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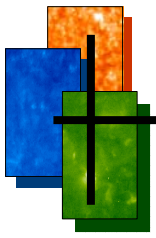
ARTICLE I

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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J. Chromatogr. A 1059 (2004) 33

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Journal of Chromatography A, 1059 (2004) 33–42
JOURNAL OF CHROMATOGRAPHY A
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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

Xavier Subirats, Elisabeth Bosch, Martí Rosés*

Departament de Química Analítica, Universitat de Barcelona, Diagonal 647, E-08028 Barcelona, Spain

Received 20 April 2004; received in revised form 17 September 2004; accepted 22 September 2004

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-

* Corresponding author. Tel.: +34 93 402 17 96; fax: +34 93 402 12 33.
E-mail address: marti@apolo.qui.ub.es (M. Rosés).

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doi:10.1016/j.chroma.2004.09.085

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-

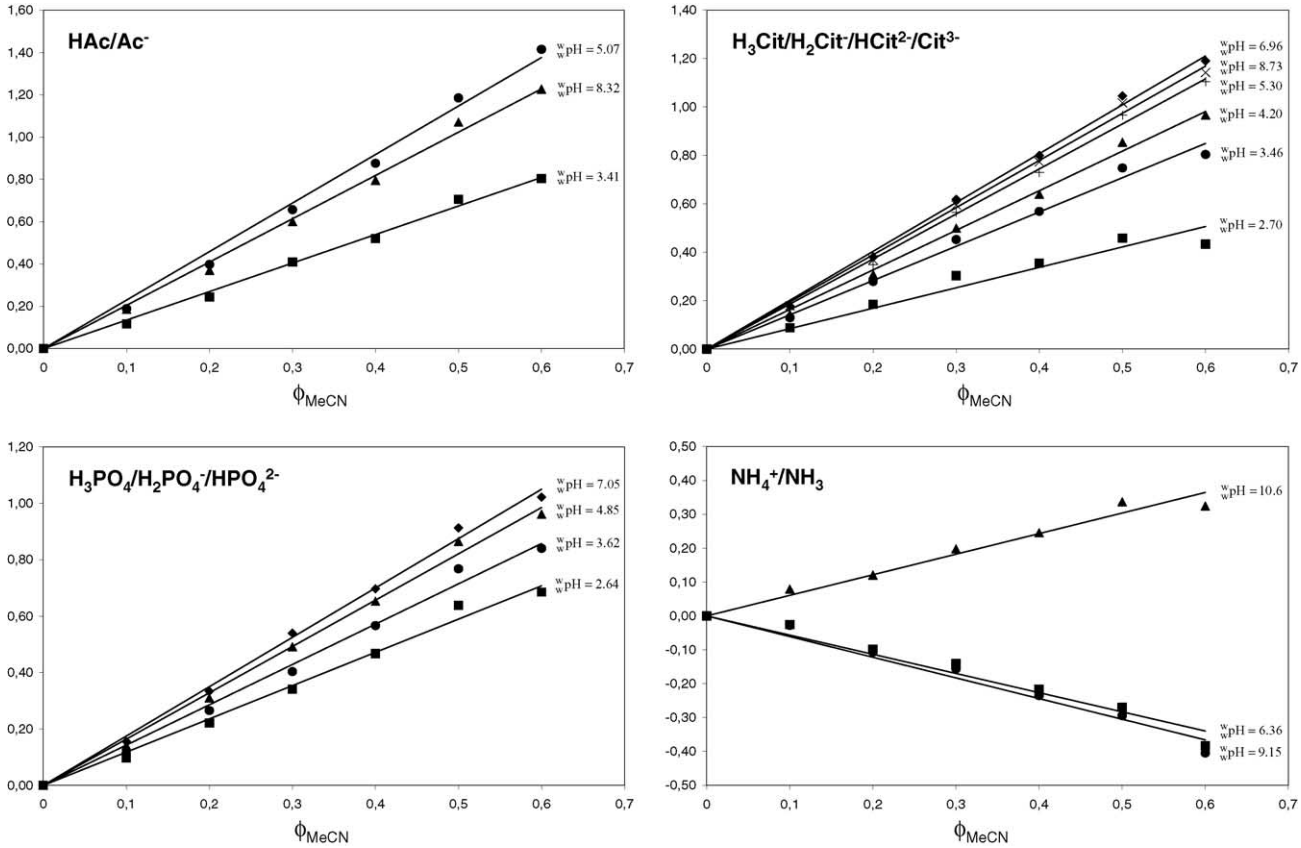

Fig. 1. Variation of the pH values (${}^{s}_{w}\mathrm{pH} - {}^{w}_{w}\mathrm{pH}$) of several studied solutions depending on the acetonitrile fraction added to aqueous buffer 0.01 M and initial aqueous ${}^{w}_{w}\mathrm{pH}$.

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

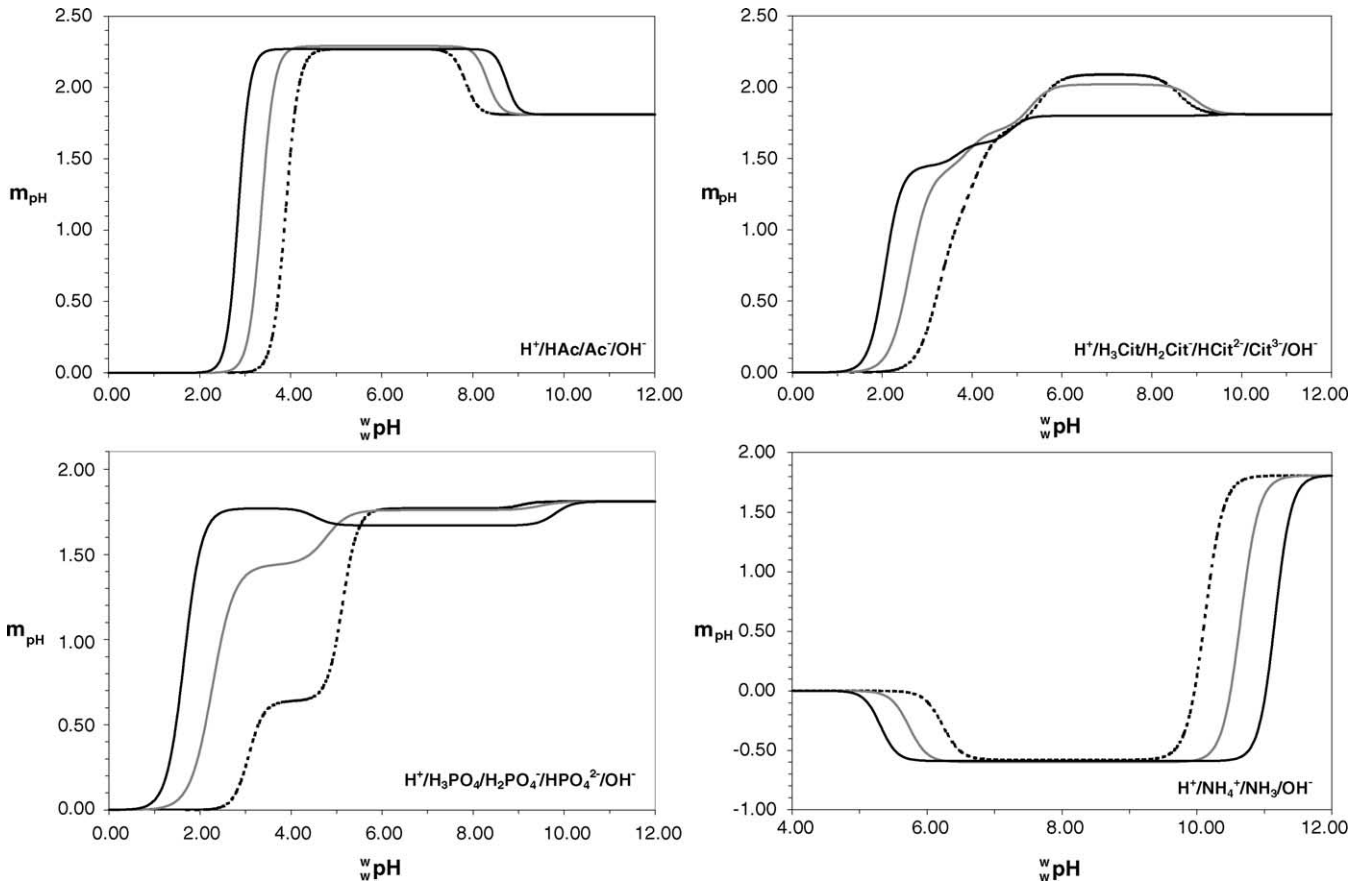

Fig. 2. Variation of the slope (m_{pH}) of Eq. (1) vs. volume fraction of acetonitrile with the initial aqueous pH of the buffer ($^{w}_{w}$pH). Dashed lines represent buffer aqueous concentrations at 0.001 mol L^{-1}, grey continuous lines 0.01 mol L^{-1} and black continuous lines 0.1 mol L^{-1}.

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

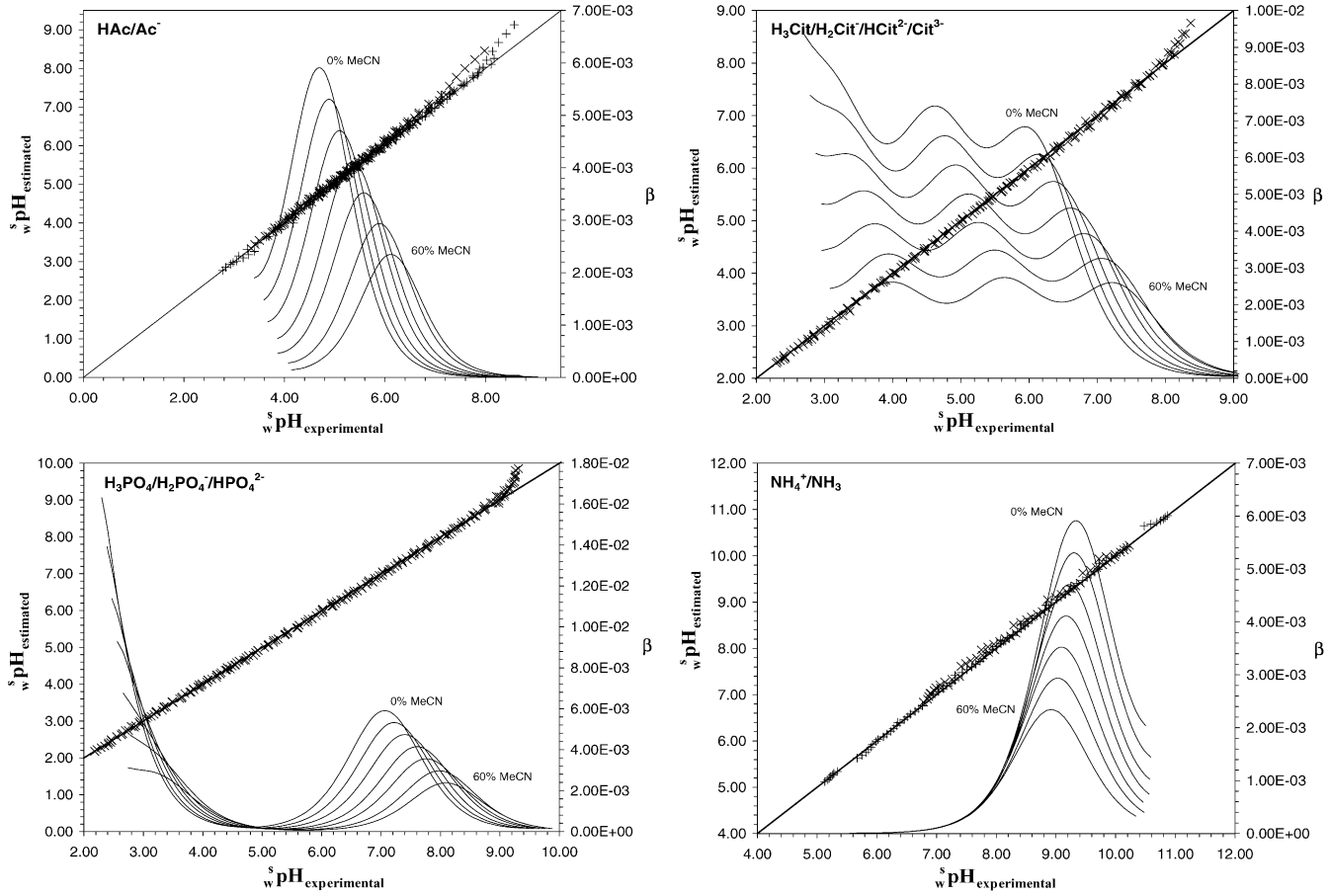

Fig. 3. Estimated $^{s}_{w}$pH values vs. experimental $^{s}_{w}$pH values plot. Straight line of unitary slope and null origin ordinate is also given. Buffer capacity variation is also shown for 0, 10, 20, 30, 40, 50 and 60% (v/v) acetonitrile–water compositions and an initial buffer concentration of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

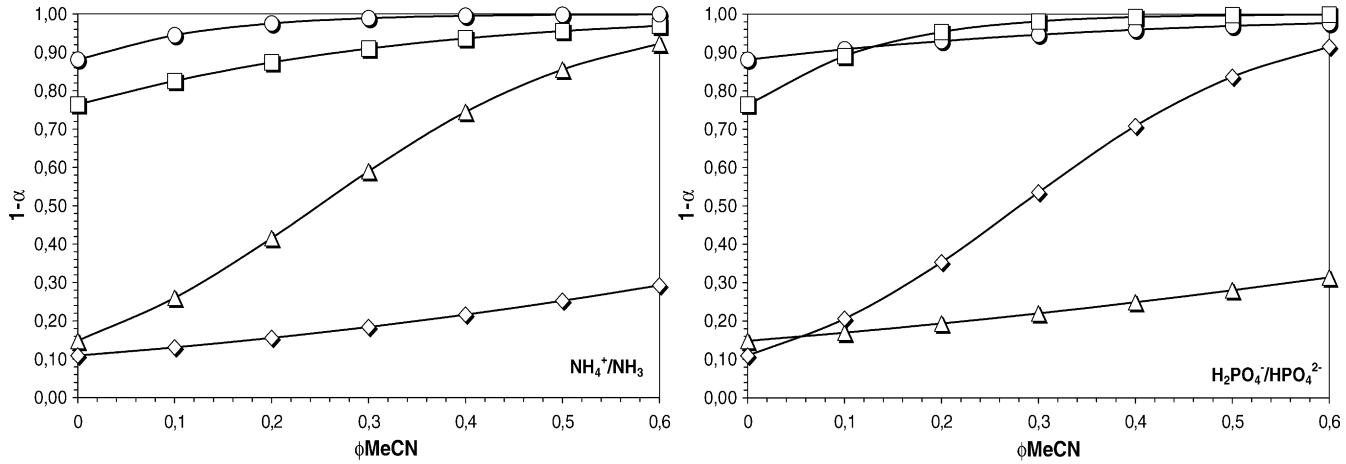

Fig. 4. Variation of the association degree of acid/base compounds with the addition of acetonitrile to NH_4^+–NH_3 and $H_2PO_4^-$–HPO_4^{2-} aqueous buffers of $^{w}_{w}$pH 8. Compounds: (◯) 3-bromophenol; (□) 2,4,6-trimethylpyridine; (△) 2-nitrophenol; (◊) *N*,*N*-dimethylbenzylamine.

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

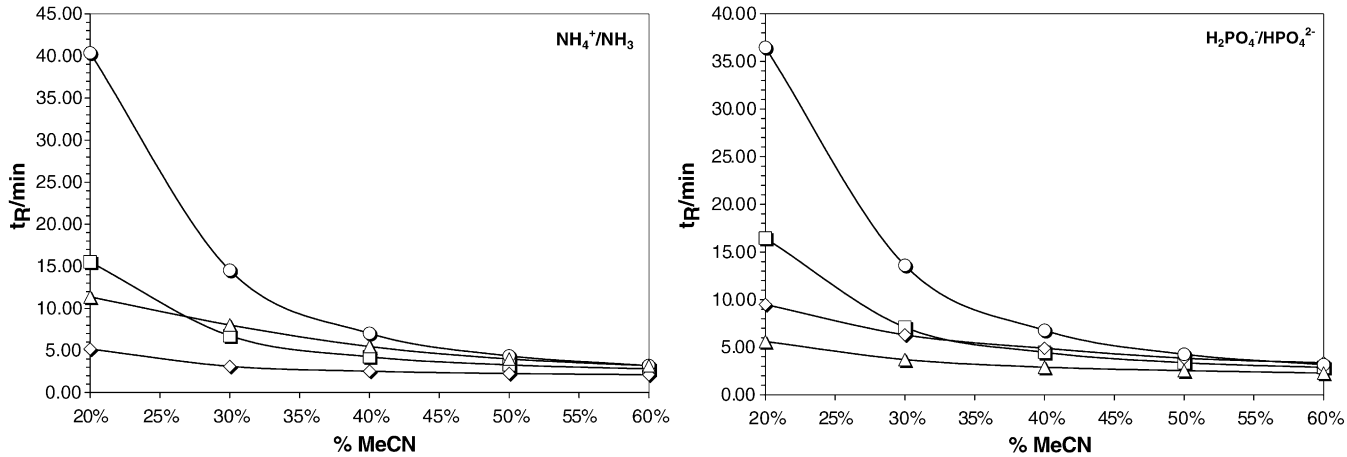

Fig. 5. Retention times of individual ionisable compounds at 20, 30, 40, 50 and 60% (v/v) of acetonitrile prepared from $H_2PO_4^-$–HPO_4^{2-} and NH_4^+–NH_3 aqueous buffers of $^{w}_{w}$pH 8. Symbols as in Fig. 4.

