbenzylamine. It is, in each case, the most ionised analyte from among the ones that have similar hydrophobicity. In both cases, the last eluted analyte is 3-bromophenol, since it is only slightly dissociated and the most hydrophobic compound.

4. Conclusions

The pH variation of commonly used aqueous buffers in RP-HPLC with addition of acetonitrile depends on the particular buffer and the hydro-organic composition. A model has been proposed to allow an accurate prediction of this pH change for several buffers (acetic, citric and phosphoric acid and ammonium systems) up to 60% of acetonitrile, and from initial aqueous buffer concentrations included between 0.001 and 0.1 mol L^{-1}. The buffer capacity decreases when acetonitrile is added, due to the dilution effect of the mixture, and their maximum values shift jointly with the pK_a variation of the buffer species. The pH of the mobile phase determines the dissociation degree of ionisable analytes, and this, together with hydrophobicity, determines the analytes retention times. The model can be used to choose which is the most appropriate buffer to reach the best pH value in a particular acetonitrile–water mobile phase composition, and it allows explanation, in terms of hydrophobicity and ionisation degree, of analyte retention behaviour with the change of acetonitrile percentage in the mobile phase.

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