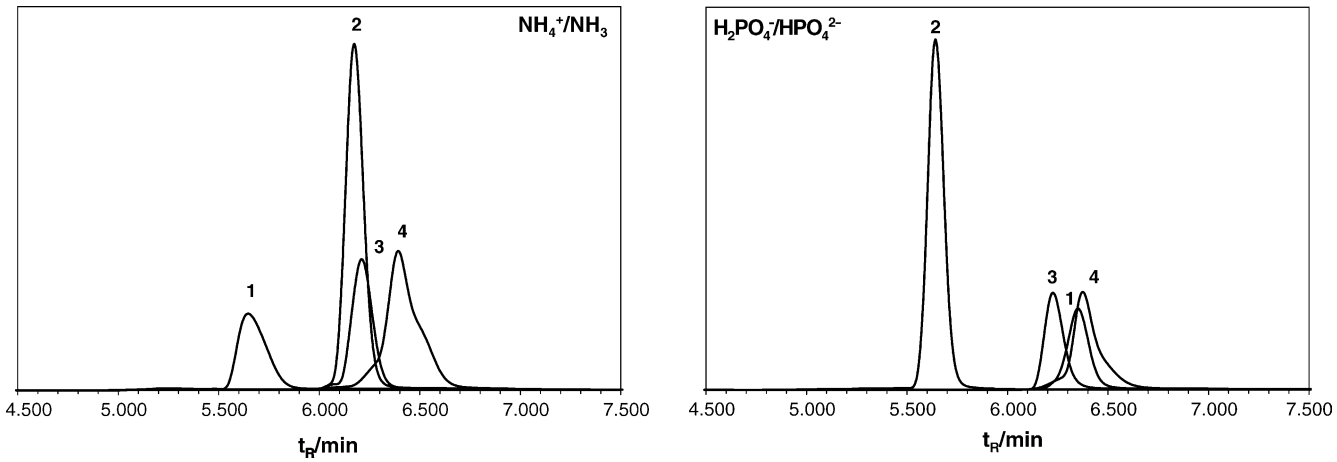

Fig. 6. Chromatograms for individual ionisable compounds, corresponding to the elution of their mixture, in a fast gradient prepared from $H_2PO_4^-$–HPO_4^{2-} and NH_4^+–NH_3 aqueous buffers of ${}^{w}_{w}$pH 8. Compounds: (1) *N*,*N*-dimethylbenzylamine; (2) 2-nitrophenol; (3) 2,4,6-trimethylpyridine;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

Acknowledgements

We thank for financial support from the MCYT of the Spanish Government and FEDER of EU (projects CTQ2004-00633/BQU and CTQ2004-00965/BQU), and from the Catalan Government (Grant 2001SGR00055).

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Erratum

Erratum to: "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" [J. Chromatogr. A 1059 (2004) 33]

Xavier Subirats, Elisabeth Bosch, Martí Rosés *

Departament de Química Analítica, Universitat de Barcelona, Diagonal 647, E-08028 Barcelona, Spain

Received 20 June 2005; accepted 30 June 2005
Available online 1 August 2005

Eq. (1) should replace Eq. (11) (page 40) in the article.

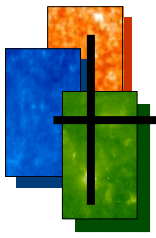
$$b_s = \frac{b_{s1}\varphi_{MeCN} + b_{s2}\varphi_{MeCN}^2}{1 + b_{s3}\varphi_{MeCN} + b_{s4}\varphi_{MeCN}^2} \quad (1)$$

DOI of original article:10.1016/j.chroma.2004.09.085.
* Corresponding author. Tel.: +34 93 402 17 96; fax: +34 93 402 12 33.
E-mail address: marti@apolo.qui.ub.es (M. Rosés).

doi:10.1016/j.chroma.2005.06.095

ARTICLE II

Retention of ionisable compounds on high-performance liquid chromatography XVI. Estimation of retention with acetonitrile/water mobile phases from aqueous buffer pH and analyte pK_a



X. Subirats, E. Bosch and M. Rosés

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Retention of ionisable compounds on high-performance liquid chromatography XVI. Estimation of retention with acetonitrile/water mobile phases from aqueous buffer pH and analyte pK_a

Xavier Subirats, Elisabeth Bosch, Martí Rosés *

Department of Analytical Chemistry, University of Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain

Received 25 December 2005; received in revised form 30 March 2006; accepted 31 March 2006
Available online 6 June 2006

Abstract

In agreement with our previous studies and those of other authors, it is shown that much better fits of retention time as a function of pH are obtained for acid–base analytes when pH is measured in the mobile phase, than when pH is measured in the aqueous buffer when buffers of different nature are used. However, in some instances it may be more practical to measure the pH in the aqueous buffer before addition of the organic modifier. Thus, an open methodology is presented that allows prediction of chromatographic retention of acid–base analytes from the pH measured in the aqueous buffer. The model presented estimates the pH of the buffer and the pK_a of the analyte in a particular acetonitrile/water mobile phase from the pH and pK_a values in water. The retention of the analyte can be easily estimated, at a buffer pH close to the solute pK_a, from these values and from the retentions of the pure acidic and basic forms of the analyte. Since in many instances, the analyte pK_a values in water are not known, the methodology has been also tested by using Internet software, at reach of many chemists, which calculates analyte pK_a values from chemical structure. The approach is successfully tested for some pharmaceutical drugs.

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

1. Introduction

Reproducible and successful chromatographic studies of ionisable compounds require a proper pH measurement. As we have extensively discussed in previous works [1–8], there are three ways of measuring the pH for a chromatographic system. Commonly [9], the pH is measured in the aqueous buffer before mixing it with the organic modifier ($^{w}_{w}$pH scale [10]). However, we recommend measuring the pH in the mobile phase after mixing aqueous buffer and organic modifier. The electrode system can be calibrated with buffers of known pH in the same organic-water mixture used as mobile phase [11], $^{s}_{s}$pH scale [10], or with commercial aqueous pH standards in water [12], and thus the pH readings directly provide the $^{s}_{w}$pH values [10] of the mobile phase (i.e. the pH value in the hydroorganic solvent (s) relative to water (w) as standard state solvent [10]).

The shortcomings of measuring the pH variation in the aqueous buffer are clear: the pH variation when adding methanol or acetonitrile to the aqueous buffer depends on the particular buffered system, on its concentration, and on the fraction of organic solvent in the mixture [11–13]. Buffered solutions prepared from anionic and neutral (uncharged) acids (e.g. HAc/Ac^-, $H_2PO_4^-$/HPO_4^{2-} buffers) increase their pH value when acetonitrile or methanol is added, whereas buffers from cationic acids (e.g. NH_4^+/NH_3 buffers) show the reverse trend. The pK_a variation of analytes follows similar tendencies. Thus, fits of retention to aqueous pH may show a big disparity when buffers of different nature are used, and the pH of the inflection point does not agree with the pK_a of the analyte [9]. In this paper we demonstrate this effect and evaluate its importance for some common buffers (acetic, phosphoric, citric and ammonium buffers) in acetonitrile/water mobile phases.

* Corresponding author. Tel. +34 93 403 92 75; fax +34 93 402 12 33.
E-mail address: marti@apolo.qui.ub.es (M. Rosés).

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doi:10.1016/j.chroma.2006.03.126

The proper pH measurement is crucial to model chromatographic retention as a function of pH and to get reliable predictions. Different authors have realized that better models are obtained when the pH in the mobile phase is considered [1,3,4,8,14–17] instead of the aqueous pH of the buffer. In a recent work Törnblom et al. [17] has concluded that improvements in prediction of retention times of phenols in acetonitrile/phosphate buffering system are obtained if an estimation of the mobile phase pH is used instead of the aqueous pH of the buffer. In that work, retention times are predicted using the commercial LC simulator from Advanced Chemistry Development [18], which requires input of experimental retention of full acidic and basic forms of the analyte, pH, nature and fraction of the organic modifier, and molecular structure of the analyte. The structure allows the estimation of the aqueous pK_a of the analyte in case there is no experimental value in the ACD pK_a library. However, in some instances the predictions of retention are not very accurate because the variation of the pK_a of the analyte with the addition of acetonitrile is not considered. The aim of our present work is to present an open methodology (that does not require commercial software) within reach of all researchers to predict the retention times of simple and complex molecules (e.g. commercial drugs) belonging to the most common families of compounds (phenols, amines, pyridines, carboxylic aromatic and aliphatic acids) in several acetonitrile/water buffering systems (phosphate, citrate, acetate and ammonia). In this paper we present a model based on equations that estimate the ${}^{s}_{w}pH$ of any acetonitrile/aqueous buffer mobile phase up to 60% in volume for the most common HPLC buffers, and on equations that estimate the ${}^{s}_{w}pK_a$ of ionisable analytes with common acid–base functional groups in the same mobile phases. This model is tested with complex ionisable molecules (pharmaceutical drugs) using two different buffering systems to cover the pH range of interest. To overcome the frequent lack of ${}^{w}_{w}pK_a$ literature values for drugs needed to estimate the ${}^{s}_{w}pK_a$ of the analyte in the mobile phase, two different computational programs are used, and results obtained from ${}^{w}_{w}pK_a$ values are compared. These programs are the commercial ACD/Labs, embedded in the SciFinder Scholar 2006, and SPARC, from the University of Georgia, which is freely accessed through Internet.

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 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 C18 5-μm from Waters (Milford, MA, USA) column with a flow rate of 1 mL/min in isochratic mode. A Shimadzu (Kyoto, Japan) HPLC system consisting of two LC-10ADvp dual reciprocating plunger solvent delivery modules, a SIL-10ADvp auto injector 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 (Darmstadt, Germany) and water purified by the Milli-Q plus (to 18 MΩ) system from Millipore (Bedford, MA, USA). The studied buffers were prepared from acetic acid, sodium acetate, sodium hydrogenphosphate, citric acid, potassium dihydrogencitrate and sodium citrate, using hydrochloric acid and potassium hydroxide to adjust the pH to the wanted value, when it was necessary. The chromatographed compounds were 4-*tert*-butylpyridine (99%) and 4-*tert*-butylbenzoic acid (99 + %), papaverine hydrochloride (>98%), 4-*tert*-butylaniline (>99%) from Sigma–Aldrich (Steinheim, Germany), aniline (p.a.) from Merck (Darmstadt, Germany), and benzoic acid (p.a.) from Scharlau (Barcelona, Spain). Codeine, trazodone hydrochloride, imipramine hydrochloride, nortriptyline hydrochloride and maprotiline hydrochloride were purchased from Sigma–Aldrich (Steinheim, Germany). Diclofenac, naproxen and ibuprofen were supplied by the firms Prodesfarma (Barcelona, Spain), Sintex (Barcelona, Spain) and Dr. Esteve (Barcelona, Spain), respectively. All these drugs were used without further purification.

2.3. Procedure

The required aqueous acid and base concentrations for the selected pH were calculated before the preparation of the buffer, to give a total buffer aqueous concentrations of 0.01 mol L^{-1}. The pH was finally adjusted to the desired value by addition of small amounts of concentrated solutions of potassium hydroxide or hydrochloric acid. The pH meter was calibrated with the usual aqueous standard reference buffers (${}^{w}_{w}pH$ 4.01 and 7.00 at 25 °C), while the ${}^{s}_{w}pH$ was measured after mixing with the desired volume of MeCN. Chromatographic data were obtained isocratically at 40 and 60% of MeCN (v/v).

3. Theory

3.1. Chromatographic retention of acid–base compounds

In case of a weak monoprotic acid–base compound, two different species are retained, the acid (HA) and the conjugate base (A). The chromatographic retention of this compound is an average of the retention of those species. The retention factor is given by:

$$k = \frac{k_A + k_{HA}10^{pK_a - pH}}{1 + 10^{pK_a - pH}} \quad (1)$$

where HA is the protonated acid form (uncharged or cationic) and A is the deprotonated basic form (anionic or uncharged).

Applying the classical approach that both species have the same hold-up time, Eq. (1) becomes:

$$t_R = \frac{t_{R(A)} + t_R 10^{pK_a - pH}}{1 + 10^{pK_a - pH}} \quad (2)$$

From Eqs. (1) and (2) it can be seen that the retention of a weak acid depends on three constant parameters: the dissociation constant of the acid, the retention time of the acid and the retention time of the conjugate base. The only variable is the pH of the mobile phase. This mobile phase pH can be measured in different ways as pointed out in the introduction: in the aqueous buffer before adding the organic modifier ($^{w}_{w}$pH) or directly in the mobile phase with water or mobile phase calibration ($^{s}_{w}$pH or $^{s}_{s}$pH, respectively). There are different relationships between this three pH scales and the results obtained in the fits of Eqs. (1) and (2) to the pH are also different [4,5,19].

3.2. pH and pK_a variation with mobile phase composition

In a previous work [13] a model was developed for common buffers used in HPLC to estimate the pH variation in acetonitrile/water mixtures containing up to 60% of organic modifier from the pH of the aqueous buffer (i.e. the difference between the $^{w}_{w}$pH and the $^{s}_{w}$pH scales). This pH variation can be approximately fitted to a linear equation:

$$^{s}_{w}pH - {}^{w}_{w}pH = m_{pH}\varphi_{MeCN} \quad (3)$$

where φ_{MeCN} is the volume fraction of acetonitrile in the mixture, m_{pH} depends on the particular buffer used and on the initial $^{w}_{w}$pH of this buffer. The variation of m_{pH} in acetonitrile/water mixtures with initial $^{w}_{w}$pH is described by the following model:

$$m_{pH} = \frac{a_0 + \sum_{i=1}^{n} a_i 10^{s(i pH - b_i)} + a_{n+1}{}^{s((n+1)pH - b_{n+1})}}{1 + \sum_{i=1}^{n} 10^{s(i pH - b_i)} + 10^{s((n+1)pH - b_{n+1})}} \quad (4)$$

where n is the number of acid/base equilibria, $a_0 = 0$ and $a_{n+1} = 1.81$. These a_i and b_i parameters depend on the buffer used and on the initial aqueous concentration of the buffer, before adding the acetonitrile, as shown in Table 1.

Finally, and in relation to pH scales, conversion between $^{s}_{w}$pH and $^{s}_{w}$pH values can be easily through the following equation:

$$^{s}_{w}pH - {}^{s}_{s}pH = \delta \quad (5)$$

where δ is a constant parameter for each acetonitrile/water composition, that can be calculated by means of: [4]

$$\delta = (-3.81 \pm 0.15) x_{MeCN}^2 \quad (6)$$

where x_{MeCN} is the mole fraction of acetonitrile in the mixture. We must take into account that the general conversion between the pH scales mentioned above is only possible if the electrode system is designed to have a negligible residual liquid-junction potential.

In relation to pK_a variation, Espinosa and co-workers [13,20] developed equations to estimate the acetonitrile/water pK_a values of pyridines, amines, carboxylic aromatic acids, carboxylic aliphatic acids and phenols:

$$^{s}_{s}pK_a = a_s {}^{w}_{w}pK_a + b_s \quad (7)$$

where $^{s}_{s}pK_a$ is the dissociation constant of the compound in the hydroorganic solvent referred to the own solvent, $^{w}_{w}pK_a$ the dissociation constant in water, and a_s and b_s are sets of values obtained for each family of compounds related to solvent composition through polynomials:

$$a_s = \frac{1 + a_{s1}\varphi_{MeCN} + a_{s2}\varphi_{MeCN}^2}{1 + a_{s3}\varphi_{MeCN} + a_{s4}\varphi_{MeCN}^2} \quad (8)$$

$$b_s = \frac{b_{s1}\varphi_{MeCN} + b_{s2}\varphi_{MeCN}^2}{1 + b_{s3}\varphi_{MeCN} + b_{s4}\varphi_{MeCN}^2} \quad (9)$$

where φ_{MeCN} is the volume fraction of MeCN in the hydroorganic mobile phase, and a_{s1}, a_{s2}, a_{s3}, a_{s4}, b_{s1}, b_{s2}, b_{s3} and b_{s4} are fitting constants for all acids of the same family at any acetonitrile/water composition up to 60% (v/v) of acetonitrile (100% for pyridines). These values are shown in Table 2.

Then from $^{w}_{w}pK_a$and Eq. (7) we can easily estimate the $^{s}_{s}pK_a$ and, through Eq. (10) and the already known δ parameter, the $^{s}_{w}pK_a$ [4,21,22]:

$$^{s}_{w}pK_a - {}^{s}_{s}pK_a = \delta \quad (10)$$

Table 1
Linear variation of the s, a_i and b_i parameters for the acetic acid/acetate, ammonium/ammonia, phosphoric acid/dihydrogenphosphate/hydrogenphosphate and citric acid/dihydrogencitrate/hydrogencitrate/citrate buffer system depending on the aqueous buffer concentration (c_T).

	Acetic acid	Ammonium	Phosphoric acid	Citric acid
s	$0.20 \log c_T + 3.56$	$0.20 \log c_T + 3.71$	$-0.04 \log c_T + 1.99$	$0.29 \log c_T + 2.59$
a_0	0.00	0.00	0.00	0.00
a_1	2.28	−0.60	$0.53 \log c_T + 2.40$	$0.14 \log c_T + 1.63$
a_2	1.81	1.81	$-0.06 \log c_T + 1.63$	$-0.06 \log c_T + 1.56$
a_3	–	–	1.81	$-0.16 \log c_T + 1.67$
a_4	–	–	–	1.81
b_1	$-0.52 \log c_T + 2.33$	$-0.45 \log c_T + 4.84$	$-0.69 \log c_T + 0.93$	$-0.58 \log c_T + 1.47$
$b_2 - b_1$	$0.45 \log c_T + 9.20$	$0.52 \log c_T + 11.67$	$-0.29 \log c_T + 4.22$	$-0.21 \log c_T + 3.47$
$b_3 - b_2$	–	–	$0.36 \log c_T + 10.18$	$-0.34 \log c_T + 4.58$
$b_4 - b_3$	–	–	–	$0.38 \log c_T + 9.72$

Table 2
Parameters for prediction of the slope (a_{si} and b_{si}) of the linear correlations between ${}^{s}_{s}pK_a$ values in acetonitrile–water and the ${}^{w}_{w}pK_a$ values in pure water [13,20]

	a_{s1}	a_{s2}	a_{s3}	a_{s4}	SD	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
	b_{s1}	b_{s2}	b_{s3}	b_{s4}	SD	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

4. Results and discussion

4.1. Effect of the pH scale on retention fits

As described and modelled in previous papers [12,13], buffer solutions of the same ${}^{w}_{w}pH$ prepared from different components lead to different values of ${}^{s}_{w}pH$ after the addition of acetonitrile as organic modifier. These pH changes can be very important in case of weak acid solutes since they cause a change in their ionisation degree and therefore in their chromatographic retention. To optimize successfully the experimental conditions for chromatographic analysis of ionisable analytes, these pH variations should not be underestimated.

For example, Fig. 1 shows the measured chromatographic retention times for several uncharged acids with conjugate anionic bases (benzoic and 4-*tert*-butylbenzoic acid) and cationic acids with conjugate uncharged bases (4-*tert*-butylaniline, papaverine, 4-*tert*-butylpyridine and aniline) in different acetonitrile/aqueous buffer systems (acetic acid/acetate and citric acid/dihydrogencitrate/hydrogencitrate/citrate) as a function of the aqueous pH of the mobile phase. We can clearly notice that starting from the same aqueous pH there are different chromatographic retention times for the same analyte at the same fraction of organic modifier depending on the nature of the buffering system. It can be also seen that this retention differences are higher as the MeCN fraction increases. For example, a mobile phase of ${}^{w}_{w}pH = 3.00$ and 60% of MeCN leads to significantly different retention times for 4-*tert*-butylaniline depending on the buffer used. If a citric acid buffer is used, the corresponding retention time is 2.69 min, whereas in the acetic acid buffer is only 1.89 min. This is a significant difference, specially taking into account that the retention time of the neutral species at 60% of MeCN is about 4.5 min.

However, if we plot these chromatographic retention values as a function of the measured ${}^{s}_{w}pH$ in the acetonitrile/water mobile phase (Fig. 2), there is a good agreement with the

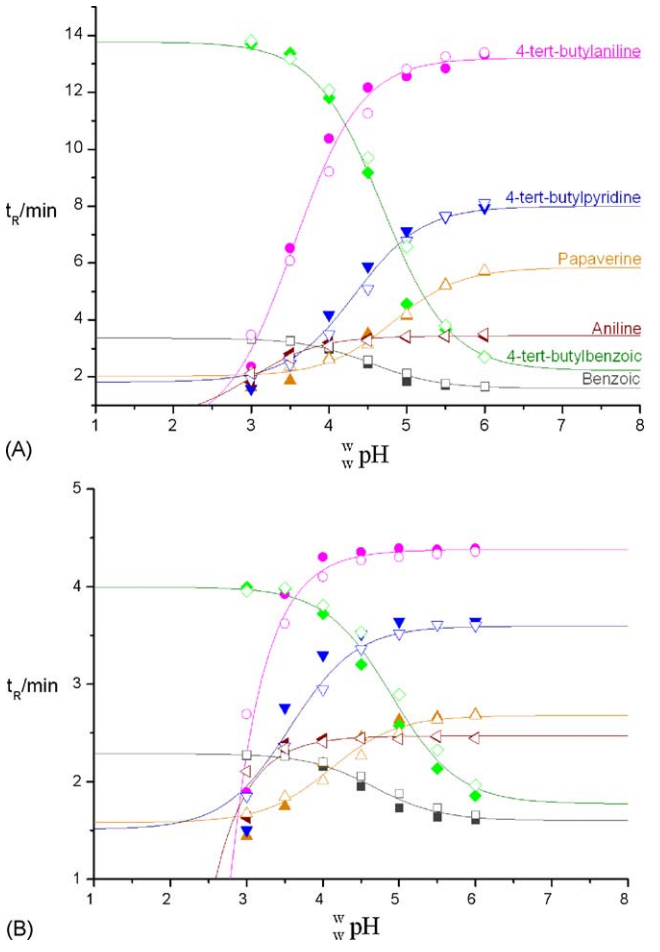

Fig. 1. Measured retention plots for some weak neutral and cationic acids in $H_3Cit/H_2Cit^-/HCit^{2-}/Cit^{3-}$ 0.01 M buffer (empty symbols) and HAc/Ac^- 0.01 mol L^{-1} buffer (filled symbols) at 40% (A) and 60% (B) of MeCN (v/v) as a function of the initial aqueous ${}^{w}_{w}pH$, measured in the mobile phase before adding the organic modifier. Legend: (●, ◌) 4-*tert*-butylaniline, (▼, ▽) 4-*tert*-butylpyridine, (▲, △) papaverine, (◀, ◁) aniline, (◇, ◊) 4-*tert*-butylbenzoic acid and (■, □) benzoic acid. Lines: fittings to Eq. (2).

retention points obtained with both buffers, the acetic and the citric, because of the different behaviour of these buffered systems when adding MeCN. With the purpose of comparing the fittings related to the hydroorganic and the aqueous pH values, these retention times were fitted all together to Eq. (2) against the ${}^{w}_{w}pH$ values of citric and acetic acid buffers employed (Table 3), and again to the corresponding ${}^{s}_{w}pH$ values of both buffers at 40 and 60% of MeCN (Table 4). It is noteworthy that better fittings are obtained when using the hydroorganic pH values, the ones measured directly in the acetonitrile/water mobile phase. The mean relative error of all retention points in relation to the values obtained from the fittings are 2.3 and 3.7% relative to ${}^{s}_{w}pH$ and ${}^{w}_{w}pH$, respectively, being the error at 40% of MeCN (3.2 and 4.2%, in relation to ${}^{s}_{w}pH$ and ${}^{w}_{w}pH$) higher than at 60% (3.2 and 1.3%). This is not surprising, because mobile phases with a high fraction of organic modifier and high eluotropic strength present less pronounced differences on analytes partition between the hydrophobic stationary phase and the hydroorganic mobile phase in relation to mobile phases with a low fraction of acetonitrile. For that reason, differences

Table 3
Statistical and fitted ${}^{w}_{w}pK_a$ parameters for Eq. 2 at 40 and 60% of MeCN, considering the measured ${}^{w}_{w}pH$ values in the mobile phase before adding the organic modifier

Compound	40% MeCN					
	$t_{R(A)}$	$t_{R(HA)}$	${}^{w}_{w}pK_a^{40\%}$	r^2	SD	F
Benzoic acid	1.58 ± 0.05	3.42 ± 0.05	4.51 ± 0.06	0.989	0.08	512
4-*tert*-Butylbenzoic acid	2.13 ± 0.33	14.06 ± 0.27	4.66 ± 0.06	0.990	0.49	541
4-*tert*-Butylaniline	13.21 ± 0.18	0.07 ± 0.72	3.55 ± 0.07	0.989	0.43	502
Papaverine	5.90 ± 0.16	1.96 ± 0.11	4.82 ± 0.08	0.982	0.21	303
4-*tert*-Butylpyridine	8.06 ± 0.15	1.54 ± 0.19	4.29 ± 0.06	0.990	0.27	531
Aniline	3.44 ± 0.03	0.55 ± 0.54	3.01 ± 0.15	0.977	0.09	238
	60% MeCN					
	$t_{R(A)}$	$t_{R(HA)}$	${}^{w}_{w}pK_a^{60\%}$	r^2	SD	F
Benzoic acid	1.60 ± 0.03	2.31 ± 0.03	4.62 ± 0.10	0.973	0.05	197
4-*tert*-Butylbenzoic acid	1.76 ± 0.09	4.03 ± 0.06	4.88 ± 0.08	0.984	0.11	342
4-*tert*-Butylaniline	4.38 ± 0.070	−285 ± 13377	0.86 ± 20	0.945	0.19	94
Papaverine	2.68 ± 0.04	1.46 ± 0.08	3.98 ± 0.11	0.966	0.09	158
4-*tert*-Butylpyridine	3.60 ± 0.08	1.51 ± 0.10	3.66 ± 0.07	0.940	0.18	189
Aniline	2.47 ± 0.04	−202 ± 48692	0.45 ± 104	0.807	0.11	23

($N = 14$).

in retention times between citrate and acetate buffer are lower at 60% of MeCN than at 40%, although variations at 60% in pH of the mobile phase and pK_a of the analytes in relation to the aqueous values are more marked than at 40%. On the other hand, fitting parameters for both mobile phases to ${}^{w}_{w}pH$ for aniline and 4-*tert*-butylaniline are meaningless, since the retention times of the fully ionised species in 40% MeCN are lower than the retention time of the KBr (∼1.5 min) used as hold-up marker, or even negative in 60% MeCN. These meaningless fittings are due to the different retention times obtained for these substances for the diverse buffers at ${}^{w}_{w}pH$ 3.

More interesting is the discussion about the physical meaning of the fitted pK_a values. In fact, the pK_a values obtained when considering ${}^{w}_{w}pH$ (Table 3) do not agree with the aqueous pK_a values found in the literature nor with the ${}^{s}_{w}pK_a$ in the mobile phase (Table 5). This lack of concurrence is more significant as the acetonitrile fraction increases in the mobile phase. As expected [2,4,6,7,12,13,20,23], these deviations from literature aqueous pK_a are positive for uncharged acids and negative for cationic acids. The conclusion is that using the aqueous pH values leads us to a fitting pK_a value that has no physical interpretation.

However, if we fit retention to pH in the mobile phase (${}^{s}_{w}pH$) and we use Eqs. (7)–(10) to estimate the pK_a variation in acetonitrile/water mixtures when increasing the fraction of organic modifier [13,20], very good matching pK_a values are obtained. In fact, there are only slight deviations comparing the estimated pK_a values shown in Table 5 with the fitted ones in

Table 4
Statistical and fitting parameter for Eq. (2) at 40 and 60% of MeCN, considering the measured ${}^{s}_{w}pH$ values in the mobile phase

Compound	40% MeCN					
	$t_{R(A)}$	$t_{R(HA)}$	${}^{s}_{w}pK_a^{40\%}$	r^2	SD	F
Benzoic acid	1.60 ± 0.03	3.37 ± 0.03	5.31 ± 0.04	0.996	0.05	1375
4-*tert*-Butylbenzoic acid	2.22 ± 0.20	13.77 ± 0.16	5.47 ± 0.04	0.996	0.31	1361
4-*tert*-Butylaniline	13.12 ± 0.15	2.13 ± 0.33	4.33 ± 0.05	0.993	0.36	731
Papaverine	5.84 ± 0.17	2.03 ± 0.11	5.62 ± 0.09	0.980	0.22	269
4-*tert*-Butylpyridine	8.00 ± 0.11	1.82 ± 0.12	5.07 ± 0.05	0.994	0.21	901
Aniline	3.42 ± 0.02	1.61 ± 0.09	3.88 ± 0.07	0.987	0.06	431
	60% MeCN					
	$t_{R(A)}$	$t_{R(HA)}$	${}^{s}_{w}pK_a^{60\%}$	r^2	SD	F
Benzoic acid	1.60 ± 0.01	2.29 ± 0.01	5.86 ± 0.04	0.994	0.02	982
4-*tert*-Butylbenzoic acid	1.77 ± 0.02	4.00 ± 0.01	6.11 ± 0.02	0.999	0.03	5596
4-*tert*-Butylaniline	4.36 ± 0.02	1.59 ± 0.10	3.86 ± 0.05	0.993	0.07	792
Papaverine	2.68 ± 0.03	1.58 ± 0.04	5.21 ± 0.08	0.981	0.06	286
4-*tert*-Butylpyridine	3.59 ± 0.04	1.51 ± 0.10	4.47 ± 0.08	0.979	0.11	261
Aniline	2.45 ± 0.00	1.27 ± 0.05	3.30 ± 0.05	0.997	0.01	1672

($N = 14$).

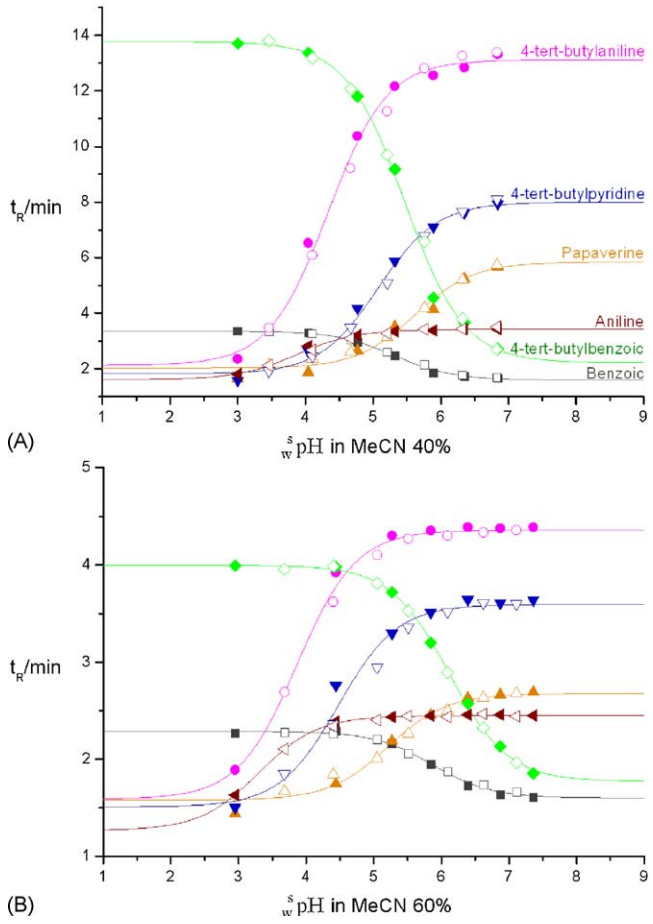

Fig. 2. Measured retention plots for some weak neutral and cationic acids in $H_3Cit/H_2Cit^-/HCit^{2-}/Cit^{3-}$ 0.01M buffer (empty symbols) and HAc/Ac^- 0.01M buffer (filled symbols) at 40% (A) and 60% (B) of MeCN (v/v) as a function of the measured ${}^{s}_{w}$pH of the hydroorganic mobile phase. Legend: (●, ○) 4-*tert*-butylaniline, (▼, ▽) 4-*tert*-butylpyridine, (▲, △) papaverine, (◀, ◁) aniline, (◊, ◊) 4-*tert*-butylbenzoic acid, (■, □) benzoic acid.

Table 4: the average of the scattering, in absolute values, is less than 0.1 pK_a units for both percentages of MeCN. The fitted pK_a values then have a truly useful physical meaning.

These examples show that it is convenient to measure the pH in the mobile phase, rather than in the aqueous buffer. However, pH measurement of the mobile phase may not be convenient, e.g. in the case of an HPLC experiment where independent reservoirs of buffer and organic solvent are pumped into and mixed within the apparatus. In these instances the equations presented in the theory section can be used to estimate the pH in the mobile phase from the aqueous pH in the buffer.

Table 5
Literature and estimated ${}^{s}_{w}pK_a$ values at 40 and 60% of MeCN (v/v) of the chromatographed compounds by means of Eqs. (7)–(10)

Compound	${}^{w}_{w}pK_a$ [a]	${}^{s}_{w}pK_a^{40\%}$	${}^{s}_{w}pK_a^{60\%}$
Benzoic acid	4.20	5.19	5.86
4-*tert*-Butylbenzoic acid	4.38	5.42	6.13
4-*tert*-Butylaniline	4.95	4.23	3.80
Papaverine	6.40	5.62	5.02
4-*tert*-Butylpyridine	5.99	5.21	4.62
Aniline	4.61	3.87	3.44

[a] From references [24], [30] and [31].

Table 6
Aqueous ${}^{w}_{w}$pH values of the buffered systems used, estimated and measured ${}^{s}_{w}$pH values at 60% MeCN in volume

Buffer	${}^{w}_{w}$pH	${}^{s}_{w}$pH	${}^{s}_{w}pH^{est}$	Δ^{s}_{w}pH
H_3PO_4	2.33	2.74	2.75	−0.01
$H_2Cit^-/HCit^{2-}$	4.04	4.98	4.98	0.00
$H_2Cit^-/HCit^{2-}$	4.92	5.98	5.96	0.02
$HCit^{2-}/Cit^{3-}$	5.93	7.03	7.12	−0.09
$H_2PO_4^-/HPO_4^{2-}$	6.96	7.97	8.01	−0.04
$H_2PO_4^-/HPO_4^{2-}$	7.93	8.91	8.98	−0.07

The aqueous buffer concentration is, in all cases, 0.01 mol L^{-1} $\Delta^{s}_{w}pH = {}^{s}_{w}pH - {}^{s}_{w}pH^{est}$.

4.2. Retention times estimation

From the equations presented in Section 3, we are able to estimate the ${}^{s}_{w}$pH in acetonitrile/water mobile phases at any fraction of organic modifier up to 60% in volume from the aqueous ${}^{w}_{w}$pH for the most commonly used buffers in HPLC, and the ${}^{s}_{w}pK_a$ values for a wide set of compounds. Then using Eq. (2), we can estimate the analyte retention times as a function of the pH in the hydroorganic mobile phases. We only need to measure the ${}^{w}_{w}$pH of the buffered system and ${}^{w}_{w}pK_a$ of the analyte, and also the retention times of the fully ionised and the neutral forms. These retention times can be measured by injecting the compounds using buffered mobile phases with pH two or three units higher and lower than the pK_a of the analyte.

Several acidic and basic drugs with known aqueous pK_a were studied to test this retention time estimation model: diclofenac, ibuprofen and naproxen (nonsteroidal anti-inflammatory drugs), codeine (narcotic analgesic), trazodone, imipramine, nortriptyline and maprotiline (antidepressants). To cover the pH range of interest different buffered systems at 60% MeCN (v/v) were used: $H_3PO_4/H_2PO_4^-/HPO_4^{2-}/PO_4^{3-}$ and $H_3Cit/H_2Cit^-/HCit^{2-}/Cit^{3-}$. In all cases, the concentration of the aqueous buffer was 0.01 mol L^{-1}. As shown in Table 6, there is a good agreement between the measured pH in the mobile phase and their corresponding pH values estimated by Eq. (3). We must take into account that over ${}^{w}_{w}$pH 8 the predicted ${}^{s}_{w}$pH values in the phosphoric acid buffer system is not as accurate as at lower pH because of the solubility of the PO_4^{3-} species in mixtures with a high content of organic modifier.

Retention was estimated by means of Eq. (2) taking as $t_{R(HA)}$ and $t_{R(A)}$ the retention times measured at low and high pH, corresponding to the neutral and fully ionised species. Therefore, $t_{R(HA)}$ and $t_{R(A)}$ for diclofenac, ibuprofen, naproxen and trazodone were measured at ${}^{w}_{w}$pH 2.33 (H_3PO_4 buffer) and 8.82 ($H_2PO_4^-/HPO_4^{2-}$ buffer), respectively. For codeine, imipramine, nortriptyline and maprotiline, ${}^{w}_{w}$pH 4.04 ($H_2Cit^-/HCit^{2-}$) and 10.48 (HPO_4^{2-}/PO_4^{3-}) were used. ${}^{s}_{w}pK_a$ were estimated from the literature ${}^{w}_{w}pK_a$ [24–28] (Table 7).

Table 7
Literature and estimated aqueous ${}^{w}_{w}pK_a$ values for the studied drugs

Compound	Literature ${}^{w}_{w}pK_a$ [a]	SPARC ${}^{w}_{w}pK_a$ [b]	ACD/Labs ${}^{w}_{w}pK_a$ [c]
Diclofenac	4.23	4.09	4.18 ± 0.20
Ibuprofen	4.40	4.52	4.41 ± 0.20
Naproxen	4.72	4.46	4.40 ± 0.20
Trazodone	6.93	5.99	6.59 ± 0.50
Codeine	8.21	9.18	8.92 ± 0.20
Imipramine	9.30	9.43	9.49 ± 0.20
Nortriptyline	10.14	10.33	10.08 ± 0.20
Maprotiline	10.45	10.33	10.63 ± 0.20

[a] From references [24], [25] and [26].
[b] From [27].
[c] From [28].

Fig. 3 shows the differences between the experimental and the estimated retention times at several measured aqueous pH. Generally, there is very good agreement between the estimated and experimental retention times. Except for ibuprofen at ${}^{w}_{w}$pH 4.92 ($\Delta t_R = -0.55$ min) and 5.93 (−0.50 min) and imipramine at 6.96 (+0.73 min) and 7.93 (+1.30 min), the largest deviations are +0.33 min and −0.10 min, and the average of the absolute error for all the analytes and studied pH values is less than 5%. The differences in retention times for imipramine and ibuprofen can be attributed to a mismatch between the chromatographically obtained ${}^{s}_{w}pK_a$ and the estimated ones by means of Eqs. (7) and (10). Presumably, when the difference in retention times of the neutral and fully ionised species are large, this pK_a mismatch has a significant effect on retention estimation. We must take into account that these drugs have a more complex structure than the substances used to set up the pK_a estimation model, and therefore it is possible that they could experience a certain bias in relation to the predicted pK_a. Moreover, they are sparely soluble in water, and their ${}^{w}_{w}pK_a$ determinations were performed through potentiometric measurements in methanol–water mixtures and subsequent extrapolation to water. Therefore, for a particular drug a certain variation of ${}^{w}_{w}pK_a$ values can be found in the literature (e.g. imipramine: 9.19–9.66 [26]; ibuprofen: 4.40–4.76 [25]).

When no experimental aqueous pK_a data is available in the literature, one can resort to computational programs, e.g. SPARC [27] and ACD/Labs [28,29], that allow calculation of pK_a values. ACD/Labs is used by many pharmaceutical companies to calculate not only acid–base ionisation constants, but also several physicochemical properties of a wide range of organic compounds. It follows a structure-fragment approach, based on an internal database of structures and related pK_a values. The ACD/Labs version used in the present work is embedded in the SciFinder Scholar 2006 data base research tool produced by the Chemical Abstracts Service of the American Chemical Society [28]. The SPARC program uses algorithms based on fundamental chemical structure theory to calculate pK_a values of organic compounds, taking into account each essential functional unit with intrinsic properties that composes the whole molecule. It is freely accessed through Internet [27].

Both programs were used to calculate the ${}^{w}_{w}pK_a$ values of the studied drugs, and the results are shown in Table 7. From these calculated aqueous acid–base constants, the ${}^{w}_{w}$pH

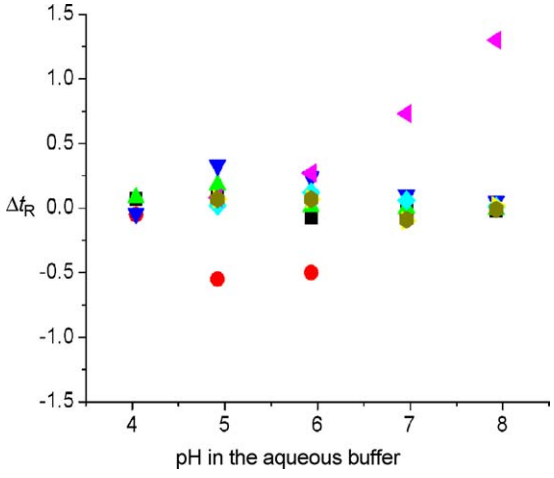

Fig. 3. Differences between the experimental and the estimated retention times (in minutes) at several measured ${}^{w}_{w}$pH ($\Delta t_R = t_R^{est} - t_R$). Estimated retention times were calculated through Eq. (2), where ${}^{s}_{w}pK_a$ were estimated from the literature ${}^{w}_{w}pK_a$ values, and ${}^{s}_{w}$pH were estimated from measured aqueous ${}^{w}_{w}$pH. Buffer aqueous concentration was, in all cases, 0.01 mol L^{-1}. Legend: (▼) trazodone, (■) diclofenac, (◆) codeine, (▲) naproxen, (●) ibuprofen, (◀) imipramine, (★) maprotiline, (▶) nortriptyline.

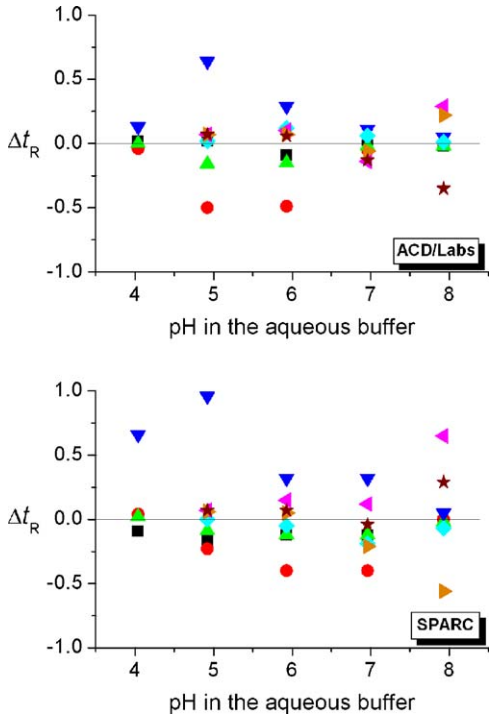

Fig. 4. Differences between the experimental and the estimated retention times (in minutes) at several measured ${}^{w}_{w}$pH ($\Delta t_R = t_R^{est} - t_R$). Estimated retention times were calculated through Eq. (2), where ${}^{s}_{w}pK_a$ were estimated from the calculated ${}^{w}_{w}pK_a$ values through computational programs SPARC and ACD/Labs, and ${}^{s}_{w}$pH were estimated from measured aqueous ${}^{w}_{w}$pH. Buffer aqueous concentration was, in all cases, 0.01 mol L^{-1}. Legend: (▼) trazodone, (■) diclofenac, (◆) codeine, (▲) naproxen, (●) ibuprofen, (◀) imipramine, (★) maprotiline, (▶) nortriptyline.

of the mobile phase and the measured $t_{R(HA)}$ and $t_{R(A)}$, retention times at several pH values were estimated and related to their corresponding chromatographic values. Results are shown in Fig. 4. In general, good correspondences are obtained for both programs, with the fits for ACD/Labs being slightly better than for SPARC. In fact, the average absolute error in retention times for all analytes over all pH ranges studied is less than 7% for SPARC and 5% for ACD/Labs. We find again, as expected, a negative mismatch for a couple of retention points for ibuprofen, because of its similar calculated and determined ${}^{w}_{w}pK_a$ values. Conversely, aqueous calculated pK_a values for trazodone are significantly lower than that found in literature, as shown in Table 7, and the correspondence using these calculated ${}^{w}_{w}pK_a$values is worse than the obtained when using the literature pK_a value. Better estimations were obtained for imipramine, because both calculated acid–base constants were rather similar, and slightly higher than the experimental one.

5. Conclusions

Our findings indicate that measurement of the pH of the hydroorganic mobile phase is preferable to measuring the pH of the buffer alone, because different buffers with the same aqueous pH can lead to significant differences in pH readings when adding an organic solvent. Moreover, better retention versus pH fittings are obtained this way, and through them it is possible to determine thermodynamic dissociation constants of weak acids or bases. Additionally, we can easily relate this ${}^{s}_{w}pK_a$ (hydrogen ion in water at infinite dilution as standard state) with the ${}^{s}_{s}pK_a$ (hydroorganic mixture as standard state) through a known δ parameter.

However, in some instances it may be more practical to measure the pH in the aqueous buffer before addition of the organic modifier. A methodology has been proposed to estimate both the hydroorganic pH values in usual MeCN/aqueous buffered systems mobile phases up to 60% of organic modifier, and the hydroorganic acidity constants of acid–base analytes, starting from the aqueous pH and concentration of the buffer and the aqueous acid–base constant of the compound. With the measured retention times of the neutral and fully ionised species, and both calculated pK_a and pH, the method is able to estimate the retention times of weak acids and bases at any hydroorganic pH. This methodology has been successfully tested for several acidic and basic analgesic, antidepressant and anti-inflammatory drugs with known aqueous pK_a. Alternatively, rather good retention estimations were achieved using calculated aqueous pK_a values by means of computational programs like ACD/Labs or SPARC.

Acknowledgements

We thank financial support from the MCYT of the Spanish Government and FEDER of EU (projects CTQ2004-00633/BQU and CTQ2004-00965/BQU).

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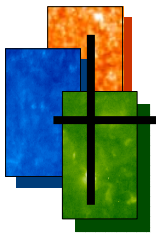
ARTICLE III

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



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J. Chromatogr. A 1138 (2007) 203

Available online at www.sciencedirect.com
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Journal of Chromatography A, 1138 (2007) 203–215
JOURNAL OF CHROMATOGRAPHY A
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Retention of ionisable compounds on high-performance liquid chromatography XVII Estimation of the pH variation of aqueous buffers with the change of the methanol fraction of the mobile phase

Xavier Subirats, Elisabeth Bosch, Martí Rosés *

Departament de Química Analítica, Universitat de Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain

Received 12 September 2006; received in revised form 24 October 2006; accepted 30 October 2006
Available online 21 November 2006

Abstract

The use of methanol–aqueous buffer mobile phases in HPLC is a common election when performing chromatographic separations of ionisable analytes. The addition of methanol to the aqueous buffer to prepare such a mobile phase changes the buffer capacity and the pH of the solution. In the present work, the variation of these buffer properties is studied for acetic acid–acetate, phosphoric acid–dihydrogenphosphate–hydrogenphosphate, citric acid–dihydrogencitrate–hydrogencitrate–citrate, and ammonium–ammonia buffers. It is well established that the pH change of the buffers depends on the initial concentration and aqueous pH of the buffer, on the percentage of methanol added, and on the particular buffer used. The proposed equations allow the pH estimation of methanol–water buffered mobile phases up to 80% in volume of organic modifier from initial aqueous buffer pH and buffer concentration (before adding methanol) between 0.001 and 0.01 mol L^{-1}. From both the estimated pH values of the mobile phase and the estimated pK_a of the ionisable analytes, it is possible to predict the degree of ionisation of the analytes and therefore, the interpretation of acid–base analytes behaviour in a particular methanol–water buffered mobile phase.

Keywords: Mobile phase composition; Methanol–water mixtures; pH; Buffers; Chromatographic retention; Ionisation degree

1. Introduction

Chromatographic retention of acid–base analytes in reversed phase high performance liquid chromatography (RP-HPLC) depends on the hydrophobicity of the analytes and on their ionisation degree, which in turn depends on the pH and analyte acid–base constant (pK_a) in the particular mobile phase used. The effect of both the pH and the pK_a on ionisation degree and therefore, on retention times in HPLC has been already extensively reported [1–22]. To achieve reproducible and successful chromatographic analysis, a careful control and accurate measurement of pH is essential. We recommend the measurement of pH in the hydroorganic mobile phase, rather than in the aqueous buffer, because the pH variation when adding an organic modifier depends on the particular buffering system, on its concentration and on the fraction of organic solvent in the mixture [2,3,7,8,10,12–14]. When the measurement of pH in the mobile phase is not easy, e.g. in the case of highly automated HPLC experiments where independent reservoirs of buffer and organic solvent are pumped into and mixed within the apparatus, it may be very useful to estimate the pH variation for a particular buffer when the organic modifier is added. This pH modelling may also be useful to provide the chromatographers with a buffered mobile phase adequate to solve a particular separation problem. This is useful in case of mixtures of analytes with similar acid–base properties, because without performing any measurement it is possible to predict the particular composition of the mobile phase in which the differences on ionisation degree between the analytes are significant enough. This *a priori* optimization could avoid fruitless time and reagent consuming experiments. On the basis of previous works, on pH estimation in acetonitrile–aqueous buffer mobile phases [11,18], we present in this paper a model developed for the pH variation of the most commonly used buffers in methanol–water mobile phases. From

* Corresponding author. Tel.: +34 93 403 92 75; fax: +34 93 402 12 33.
E-mail address: marti.roses@ub.edu (M. Rosés).

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doi:10.1016/j.chroma.2006.10.087

the aqueous buffer pH and the aqueous pK_a of the analyte, we are able to predict the analyte ionisation degree in a particular hydroorganic mobile phase up to 80% (v/v) of methanol.

2. pH scales

As we have extensively discussed in previous works [2,3,7,8,10,12–14], three different procedures are used to measure the pH of hydroorganic mobile phases in HPLC. A typical one consists on calibrating the electrode systems with commercial aqueous standard buffers, and then measuring the pH of the aqueous buffer before mixing it with the organic modifier. This way the pH value is obtained in the ${}^{w}_{w}$pH scale [15]. In our opinion this is not the best option because the pH of the solution changes after dilution of the aqueous buffer with the organic modifier, according to the nature of the buffer used. If the electrode system is calibrated with standard buffers prepared in the same solvent composition used as mobile phase, and the pH is measured in this particular mobile phase composition, the ${}^{s}_{s}$pH value is obtained. Working in the ${}^{s}_{s}$pH scale requires a careful preparation and maintenance of the standard buffers and electrodes, and often these standards are not commercially available. Finally, when pH is measured in the hydroorganic mixture, although the electrode system is calibrated with aqueous buffers, the ${}^{s}_{w}$pH values are obtained. Notice that here the IUPAC nomenclature [5] has been used: the left hand superscript indicates the medium where the quantity is measured (w for water and s for hydroorganic mixture), and the subscript indicates the standard state medium (i.e., the solvent where activity coefficients are taken as equal to unity at infinite dilution), which means in practice, the solvent (w or s) in which electrode systems are calibrated. It has been widely reported that better models are obtained when the pH in the mobile phase is considered instead of the aqueous pH of the buffer [1,2,4,7–9,14,19–22]. ${}^{s}_{s}$pH can be easily converted to ${}^{s}_{w}$pH by means of δ parameter [10,23,24]:

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

The δ term is a constant value for each mobile phase composition. It includes the primary medium effect and the difference between the liquid junction potentials of the electrode system in the hydroorganic mobile phase and in water. The primary medium effect depends only on the mobile phase solvent composition, but the liquid junction potential depends also on the particular electrode system, pH standards, and sample composition. 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. In practice, this requirement is fulfilled using a combination electrode containing a reference electrode with a concentrated KCl solution in water as a salt bridge. These δ values for methanol–water mixtures were studied by various authors [7,10,25,26] and they can be estimated from the solvent composition through the empirical equation [10]:

$$ \delta = \frac{0.09\phi_{\mathrm{MeOH}} - 0.11\phi^2_{\mathrm{MeOH}}}{1 - 3.15\phi_{\mathrm{MeOH}} + 3.51\phi^2_{\mathrm{MeOH}} - 1.35\phi^3_{\mathrm{MeOH}}} \quad (2) $$

Table 1
Macroscopic properties of methanol–water mixtures at 25 °C [2,10]

ϕ_{MeOH} (v/v)	x_{MeOH}	ρ (g mL^{-1})	A	a_0B	${}^{s}_{s}$pK_{ap}	δ
0.0	0.000	0.9948	0.53	1.50	14.00	0.00
0.1	0.047	0.9826	0.56	1.53	14.08	0.01
0.2	0.100	0.9693	0.60	1.57	14.08	0.02
0.3	0.160	0.9548	0.64	1.62	14.07	0.04
0.4	0.229	0.9388	0.70	1.67	14.09	0.08
0.5	0.308	0.9209	0.78	1.73	14.14	0.13
0.6	0.400	0.9008	0.88	1.80	14.23	0.19
0.7	0.509	0.8780	1.02	1.88	14.39	0.22
0.8	0.640	0.8518	1.21	1.99	14.63	0.10
0.9	0.800	0.8216	1.48	2.13	15.04	−0.28
1.0	1.000	0.7870	1.87	2.31	16.77	−2.24

x_{MeOH}, molar fraction of methanol in the mixture; A and a_0B, Debye–Hückel equation parameters; ${}^{s}_{s}$pK_{ap}, autoprotolysis constant of the solvent mixture; ρ, solvent density.

where ϕ_{MeOH} is the volume fraction of methanol in the hydroorganic mixture. The relationship between ${}^{s}_{s}$pH and ${}^{s}_{w}$pH mainly depends on the methanol fraction in the mixture, whereas the difference between ${}^{w}_{w}$pH and ${}^{s}_{s}$pH (or ${}^{s}_{w}$pH) depends not only on the mobile phase composition but also on the particular buffering solution employed. Table 1 reports δ values for some methanol–water mixtures, together with other macroscopic properties of interest for pH estimation. These δ values are also useful to convert ${}^{s}_{w}$pK_a values to ${}^{s}_{s}$pK_a, and ${}^{s}_{w}$pK_{ap} to ${}^{s}_{s}$pK_{ap}, where pK_a refers to the analyte acid–base constant and pK_{ap} to the autoprotolysis constant of the solvent (methanol–water mixture). ${}^{s}_{w}$pK and ${}^{s}_{s}$pK indicate the negative logarithm of the constant (pK_a or pK_{ap}) when the pH is measured in ${}^{s}_{w}$pH or ${}^{s}_{s}$pH scale, respectively.

3. Experimental

3.1. Apparatus

Potentiometric measurements were taken with a Crison 5014 combination electrode (glass electrode and a reference electrode with a 3.0 mol L^{-1} KCl solution in water as salt bridge) in a Crison GLP22 pH meter with a precision of ±0.1 mV (±0.002 pH unit). 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 C18 5 μm column from Waters (Milford, MA, USA), externally thermostated with a water jacket at 25 ± 0.1 °C, with a flow rate of 1 mL min^{-1} in isocratic mode in an ISCO model 2350 dual-pump system (Lincoln, NE, USA) with a 10 μL injection loop. An ISCO V^4 ultra-violet visible spectrophotometric detector set at 254 nm was employed, and data were collected through ISCO ChemResearch data management program.

3.2. Chemicals

Methanol was RP-HPLC gradient grade from Merck and water purified by the Milli-Q plus system (to 18 MΩ) from Millipore (Bedford, MA, USA). The studied buffers were prepared from acetic acid (Merck, glacial, for analysis), sodium

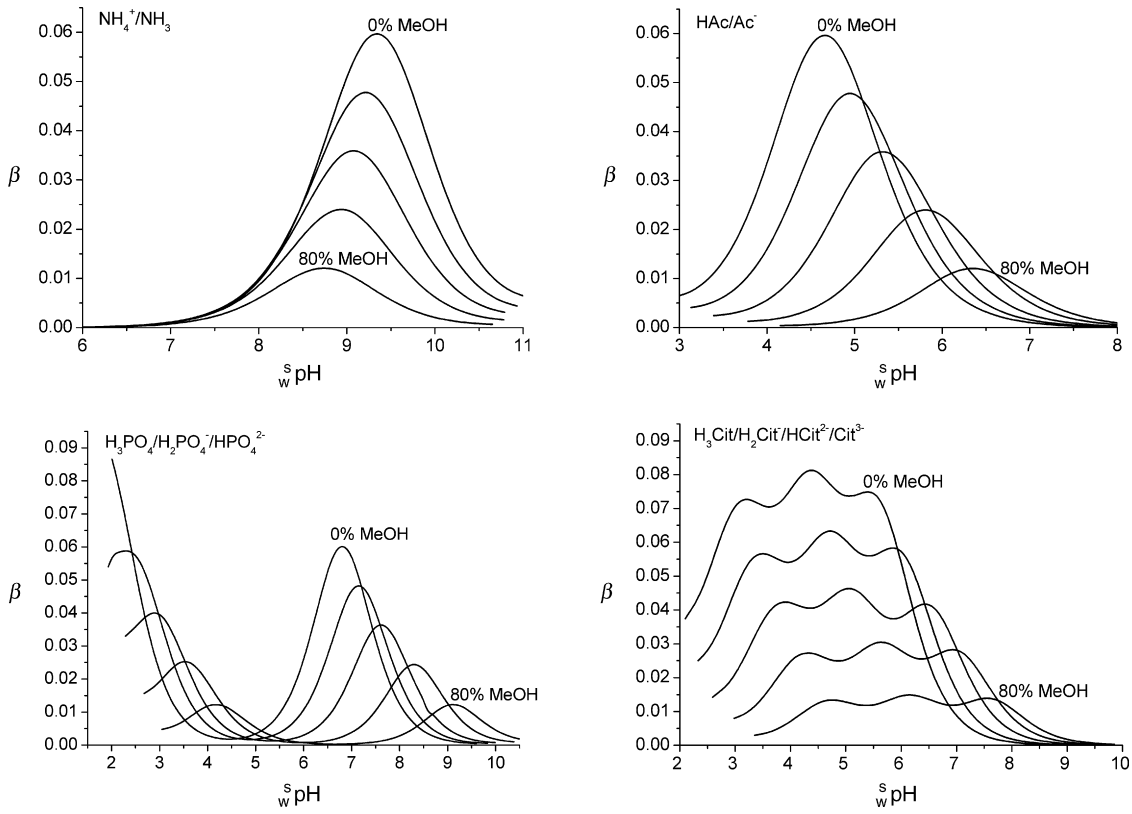

Fig. 1. Buffer capacity variation of the studied systems for 0, 20, 40, 60 and 80% (v/v) methanol–water compositions and an initial aqueous buffer concentration of 0.1 mol L^{-1}.

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 when it was necessary. The chromatographied compounds were 2-nitrophenol (Fluka, >99%), 3-nitrophenol (Aldrich, 99%), 4-nitrophenol (Fluka, for analysis), 2-chlorophenol (Aldrich, 99+%), 3-bromophenol (Aldrich, 98%), 2,4,6-trimethylpyridine (Merck, for analysis), *N*,*N*-dimethylbenzylamine (Merck–Schuchardt, for synthesis).

3.3. Procedure

The required aqueous acid and base concentrations for the selected pH was calculated to provide a total buffer aqueous concentrations of 0.001, 0.01 and 0.1 mol L^{-1}. If necessary, the pH was finally adjusted by addition of small amounts of concentrated solutions of potassium hydroxide or hydrochloric acid. Methanol–water buffers were prepared by addition of methanol 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 methanol. Chromatographic data were obtained isocratically in a mobile phase with a 60% of methanol in volume.

4. Results and discussion

4.1. Variation of buffer capacity with solvent composition

How much ability the buffer has to keep pH in front of the addition of acids or bases (buffer capacity) when it is mixed with methanol is a crucial consideration in the preparation of an HPLC buffer. Quantitative measurement of buffer ability to keep pH can be expressed in terms of buffer capacity (β), which can be calculated by means of the following differential equation [23,24]:

$$\beta = \frac{\mathrm{d}c_\mathrm{b}}{\mathrm{d(pH)}} = -\frac{\mathrm{d}c_\mathrm{a}}{\mathrm{d(pH)}} \quad (3)$$

where c_b and c_a are the concentrations of the buffering base and acid, respectively. Buffer capacity is, in rough terms, the strong base or strong acid amount (expressed in equivalents) required to produce one pH unit change in the buffer solution. For a weak acid–weak base buffer, maximum buffer capacity of a protolyte occurs when the acid species concentration is equal to the concentration of conjugate base.

Fig. 1 shows the calculated buffer capacity variation of the studied systems for 0, 20, 40, 60 and 80% (v/v) methanol–water compositions and an initial buffer concentration of

0.1 mol L^{-1}. It has been calculated by means of the algorithms used to determine the pH of the hydroorganic solutions, calculating the pH change produced by a 0.1% variation of the base or the acid concentration. According to Eq. (3) the ratio between this small change of concentration and the resulting variation produced in pH is assumed to be the buffer capacity. A decrease on buffer capacity is observed when the methanol fraction in the hydroorganic mixture increases, due to the dilution effect. Moreover, the addition of methanol 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 values for the cationic acid buffer (NH_4^+–NH_3). The broad poorly buffered zone between the first and the second pK_a of the phosphoric acid system, around ${}^{w}_{w}$pH 4.5, and the wide range of excellent buffer capacity of citric acid system up to ${}^{w}_{w}$pH 6.5.

4.2. Variation of pK_a and pH of buffers with solvent composition

In previous studies [27,28], linear relationships between pK_a values in acetonitrile–water mixtures (${}^{s}_{w}pK_a$) and solvent composition were established:

$$ {}^{s}_{w}pK_a - {}^{w}_{w}pK_a = m_{pK}\phi_{MeCN} \quad (4) $$

where ϕ_{MeCN} is the volume fraction of acetonitrile in the mixture and m_{pK} the proportionality coefficient between pK_a change and mobile phase composition. Eq. (4) led us to linear relationships for the pH variation of HPLC acetonitrile–aqueous buffers with the volume fraction of the organic modifier:

$$ {}^{s}_{w}pH - {}^{w}_{w}pH = m_{pH}\phi_{MeCN} \quad (5) $$

where m_{pH} is the proportionality coefficient for the pH change. The same type of relationships has been tested here for methanol–aqueous buffer mobile phases. The pK_a variation of some neutral, anionic and cationic acids commonly used to prepare HPLC buffers (acetic, phosphoric – first and second pK_a – and citric acids and ammonium) has been studied with the addition of methanol. The variation of the third pK_a value of phosphoric acid has not been studied because it is not available in the literature. It seems to be two reasons for this lack of data: one is the poor stability of traditional silica phases at high pH values, and the other is the well known insolubility of phosphate

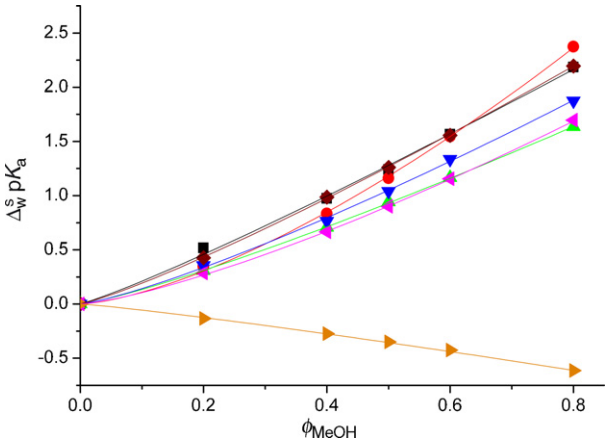

Fig. 2. Variation of the pK_a values (${}^{s}_{w}pK_a - {}^{w}_{w}pK_a$) of the studied buffers with the addition of methanol: (■) phosphoric acid–dihydrogenphosphate, (●) dihydrogenphosphate–hydrogenphosphate, (▲) citric acid–dihydrogencitrate, (▼) dihydrogencitrate–hydrogencitrate, (♦) hydrogencitrate–citrate, (◄) acetic acid–acetate, and (►) ammonium–ammonia.

in methanol–water mixtures at high percentages of methanol and high pH [2,10,29–31]. ${}^{s}_{w}pK_a$ values were taken from literature [10], and their variation (${}^{s}_{w}pK_a - {}^{w}_{w}pK_a$) with the volume fraction of methanol is presented in Fig. 2. The fittings of these values to a linear equation analogous to Eq. (4) are not as good as expected in relation to the ones obtained for acetonitrile–water mixtures. Therefore, a new curvilinear equation was assayed to describe the ${}^{s}_{w}pK_a$ variation for the studied buffers:

$$ {}^{s}_{w}pK_a - {}^{w}_{w}pK_a = m_{pK}\phi_{MeOH}^{d_{pK}} \quad (6) $$

where ϕ_{MeOH} is the volume fraction of methanol in the mixture and m_{pK} and d_{pK} are empirical fitting parameters. m_{pK} values must be positive for neutral (acetic, phosphoric, citric) and anionic (dihydrogenphosphate, dihydrogencitrate, hydrogencitrate) acids and negative for cationic acids (ammonium), since the pK_a of neutral and anionic acids increases when the methanol content in the mobile phase increases and cationic acids show the reversed behaviour. These trends have been already explained in terms of electrostatic interactions that contribute to the pK_a value [32,33]. The ${}^{s}_{w}pK_a$ values of the studied acids are shown in Table 2 together with the best fits for both m_{pK} and d_{pK} parameters from Eq. (6). The ${}^{s}_{w}pK_a$ values used in the fittings of Eq. (6) have been calculated from their corresponding literature ${}^{s}_{s}pK_a$ values [10] using the δ parameter for each particular

Table 2
${}^{s}_{w}pK_a$ values of the studied buffers in methanol–water mixtures [10] and m_{pK} and d_{pK} parameters of Eq. (6)

Acid–base pair	${}^{s}_{w}pK_a$ in % of methanol by volume						m_{pK}	d_{pK}
	0	20	40	50	60	80		
H3PO4–H2PO4$^-$	2.11	2.63	3.09	3.35	3.68	4.30	2.78	1.12
H2PO4$^-$–HPO4^{2-}	7.20	7.55	8.04	8.36	8.75	9.58	3.29	1.48
H3Cit–H2Cit$^-$	3.13	3.44	3.84	4.07	4.30	4.77	2.14	1.20
H2Cit$^-$–HCit^{2-}	4.76	5.12	5.53	5.80	6.10	6.64	2.48	1.24
HCit^{2-}–Cit^{3-}	6.40	6.83	7.39	7.66	7.96	8.60	2.85	1.17
HAc–Ac$^-$	4.76	5.05	5.43	5.66	5.92	6.46	2.27	1.33
NH4$^+$–NH3	9.24	9.11	8.97	8.89	8.82	8.63	−0.79	1.15

Table 3
Calculated d_{pH} parameter values of Eq. (7), including standard deviation

mmol L^{-1}	Acetic	N	Phosphoric	N	Citric	N	Ammonium	N
1	1.29 ± 0.05	45	1.40 ± 0.05	88	1.25 ± 0.07	132	0.99 ± 0.04	40
3	1.29 ± 0.04	45	1.36 ± 0.04	88	1.24 ± 0.07	132	1.03 ± 0.02	40
5	1.29 ± 0.04	45	1.33 ± 0.05	88	1.24 ± 0.06	132	0.99 ± 0.02	40
7	1.29 ± 0.04	45	1.32 ± 0.06	88	1.24 ± 0.06	132	0.99 ± 0.02	40
10	1.33 ± 0.03	45	1.30 ± 0.07	88	1.23 ± 0.06	132	0.99 ± 0.02	40
20	1.33 ± 0.03	45	1.28 ± 0.09	88	1.23 ± 0.05	132	0.99 ± 0.02	40
30	1.33 ± 0.02	45	1.27 ± 0.09	88	1.23 ± 0.05	132	0.99 ± 0.02	40
40	1.33 ± 0.02	45	1.27 ± 0.10	88	1.23 ± 0.05	132	0.99 ± 0.02	40
50	1.33 ± 0.02	45	1.26 ± 0.10	88	1.23 ± 0.05	132	0.99 ± 0.02	40
60	1.33 ± 0.02	45	1.26 ± 0.10	88	1.23 ± 0.05	132	0.99 ± 0.02	40
70	1.33 ± 0.02	45	1.26 ± 0.11	88	1.23 ± 0.05	132	0.99 ± 0.02	40
80	1.33 ± 0.02	45	1.26 ± 0.11	88	1.23 ± 0.05	132	0.99 ± 0.02	40
90	1.33 ± 0.02	45	1.26 ± 0.11	88	1.23 ± 0.05	132	1.03 ± 0.02	40
100	1.33 ± 0.02	45	1.25 ± 0.11	88	1.23 ± 0.05	132	0.99 ± 0.02	40
All	1.32 ± 0.03	630	1.29 ± 0.10	1232	1.23 ± 0.05	1848	0.99 ± 0.02	560

methanol–water composition. The d_{pK} values shown in Table 2 for the studied buffers are different from the unity value we had in the acetonitrile–water model Eq. (5), and their values are between 1.1 and 1.5. The lowest d_{pK} values were found for phosphoric acid – first pK_a –, ammonium and hydrogencitrate, and the highest one for dihydrogenphosphate. Intermediate d_{pK} values were calculated for citric acid – first pK_a– dihydrogencitrate and acetic acid.

In previous works [11,18], we proposed equations accounting for a linear variation of the pH in the hydroorganic mixture when adding acetonitrile to an aqueous buffer Eq. (5). The same procedure [11,18] was tested for methanol–water mixtures up to 80% in volume of organic modifier. From the ${}^{w}_{w}$pH and aqueous concentration of the buffer, ${}^{s}_{s}$pH values at 0, 20, 40, 50, 60 and 80% (v/v) of methanol were calculated through a similar procedure to that described by De Levie [34] for titrations of acid–base mixtures. All calculations were performed taking into account the corresponding dilution coefficient when increasing the methanol fraction, the molar activity coefficient (by means of Debye–Hückel equation), and the ${}^{s}_{s}$pK_a values of each buffer component at the corresponding hydroorganic composition [11,18]. The autoprotolysis constants of each solvent composition (${}^{s}_{s}$pK_{ap}) were also considered. pH calculations were carried out for fourteen different initial aqueous buffer concentrations: 0.001, 0.003, 0.005, 0.007, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.1 mol L^{-1}. Finally, calculated ${}^{s}_{s}$pH values were converted to the ${}^{s}_{w}$pH scale by means of δ values Eq. (2). The dielectric constants of the studied solvent mixtures are higher than 40 [35] and thus, ion pairing should be insignificant in them [36] and was not considered in pH calculation. After all, a quite similar relation to that for pK_a variation was found for pH variation in methanol–water mixtures, expressed through the equation:

$$ {}^{s}_{w}\mathrm{pH} - {}^{w}_{w}\mathrm{pH} = m_{pH}\phi_{MeOH}^{d_{pH}} \quad (7) $$

where ${}^{s}_{w}$pH is the pH of the hydroorganic mobile phase, ${}^{w}_{w}$pH is the pH of the aqueous buffer before adding the methanol, m_{pH} is a proportionality coefficient, and d_{pH} is an empirical parameter to optimize the fit of the data. The d_{pH} values have been determined from the calculated pH variations in Eq. (7) for all the studied buffers and concentrations between 0.001 and 0.1 mol L^{-1} in a wide range of initial ${}^{w}_{w}$pH values, and the averaged values for each concentration are shown in Table 3. The d_{pH} values are quite similar within a buffer, nearly independent of the concentration of the buffer and the initial aqueous pH. Table 3 shows also the average of the d_{pH} values resulting on considering all calculated particular d_{pH} values, which as expected are very similar to the d_{pK} values of Table 2 for each particular buffer component. It is noteworthy that we can clearly differentiate the d_{pH} values of neutral and anionic acids (HAc–Ac^-, H_3PO_4–$H_2PO_4^-$–HPO_4^{2-}, H_3Cit–H_2Cit^-–$HCit^{2-}$–Cit^{3-}) and the cationic ammonium (NH_4^+–NH_3). From all the particular d_{pH} values of neutral and anionic acids, independently of the buffer (N = 3710), the average is 1.27 ± 0.08 (≈5/4). The corresponding average for ammonium is 0.99 ± 0.02 (≈1). Then, for the sake of simplicity we assume that d_{pH} is equal to 1 for cationic acids, and 5/4 for neutral and anionic acids.

The variation of the slope (m_{pH}) of Eq. (7) with the initial aqueous ${}^{w}_{w}$pH of the buffer is described by means of an equation very similar to the one proposed in a previous work [11] to estimate the pH variation in acetonitrile–aqueous buffer mixtures, except for the variation of the s_i parameters (constant s_i values were taken in the previous work, i.e. $s_1 = s_2 = s_3 = \ldots = s_n$):

$$ m_{pH} = \frac{a_0 + \sum_{i=1}^{n} a_i 10^{s_i({}^{w}_{w}\mathrm{pH} - b_i)} + a_{n+1} 10^{s_i((n+1){}^{w}_{w}\mathrm{pH} - b_{n+1})}}{1 + \sum_{i=1}^{n} 10^{s_i({}^{w}_{w}\mathrm{pH} - b_i)} + 10^{s_i((n+1){}^{w}_{w}\mathrm{pH} - b_{n+1})}} \quad (8) $$

where 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. The $n+1$ term predominates at very basic pH values (buffers with strong bases). 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 a_i values should be close to the m_{pK} values Eq. (6) of buffering species reported in Table 2, and b_i values should be a combination of the aqueous pK_a values of the corresponding acid–base pairs of the system. s_i are fitting parameters that account for the sharpness of the transition between the different pH zones buffered by the different acid–conjugate base pairs of the system. Eq. (8) is also analogous to the equation used to fit retention time and chromatographic hydrophobicity index (CHI) to aqueous pH during gradient elution [37,38]. A similar equation with two terms and $s = 1$ was used to fit the ${}^{s}_{w}$pH change of ammonium acetate buffers in methanol–water mobile phases [37].

a_0 value can be calculated through the pH variation of a solution prepared from a strong monoprotic acid. Neglecting the volume contraction when adding methanol to water and the differences between the activity coefficients in the mixture and in water (<0.02 pH units at 80% (v/v) of methanol), the pH variation of a strong acid in the ${}^{s}_{w}$pH scale can be calculated [11] from:

$$ {}^{s}_{s}\mathrm{pH} - {}^{w}_{w}\mathrm{pH} = -\log\,\phi_{H_2O} \tag{9} $$

and Eq. (1):

$$ {}^{s}_{w}\mathrm{pH} - {}^{w}_{w}\mathrm{pH} = \delta - \log\,\phi_{H_2O} \tag{10} $$

where ϕ_{H_2O} is the volume fraction of water in the mixture. Up to 80% in volume of methanol, there is a good linear correlation between this $\delta - \log\phi_{H_2O}$ term and the volume fraction of methanol, ϕ_{MeOH}, in the solvent mixture, which leads to:

$$ {}^{s}_{w}\mathrm{pH} - {}^{w}_{w}\mathrm{pH} = (0.91 \pm 0.05)\,\phi_{MeOH} $$
$$ (r = 0.995;\ \mathrm{SD} = 0.05;\ F = 773) \tag{11} $$

Better fitting is obtained considering this pH variation against the volume fraction of methanol at 5/4 exponent, $\phi^{5/4}_{MeOH}$.

$$ {}^{s}_{w}\mathrm{pH} - {}^{w}_{w}\mathrm{pH} = (1.03 \pm 0.02)\,\phi^{5/4}_{MeOH} $$
$$ (r = 0.999;\ \mathrm{SD} = 0.02;\ F = 2895) \tag{12} $$

Therefore, depending on the exponent of the methanol volume fraction term, the m_{pH} slope for a strong acid is 0.91 or 1.03 in relation to ϕ_{MeOH} or $\phi^{5/4}_{MeOH}$, respectively. The former value is used for the cationic ammonium buffering system, and the latter one for buffers prepared from neutral and anionic acids.

In the same manner, a_{n+1} is derived from the pH variation for a strong base, which should be [11]:

$$ {}^{s}_{w}\mathrm{pH} - {}^{w}_{w}\mathrm{pH} = \left({}^{s}_{w}\mathrm{p}K_{ap} - {}^{w}_{w}\mathrm{p}K_{ap}\right) + \log\,\phi_{H_2O} $$
$$ = \Delta\mathrm{p}K_{ap} + \log\,\phi_{H_2O} \tag{13} $$

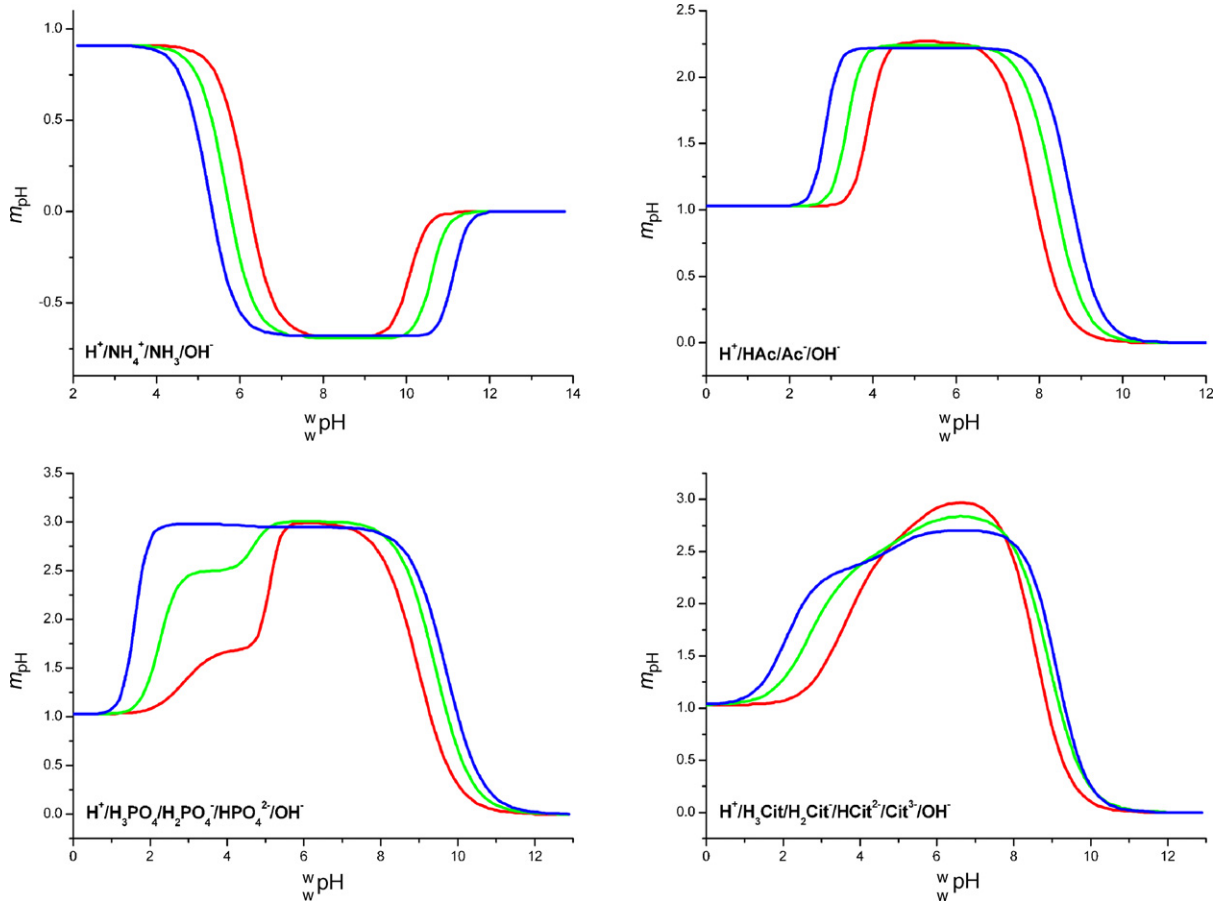

Fig. 3. Variation of the m_{pH} slope of Eqs. (6) and (7) with the initial aqueous pH of the buffer before adding the organic modifier (${}^{w}_{w}$pH). Buffer aqueous concentrations at 0.001 mol L^{-1} (inner line), 0.01 mol L^{-1} (center line) and 0.1 mol L^{-1} (outer line).

The average of this pH variation up to 80% of methanol is 0.00 ± 0.03 units, because when adding methanol to water the increasing in ΔpK_{ap} term is balanced by the log ϕ_{H_2O} term. Therefore, the m_{pH} slope for a strong base is considered to be 0.00.

The m_{pH} calculated values for the studied buffers and concentrations, together with the calculated a_0 and a_{n+1} parameters, were plotted against their corresponding initial aqueous ${}^{w}_{w}$pH value, and fitted to Eq. (8). Fig. 3 shows three of the most representative studied concentrations (0.001, 0.01 and 0.1 mol L^{-1}) for the studied systems. Table 4 shows the fitted s_i, a_i and b_i parameters corresponding to the studied buffered systems (acetic acid–acetate, citric acid–dihydrogencitrate–hydrogencitrate–citrate, phosphoric acid–dihydrogenphosphate–hydrogenphosphate, and 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 ($a_0 \approx 1.03$ Eq. (11)), 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 ($a_2 \approx 0.00$), b_1 corresponds to the ${}^{w}_{w}$pH value of the inflection point of the upward curve (an acetic acid solution alone) and $b_2 - b_1$ corresponds to the ${}^{w}_{w}$pH value of the inflection point of the downward curve (an acetate solution alone) in Fig. 3, H^+/HAc/Ac^-/OH^- system. s_i are the fitting parameters related to the sharpness of the transitions between the different a_i values.

In the citric acid system, due to the high number of polynomial variables (s_1, s_2, s_3, s_4, a_1, a_2, a_3, a_4, b_1, b_2, b_3, b_4; $a_0 \approx 1.03$ and $a_4 \approx 0.00$) and with the aim to avoid overparameterization, an averaged s value was taken for all the s_i ones, and b_i parameters were fixed before the iteration process to reach a better fitting. b_4 can be easily known because $b_4 - b_3$ agrees with the ${}^{w}_{w}$pH value corresponding to solutions of pure citrate. When hydrogencitrate is the 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 the citric acid. a_1 refers to the m_{pH} value of citric acid–dihydrogencitrate solutions, a_2 to dihydrogencitrate–hydrogencitrate and a_3 to hydrogencitrate–citrate (Table 4).

In the calculation of the pH involved in the phosphoric acid buffer system, we have only considered the contribution of the phosphoric acid, dihydrogenphosphate and hydrogenphosphate. Similarly to the citric acid system, $b_3 - b_2$ 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. On the other hand, a_1 refers to m_{pH} of phosphoric acid–dihydrogenphosphate solutions and a_2 to dihydrogenphosphate–hydrogenphosphate. For aqueous concentrations of the buffer above 0.08 mol L^{-1}, the system is clearly overparameterizated, and the results obtained in the polynomial fit are meaningless.

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

A linear tendency is observed in the graphical representations of the parameters s_i, a_i and b_i value against the logarithm of the

Table 4
Parameters of Eq. (8) for the calculation of the slope (m_{pH}, Eq. (7)) of the ${}^{s}_{w}$pH variation with the addition of methanol, at three representative initial aqueous buffer concentrations

Parameter	Acetic acid			Citric acid			Phosphoric acid			Ammonium		
	1 mM	10 mM	50 mM	1 mM	10 mM	50 mM	1 mM	10 mM	50 mM	1 mM	10 mM	50 mM
s_1	2.43	2.59	2.75	1.04	0.96	0.99	1.18	1.91	2.40	1.32	1.34	1.39
s_2	1.80	1.89	2.00	1.04	0.96	0.99	2.12	2.03	2.11	1.70	1.85	1.96
s_3	–	–	–	1.04	0.96	0.99	1.72	1.67	1.72	–	–	–
s_4	–	–	–	1.04	0.96	0.99	–	–	–	–	–	–
a_0	1.03	1.03	1.03	1.03	1.03	1.03	1.03	1.03	1.03	0.91	0.91	0.91
a_1	2.27	2.24	2.23	1.87	2.21	2.29	1.70	2.50	2.80	−0.69	−0.69	−0.68
a_2	0.00	0.00	0.00	2.59	2.51	2.43	3.00	3.01	2.96	0.00	0.00	0.00
a_3	–	–	–	2.93	2.92	2.78	0.00	0.00	0.00	–	–	–
a_4	–	–	–	0.00	0.00	0.00	–	–	–	–	–	–
b_1	3.90	3.38	3.02	3.24	2.62	2.24	2.90	2.25	1.80	6.16	5.68	5.37
b_2	10.37	9.89	9.55	7.31	6.49	6.00	8.99	7.09	5.85	17.12	17.65	17.98
b_3	–	–	–	12.79	11.78	11.06	15.88	13.98	12.45	–	–	–
b_4	–	–	–	21.38	20.70	20.13	–	–	–	–	–	–
N	45	45	45	132	132	132	88	88	88	45	45	45
SD	0.014	0.011	0.006	0.031	0.029	0.033	0.011	0.011	0.014	0.007	0.005	0.002
r^2	0.998	0.998	0.999	0.996	0.994	0.990	1.000	0.999	0.999	0.998	0.999	1.000
F	4192	6623	18518	10218	7707	4166	35735	21743	9944	6494	13036	61704

Table 5
Linear variation of the s_i, a_i and b_i parameters for the acetic acid–acetate buffer system depending on the aqueous buffer concentration, c_T ($0.001 < c_T < 0.1$ mol L^{-1})

Parameter	Acetic acid–acetate		
	Equation	N	SD
s_1	$0.22 \log c_T + 3.07$	14	0.055
s_2	$0.13 \log c_T + 2.19$	14	0.029
a_0	1.03	–	–
a_1	$-0.03 \log c_T + 2.18$	14	0.010
a_2	0.00	–	–
b_1	$-0.51 \log c_T + 2.35$	14	0.004
b_2	$-0.50 \log c_T + 8.86$	14	0.051

Table 6
Linear variation of the s_i, a_i and b_i parameters for the citric acid–dihydrogencitrate–hydrogencitrate–citrate buffer system depending on the aqueous buffer concentration, c_T ($0.001 < c_T < 0.1$ mol L^{-1})

Parameter	Citric acid–dihydrogencitrate–hydrogencitrate–citrate		
	Equation	N	SD
s	$0.03 \log c_T + 1.05$	14	0.047
a_0	1.03	–	–
a_1	$0.18 \log c_T + 2.52$	14	0.047
a_2	$-0.10 \log c_T + 2.30$	14	0.006
a_3	$-0.15 \log c_T + 2.57$	14	0.048
a_4	0.00	–	–
b_1	$-0.57 \log c_T + 1.51$	14	0.015
b_2	$-0.73 \log c_T + 5.05$	14	0.024
b_3	$-1.02 \log c_T + 9.73$	14	0.012
b_4	$-0.76 \log c_T + 19.13$	14	0.029

$s_1 = s_2 = s_3 = s_4 = s$.

aqueous concentration of the buffer ($\log c_T$), before adding the organic modifier. For each buffer system, the results of the linear regression are shown in Tables 5–8. This logarithmic approximation is more reasonable than the direct fitting to the concentration values, because it is well known that the solution pH is directly related to the logarithm of the concentration of the acid–base species present in the medium.

Table 7
Linear variation of the s_i, a_i and b_i parameters for the phosphoric acid–dihydrogenphosphate–hydrogenphosphate buffer system depending on the aqueous buffer concentration, c_T ($0.001 < c_T < 0.1$ mol L^{-1})

Parameter	Phosphoric acid–dihydrogenphosphate–hydrogenphosphate		
	Equation	N	SD
s_1	$0.73 \log c_T + 3.38$	12	0.027
s_2	$0.02 \log c_T + 2.11$	12	0.041
s_3	$0.02 \log c_T + 1.73$	12	0.025
a_0	1.03	–	
a_1	$0.57 \log c_T + 3.55$	12	0.076
a_2	$-0.00 \log c_T + 2.91$	12	0.014
a_3	0.00	–	–
b_1	$-0.64 \log c_T + 0.97$	12	0.005
b_2	$-1.89 \log c_T + 3.32$	12	0.063
b_3	$-2.12 \log c_T + 9.64$	12	0.110

Table 8
Linear variation of the s_i, a_i and b_i parameters for the ammonium–ammonia buffer system depending on the aqueous buffer concentration, c_T ($0.001 < c_T < 0.1$ mol L^{-1})

Parameter	Ammonium–ammonia		
	Equation	N	SD
s_1	$0.05 \log c_T + 1.45$	14	0.034
s_2	$0.16 \log c_T + 2.18$	14	0.019
a_0	0.91	–	–
a_1	$0.01 \log c_T - 0.67$	14	0.003
a_2	0.00	–	–
b_1	$-0.45 \log c_T + 4.79$	14	0.012
b_2	$0.53 \log c_T + 18.68$	14	0.126

4.3. Experimental evaluation of the model

In order to evaluate the accuracy of the model in the estimation of the pH variation of buffers with the mobile phase composition, several buffers at different composition, concentration and initial aqueous pH have been prepared and their ${}^{s}_{w}$pH values measured. To calculate the pH variation, we first determine the parameters s_i, a_i and b_i as a function of the aqueous buffer concentration (Tables 5–8). This model is suitable for aqueous buffer concentrations (i.e., before adding the organic modifier) between 0.001 and 0.1 mol L^{-1}. Then, when these parameters are fixed, the m_{pH} value can be estimated through Eq. (8) for each initial ${}^{w}_{w}$pH value. Finally, through the estimated value of m_{pH}, we can estimate the ${}^{s}_{w}$pH value corresponding to any methanol–aqueous buffer mobile phase up to 80% (v/v) Eq. (7), and compare it with the experimental value.

Fig. 4 represents the estimated ${}^{s}_{w}$pH values against the experimental ${}^{s}_{w}$pH values for all studied buffers at three different initial aqueous concentrations: 0.001, 0.01 and 0.01 mol L^{-1}. There is a good agreement between these pH values and the expected straight line of unitary slope and null intercept, especially for the two higher concentrations. Table 9 shows the statistics of the linear regressions (considering null intercept) for the three representative concentrations depicted in Fig. 4 for the studied buffers. Except for the lowest concentration in case of ammonium and phosphoric acid systems, the standard deviation of the linear fittings is lower than 0.1, and the standard deviation for the slope of these fittings is not higher than 0.002. In all cases, the slope of the linear regressions is very close to the theoretical value of 1, which means a very good correspondence between the estimated and the measured pH values.

In the ammonium–ammonia buffer, especially at low concentration, the correspondence between both pH values are not as good as expected. In fact, the measured slope (m_{pH}) of the series is lower than the corresponding estimated one. It means that the measured solution is more acidic than expected, possibly due to the low concentration of the buffer and the volatility of ammonia.

In the phosphoric acid buffer system, a slight dispersion in high methanol mixtures and at low buffer concentration is observed, especially between ${}^{s}_{w}$pH 6 and 8. We attribute this dispersion to the poor buffer capacity at high methanol contents, which as shown in Fig. 1 is really very low.

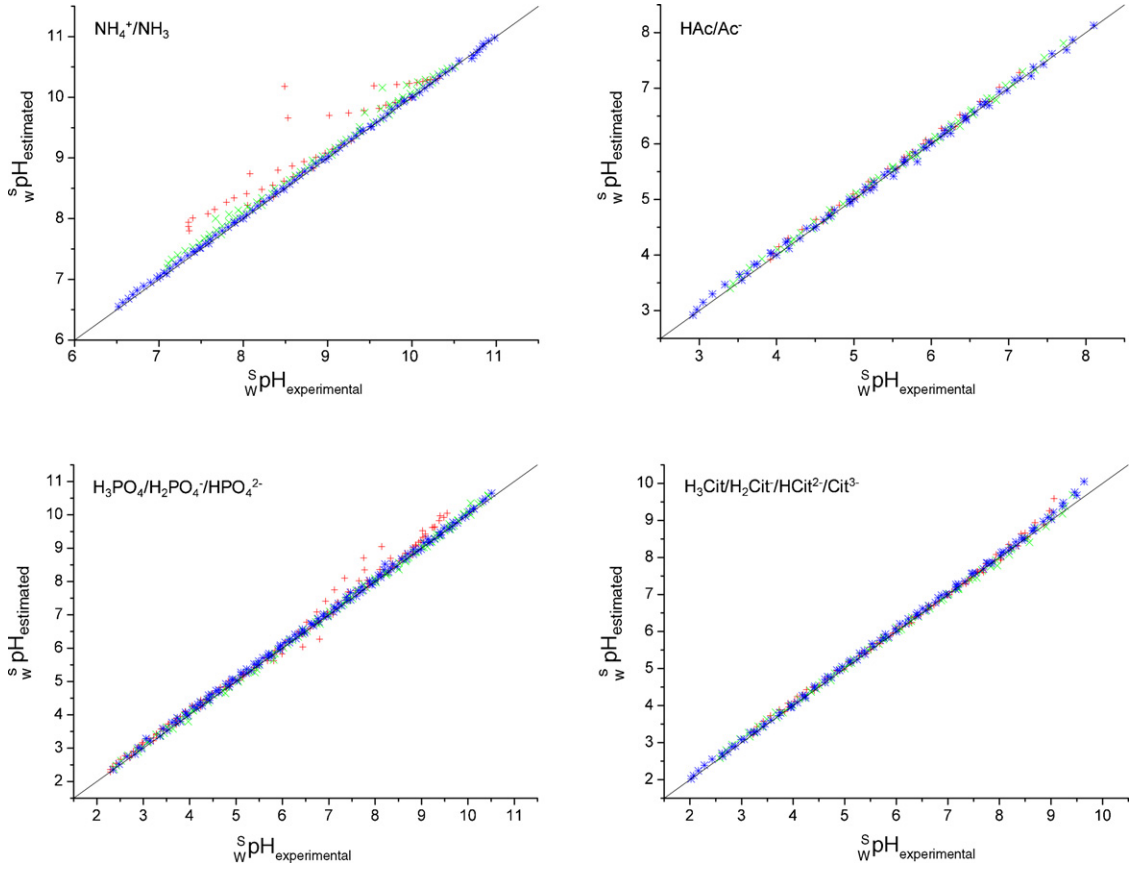

Fig. 4. Estimated $^{s}_{w}$pH values vs. experimental $^{s}_{w}$pH values plot. Straight line of unitary slope and null intercept is also give. Symbols for initial aqueous concentration: (+) 0.001 mol L^{-1}, (×) 0.01 mol L^{-1} and (*) 0.1 mol L^{-1}.

4.4. Estimation of the degree of ionisation and variation on chromatographic retention of solutes

Hydrophobicity and ionisation degree are the main parameters responsible for retention of acid–base analytes in RP-HPLC [2–4,7,10,39–44]. Whereas the hydrophobicity of a substance is a property inherent to the own nature of the analyte, the degree of ionisation depends on both analyte dissociation constant and mobile phase pH. As a general rule for analytes of similar hydrophobicity, the higher the degree of ionisation, the lower the retention.

For a compound that has a unique acid–base equilibrium ($HA^{z} - A^{z-1}$), ruled by an acidity constant (K_a), its ionisation degree (α), i.e. the mole fraction of the ionised species, can be

Table 9
Slope and statistics of the linear regressions (considering null origin ordinate) that analyze the correspondence between the estimated and the experimental $^{s}_{w}$pH values

Buffering system	*r*	SD	Slope	*F*	*N*
$HAc–Ac^{-}$					
0.001 mol L^{-1}	1.0000	0.053	1.0145 ± 0.0016	398836	36
0.01 mol L^{-1}	1.0000	0.048	1.0086 ± 0.0012	748213	54
0.1 mol L^{-1}	0.9999	0.058	1.0043 ± 0.0012	689879	72
$H_3Cit–H_2Cit^{-}–HCit^{2-}–Cit^{3-}$					
0.001 mol L^{-1}	0.9999	0.096	1.0081 ± 0.0016	373759	81
0.01 mol L^{-1}	0.9999	0.072	1.0017 ± 0.0013	595163	81
0.1 mol L^{-1}	1.0000	0.062	1.0114 ± 0.0010	1042602	99
$H_3PO_4–H_2PO_4^{-}–HPO_4^{2-}$					
0.001 mol L^{-1}	0.9996	0.192	1.0248 ± 0.0027	147407	124
0.01 mol L^{-1}	1.0000	0.069	1.0039 ± 0.0009	1359558	126
0.1 mol L^{-1}	0.9999	0.075	1.0093 ± 0.0010	1117547	128
$NH_4^{+}–NH_3$					
0.001 mol L^{-1}	0.9994	0.334	1.0339 ± 0.0056	34032	45
0.01 mol L^{-1}	1.0000	0.089	1.0113 ± 0.0012	747880	71
0.1 mol L^{-1}	1.0000	0.027	1.0027 ± 0.0003	8596425	81

calculated by:

$$\alpha_A = \frac{[A^{z-1}]}{[HA^z] + [A^{z-1}]} = \frac{1}{1 + 10^{pK_a - pH}} \quad (14)$$

or

$$\alpha_{HA} = \frac{[HA^z]}{[HA^z] + [A^{z-1}]} = \frac{1}{1 + 10^{pH - pK_a}} \quad (15)$$

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 should be ${}^{s}_{s}$pH and ${}^{s}_{s}$pK_a. However, we can use ${}^{s}_{w}$pH and ${}^{s}_{w}$pK_a values because ${}^{s}_{s}\text{pH} - {}^{s}_{s}\text{p}K_a = {}^{s}_{w}\text{pH} - {}^{s}_{w}\text{p}K_a$ since ${}^{s}_{w}\text{pH} - {}^{s}_{s}\text{pH} = {}^{s}_{w}\text{p}K_a - {}^{s}_{s}\text{p}K_a = \delta$.

With the addition of methanol, both pH of the hydroorganic mobile phase and pK_a of the analyte will change, and also the ionisation degree of the analyte. According to Eqs. (6) and (7), the difference between the pH and pK_a values that determines the degree of ionisation (Eqs. (14) and (15)) can be calculated by means of:

$${}^{s}_{w}\text{pH} - {}^{s}_{w}\text{p}K_a = \left({}^{w}_{w}\text{pH} - {}^{w}_{w}\text{p}K_a\right) + \left(m_{pH}\phi_{MeOH}^{d_{pH}} - m_{pK_a}\phi_{MeOH}^{d_{pK}}\right) \quad (16)$$

Eq. (8) and the calculated parameters from Tables 5–8 allow the estimation of the m_{pH} values of the buffers studied in the present work. As discussed before, d_{pH} is taken equal to 5/4 for neutral and anionic buffering acids, and to 1 for cationic acids. For the purpose of a qualitative discussion, d_{pK} values of neutral and cationic analytes can be considered close to the d_{pH} values corresponding to neutral and cationic buffering acids. Therefore, in the case of a neutral analyte in a neutral or anionic buffer, the second term in Eq. (16) can be rewritten as $(m_{pH} - m_{pK})\phi_{MeOH}^{5/4}$. Both m_{pH} and m_{pK} are positive in this kind of system, but depending on the value of these slopes the ionisation degree of the analyte (α_A, Eq. (14)) will increase if $m_{pH} > m_{pK}$ or decrease if $m_{pH} < m_{pK}$ when the methanol contents in the mobile phase increases. The same consideration is useful in the case of a cationic analyte (neutral base) in a cationic buffering system (e.g., NH_4^+–NH_3), when both slopes are negative, and the last term in Eq. (16) can be expressed as $(m_{pH} - m_{pK})\phi_{MeOH}$. Now the ionisation degree (α_{HA}, Eq. (15)) will decrease if $m_{pH} > m_{pK}$ or will increase if $m_{pH} < m_{pK}$ with the addition of methanol to prepare the mobile phase. In the rest of cases the simplification of Eq. (16) is not straightforward but qualitatively it is clear that a cationic analyte of pK_a close to the pH of the buffer will show a large decrease in its ionisation degree (α_{HA}) when methanol is added to an aqueous neutral or anionic buffer because $m_{pH} > 0 > m_{pK}$. Likewise, a neutral analyte will also decrease its ionisation in a cationic mobile phase because $m_{pH} < 0 < m_{pK}$.

In relation to these discussions, a representative example of the variation of ionisation degree of the analyte with the methanol fraction for different buffers is discussed here. We consider two different buffering systems of ${}^{w}_{w}$pH 8 and initial aqueous concentration of 0.01 mol L^{-1} prepared from a dihydrogenphosphate/hydrogenphosphate buffer and an ammonium/ammonia one. The m_{pH} values of these two buffers are 2.85 and −0.69, respectively. We also consider the ionisation of the following compounds of pK_a relatively close to 8 (with their corresponding ${}^{w}_{w}$pK_a values in brackets): 4-nitrophenol (7.15), 2-nitrophenol (7.23), 2,4,6-trimethylpyridine (7.43), 3-nitrophenol (8.36), 2-chlorophenol (8.56), *N*,*N*-dimethylbenzylamine (8.91), and 3-bromophenol (9.03). All pK_a values were taken from ref. [45], except the one corresponding to the *N*,*N*-dimethylbenzylamine [13]. The calculated [33] m_{pK} values are 1.74, 1.75, −1.88, 1.90, 1.93, −1.03, and 1.99, respectively. When increasing the methanol fraction all phenols are more ionized in the phosphate buffer than in the ammonia one. Clearly, the pK_a of phenols (neutral acids) increases with the addition of the organic modifier ($m_{pK} > 0$), and so does the pH of the phosphate buffer ($m_{pH} > 0$), whereas the pH of the ammonia buffer decreases ($m_{pH} < 0$). Therefore, phenols in ammonia buffer will decrease their ionisation degree because their pK_a values increases whereas the pH decreases when adding methanol. However, in phosphate buffer the ionisation increases because the pH of the buffer increases to a larger degree than the pK_a of the phenols ($m_{pH} > m_{pK} > 0$). The opposite effect can be seen in case of *N*,*N*-dimethylbenzylamine (neutral base). In the phosphate buffer the ionisation degree is reduced when adding methanol because the pH increases ($m_{pH} > 0$) whereas the pK_a decreases ($m_{pK} < 0$), and in the ammonia buffer there is only a small change in ionisation because both the pH and pK_a decrease in a similar way. Only the 2,4,6-trimethylpyridine (neutral base) has a similar ionisation profile in both buffering systems, because in the phosphate buffer $m_{pK} < 0 < m_{pH}$ and in the ammonia buffer $m_{pK} < m_{pH} < 0$.

If we want to calculate accurately the ionisation degree of the analyte in a buffered mobile phase composition, we must take into account the pK_a variation of the analyte as a function of the mobile phase composition. Literature [33,46] proposes equations that allow accurate calculation of the ${}^{s}_{s}$pK_a values of any member of the most common families of compounds (phenols, carboxylic acids, amines and pyridine derivatives) at any methanol–water mixture from the pK_a value of the compound in water (${}^{w}_{w}$pK_a). The ${}^{s}_{s}$pK_a of an analyte can be linearly related to their corresponding aqueous value (${}^{w}_{w}$pK_a) through the equation:

$${}^{s}_{s}\text{p}K_a = a_s\,{}^{w}_{w}\text{p}K_a + b_s \quad (17)$$

with

$$a_s = \frac{1 + a_{s1}\phi_{MeOH} + a_{s2}\phi_{MeOH}^2}{1 + a_{s3}\phi_{MeOH} + a_{s4}\phi_{MeOH}^2} \quad (18)$$

$$b_s = \frac{b_{s1}\phi_{MeOH} + b_{s2}\phi_{MeOH}^2}{1 + b_{s3}\phi_{MeOH} + b_{s4}\phi_{MeOH}^2} \quad (19)$$

where a_{s1}, a_{s2}, a_{s3}, a_{s4}, b_{s1}, b_{s2}, b_{s3} and b_{s4} are fitting parameters constant for all acids of the same family at all methanol–water compositions. These parameters are reported on Tables 10 and 11. These equations and Eq. (2) have been used to estimate the ${}^{s}_{w}$pK_a values of the analytes of the previous example for all the studied methanol–water compositions. These ${}^{s}_{w}$pK_a values, together with the corresponding estimated ${}^{s}_{w}$pH values of the buffering system, are used in Eqs. (14) and

Table 10
Parameters for the prediction of the slope Eq. (18) of the linear correlation between ${}^{s}_{s}pK_a$ values in methanol–water and ${}^{w}_{w}pK_a$ in water Eq. (17) [33]

Family of compounds	a_{s1}	a_{s2}	a_{s3}	a_{s4}
Phenols	−0.656	−0.030	−0.844	0.133
Aliphatic carboxylic acids	−1.406	0.680	−1.551	0.827
Aromatic carboxylic acids				
With orto-substituents	−1.189	0.190	−1.424	0.425
Without orto-substituents	−1.101	0.103	−1.516	0.518
Amines	−0.476	0.209	−0.400	0.158
Pyridines	2.617	0.000	2.809	0.000

Table 11
Parameters for the prediction of the intercept Eq. (19) of the linear correlation between ${}^{s}_{s}pK_a$ values in methanol–water and ${}^{w}_{w}pK_a$ in water Eq. (17) [33]

Family of compounds	b_{s1}	b_{s2}	b_{s3}	b_{s4}
Phenols	−0.454	0.866	−0.017	−0.865
Aliphatic carboxylic acids	1.034	−0.898	−1.250	0.277
Aromatic carboxylic acids				
With orto-substituents	0.449	−0.429	−1.674	0.677
Without orto-substituents	−0.178	0.187	−1.699	0.702
Amines	−0.458	0.477	−1.674	0.690
Pyridines	−1.733	1.763	−1.214	0.272

(15) to accurately calculate the ionisation degrees of the former example at several methanol–water mixtures. Fig. 5 shows the variation of the ionisation of these analytes as a function of the organic fraction. The results obtained fully agree with the above qualitative discussion.

This different behaviour of ionisable analytes when adding methanol to aqueous buffers of the same pH but prepared from different buffer components, may well produce relevant changes in the selectivity of RP-HPLC analysis. This phenomenon is clearly shown in Fig. 6. The same ionisable analytes studied before, namely *N*,*N*-dimethylbenzylamine, 4-nitrophenol, 3-nitrophenol, 2-chlorophenol, 2-nitrophenol, 2,4,6-trimethylpyridine, and 3-bromophenol, were eluted in a mobile phase with 60% of methanol (v/v) prepared from aqueous $H_2PO_4^-$–HPO_4^{2-} or NH_4^+–NH_3 buffers of concentration 0.01 mol L^{-1} and ${}^{w}_{w}pH = 8.00$. Just by looking the chromatograms, it is clear that better separations are achieved using the phosphate mobile phase. Although both mobile phases have the same content of methanol, aqueous pH and buffer concentration, the retention times of the analytes are dramatically different. These retention differences of the studied analytes in both buffers are consistent with their ionisation degrees, shown in Fig. 5, and with their hydrophobicity. As a well known general rule in reversed phase HPLC, we can consider that the higher the compound ionisation and the lower hydrophobicity, the lower the retention time. Taken into account the above considerations and by inspection of Fig. 5, we are able to interpret the chromatograms of Fig. 6 as follows.

The phenols increase their ionisation, and thus decrease retention, when methanol is added to the phosphate buffer because the pH of the buffer increases more than the pK_a of the phenols, but ionisation decreases and therefore, retention increases with the ammonia buffer because the pH of the cationic buffer decreases. The latter effect is more noticeable for 4- and 2-nitrophenol because they have an aqueous pK_a value (7.15 and 7.23, respectively) close to but lower than the aqueous pH of the ammonia buffer (8.00). Thus, the combined effect of increasing analyte pK_a and decreasing buffer pH reverses the pK_a–pH order. In 60% methanol and ammonia buffer, the pK_a of 4- and 2- nitrophenol is higher than the pH of the buffer and thus, ionisation is low and retention high.

N,*N*-dimethylbenzylamine shows the reversed trend than 2- and 4-nitrophenol. It has an aqueous pK_a of 8.91, higher than the aqueous pH of the buffers (8.00). When methanol up to 60% is added to the phosphate buffer its pH increases, whereas the pK_a of the *N*,*N*-dimethylbenzylamine (cationic acid) decreases and becomes much lower than the pH of the buffer. Ionisation decreases to almost 0 and chromatographic retention is high. The decrease of pH of the ammonia buffer (cationic buffer) is similar to the decrease of pK_a of the amine when methanol is added, and thus ionisation keeps high and retention low.

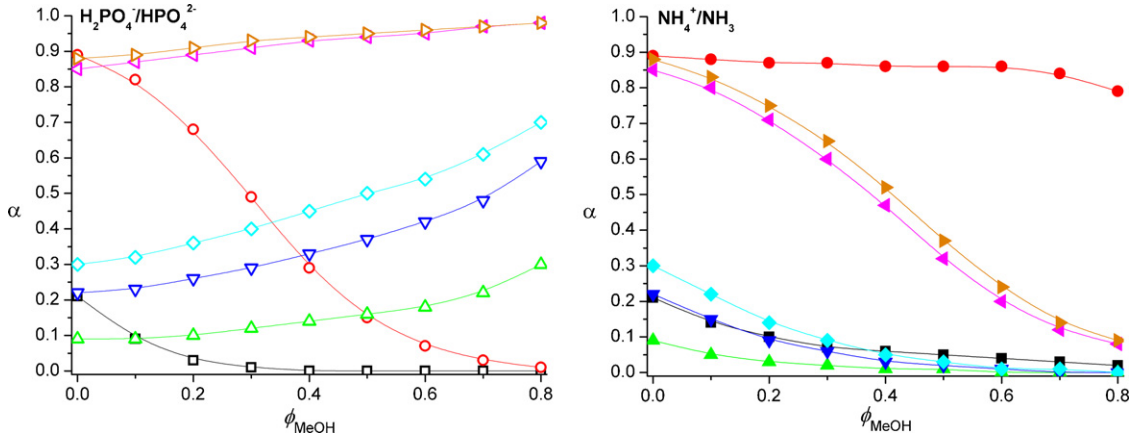

Fig. 5. Variation of the ionisation degree of acid–base compounds with the addition of methanol to $H_2PO_4^-$–HPO_4^{2-} and NH_4^+–NH_3 aqueous buffers of ${}^{w}_{w}pH$ 8 and concentration 0.01 mol L^{-1}. Legend: (■, □) 2,4,6-trimethylpyridine, (●,◯) *N*,*N*-dimethylbenzylamine, (▲, △) 3-bromophenol, (▼, ▽) 2-chlorophenol, (◀, ◁) 2-nitrophenol, (♦, ◊) 3-nitrophenol, and (▶, ▷) 4-nitrophenol.

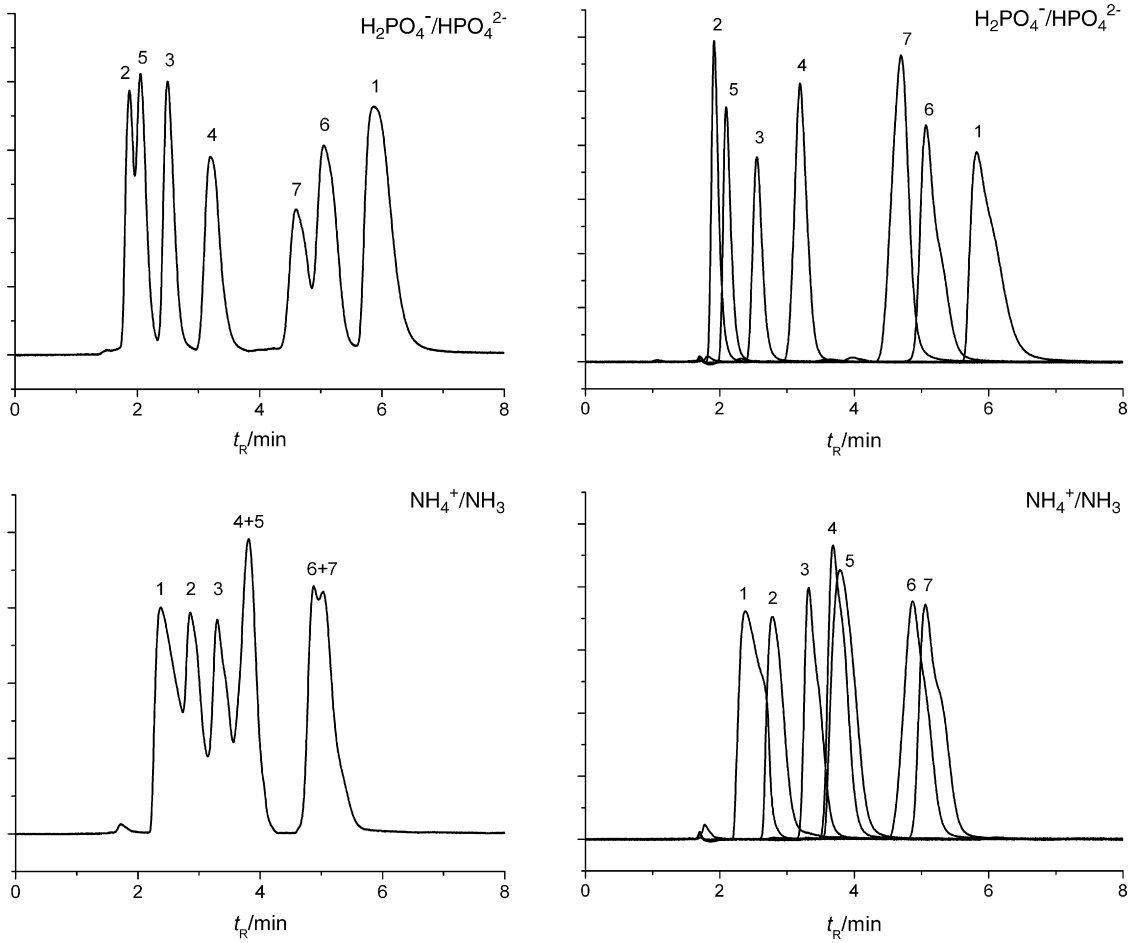

Fig. 6. Chromatograms of individual ionisable compounds and their corresponding eluted mixture in a 60% (v/v) methanol mobile phase prepared from $H_2PO_4^-$–HPO_4^{2-} and NH_4^+–NH_3· aqueous buffers of concentration 0.01 mol L^{-1} and $^{w}_{w}$pH = 8.00. Compounds: (1) *N*,*N*-dimethylbenzylamine; (2) 4-nitrophenol; (3) 3-nitrophenol; (4) 2-chlorophenol; (5) 2-nitrophenol; (6) 2,4,6-trimethylpyridine; (7) 3-bromophenol.

2,4,6-trimethylpyridine (neutral base and cationic acid) has an aqueous pK_a (7.43) lower than the pH of the buffers (8.00). Ionisation in water is low (about 20%). The addition of methanol decreases its pK_a more than the pH of the ammonia buffer, and thus ionisation at 60% of methanol is even lower (about 5%). The pH of the phosphate buffer increases when methanol is added to this buffer and since pK_a of the pyridine decreases, ionisation at 60% of methanol is very low (less than 1%). Therefore, the compound is very poorly ionised in both buffers, and its retention is quite similar and high.

5. Conclusions

Selectivity in reversed phase high performance liquid chromatography (RP-HPLC) separations of ionisable analytes depends on their hydrophobicity and on their ionisation degree in the particular mobile phase used. Hydrophobicity is inherent to the analyte nature, and therefore, it cannot be modified by the chromatographer. But the analyte ionisation degree clearly depends on the pK_a of the compound and on the pH of the mobile phase, the latter can be properly tuned to the desired value with an appropriate buffer composition. However, the variation of the analyte pK_a and buffer pH upon addition of the organic modifier the aqueous buffer must be taken into account to obtain reliable predictions. In the present work, a method for the accurate pH estimation in methanol–aqueous buffer mobile phases has been successfully developed. For the studied buffers, this method allows us to calculate the pH in any methanol–aqueous buffer mobile phase up to 80% in volume of organic modifier, and considering an initial aqueous concentration (before adding the methanol) between 0.001 and 0.1 mol L^{-1}. The selected buffers are the most commonly used in RP-HPLC, and the ones which cover a large range of useful pH values: acetic acid–acetate, phosphoric acid–dihydrogenphosphate–hydrogenphosphate, citric acid–dihydrogencitrate–hydrogencitrate–citrate and ammonium–ammonia. In addition, a previous model that allows the estimation of pK_a values of analytes belonging to the most common families of compounds (phenols, carboxylic acids, amines and pyridine derivatives) was adapted in terms of the present work. Then, with the estimation of both the pK_a of the analyte and the pH of the particular mobile phase, can be easily calculated the analyte ionisation degree, which plays an important role in the chromatographic retention of acid–base compounds.

Acknowledgements

We thank financial support from the MCYT of the Spanish Government and FEDER of EU (projects CTQ2004-00633/BQU and CTQ2004-00965/BQU).

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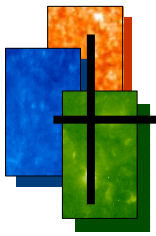
ARTICLE IV

On the effect of organic solvent composition on the pH of buffered HPLC mobile phases and the pK_a of analytes (Review)



X. Subirats, M. Rosés and E. Bosch

Sep. Purif. Rev. Accepted (2007)

Separation & Purification Reviews (accepted)

ISSN 1542-2119 print/1542-2127 online

Review

On the effect of organic solvent composition on the pH of buffered HPLC mobile phases and the pK_a of analytes

Xavier Subirats, Martí Rosés and Elisabeth Bosch

Departament de Química Analítica, Universitat de Barcelona

Martí i Franquès 1-11, E-08028 Barcelona, Spain

Abstract: A review about the analyte pK_a and buffer pH variations in RP-HPLC mobile phases with the changes in the organic modifier content (acetonitrile or methanol) is presented. A model to accurately predict the pH of particular mobile phases for several commonly used buffers (acetic, citric and phosphoric acid and ammonia systems) in acetonitrile-water and methanol-water mixtures is described. Linear relationships are also presented for several families of acid-base compounds (aromatic and aliphatic carboxylic acids, phenols, amines and pyridines) to estimate pK_a values of analytes in methanol-water and acetonitrile-water from their corresponding aqueous pK_a. From both, the estimated pH of the mobile phase and the estimated pK_a of acid-base analytes, it is possible to predict their degree of ionisation and, therefore, the analyte chromatographic retention.

Keywords: Mobile phase composition; methanol–water mixtures; acetonitrile-water mixtures; pH; pK_a; buffers; chromatographic retention; ionisation degree.

Address correspondence to Elisabeth Bosch, Departament de Química Analítica, Universitat de Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain. E-mail: e.bosch@ub.edu

1. INTRODUCTION

The use of buffered mobile phases in liquid chromatography is very common for separation of analytes with acid-base properties. For monoprotic acids there is a well known relationship between the retention factor (k), the pK_a of the analyte at the working ionic strength and the pH of the mobile phase (1):

$$k = \frac{k_{HA} + k_A 10^{pH-pK_a}}{1+10^{pH-pK_a}} \quad [1]$$

where k_{HA} and k_A are the retention factors obtained when the analyte is completely in its acidic or basic form, respectively. Eq. 1 defines a sigmoidal plot for the retention as a function of the pH of the mobile phase, with a pronounced jump around the analyte pK_a. Therefore, slight variations in the pH of the mobile phase at pH near the analyte pK_a result in significant changes in retention and, thus, two similar analytes with small differences in their pK_a values can be successfully separated by a proper control of mobile phase pH. Expressions equivalent to Eq. 1 can be obtained if retention is measured in retention time (t_R) or adjusted retention time ($t_R' = t_R - t_M$) if the holdup time (t_M) is independent of the buffer (2-4). If the analyte has more than one acid-base equilibria more complex expressions should be considered (1, 4, 5).

When an organic modifier is added to an aqueous buffer to prepare the mobile phase there is a change in the pK_a of the buffering acid and in the autoprotolysis constant of the solvent, which is responsible of the pH range of the pH scale. Consequently there is a variation in the pH of the hydroorganic mixture in relation to the aqueous pH of the buffer. Moreover, the pK_a of the analyte also changes. These variations affect the ionisation degree of acid-base analytes and, therefore, they may produce important changes in chromatographic retention and selectivity. The sign and extent of the pH variation when adding an organic solvent to an aqueous buffer depend not only on the organic fraction of the mixture, the aqueous pH and buffer concentration, but also on the nature of the buffering system (3, 6-12). The example given in Fig. 1 illustrates these statements. The order of elution of the ionisable analytes is clearly different, even though in both cases we have mobile phases containing a 60% of methanol (v/v) prepared from aqueous buffers of the same pH (8.00) and concentration (0.01 mol·L^{-1}). In this instance, the difference lies in the nature of the buffer: in one case it is ammonium/ammonia and in the other it is dihydrogenphosphate

/hydrogenphosphate. Obviously the acid-base constant of the analytes in the particular mobile phase plays an important role, but in contrast to the mobile phase pH, it only depends on the organic solvent fraction in the mobile phase. The effect of both the pH and the pK_a on ionisation degree and therefore on retention times in HPLC has been already extensively reported (3, 6-26). In this review we present the models developed in our research group to estimate the pH values of the most commonly used buffering systems in RP-HPLC at any fraction of organic solvent in a particular acetonitrile- and methanol-water medium up to 60% and 80% (v/v), respectively. The model we proposed to estimate the pK_a of a compound in a particular methanol-water or acetonitrile-water from its corresponding aqueous pK_a is also presented.

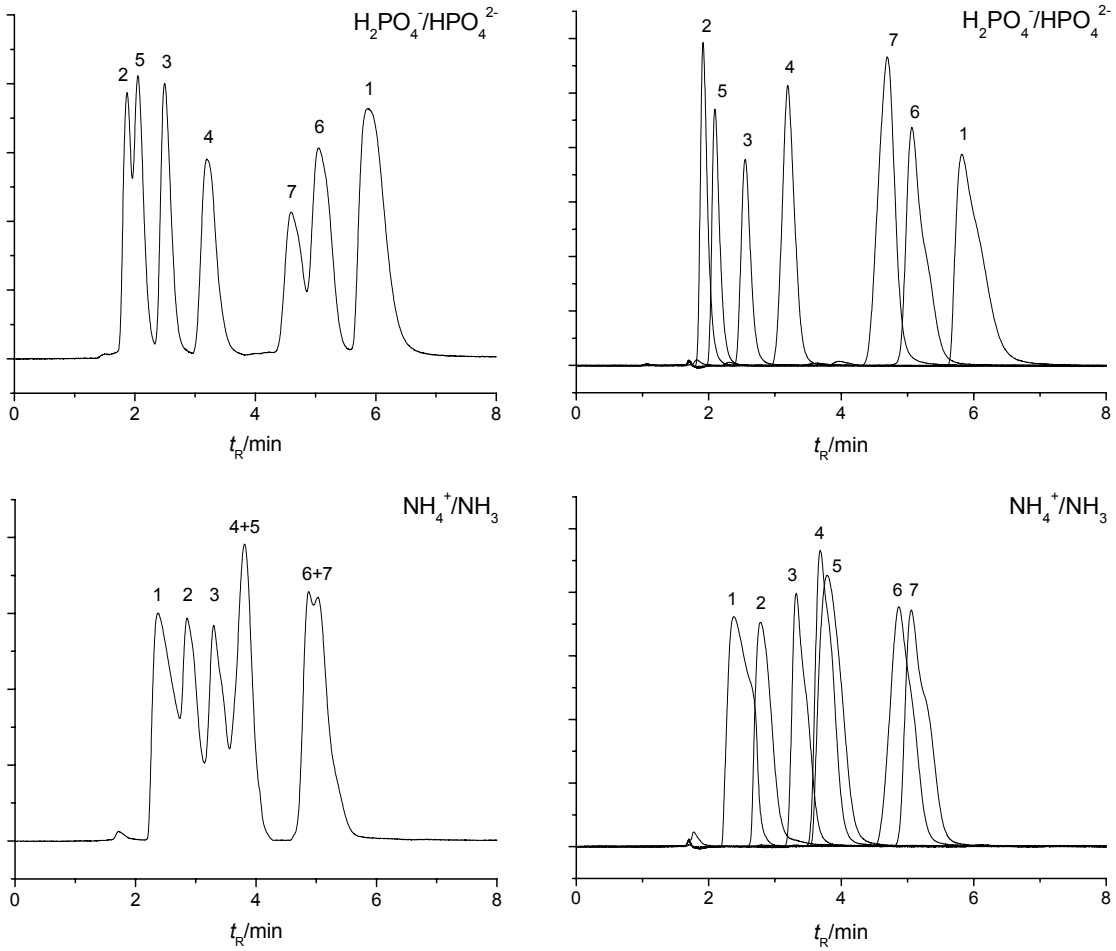


Figure 1. Chromatograms of individual acid-base compounds and their corresponding eluted mixture in a 60% (v/v) methanol mobile phase prepared from $H_2PO_4^-$-HPO_4^{2-} and NH_4^+-NH_3 aqueous buffers of concentration 0.01 mol·L^{-1} and pH=8.00. Compounds: (1) *N,N*-dimethylbenzylamine; (2) 4-nitrophenol; (3) 3-nitrophenol; (4) 2-chlorophenol; (5) 2-nitrophenol; (6) 2,4,6-trimethylpyridine; (7) 3-bromophenol. From ref. (44), with permission, © 2007 Elsevier.

2. pH DEFINITION IN ORGANIC SOLVENT-WATER MIXTURES

Looking for a friendly way to write small hydrogen ion concentrations, the pH definition was first introduced by Sørensen (27) in 1909 in terms of the negative decimal logarithm of the hydrogen ion concentration. Some years later Sørensen found that the electrodes used to measure the pH responded to hydrogen ion activity (a_H) instead of concentration, so pH was redefined as (28):

$$pH = -\log a_H \qquad [2]$$

Although activity and pH are dimensionless quantities, activity must be referred to a particular concentration scale. In fact, activity can be related to concentration through an activity coefficient (γ). This means that the same solution may have different pH values depending on the scale in which hydrogen ion concentration is measured. In analytical chemistry practice, including chromatography, the pH definition in the molarity scale (moles of hydrogen ion per litre of solvent, $mol \cdot L^{-1}$) (29, 30) is commonly used because of its simplicity for preparation of solutions. The pH definition of Eq. 2 is only notional because it involves single ion activity, which is immeasurable (29-35). Therefore an operational definition of pH was established. The pH of a solution is obtained by comparison of the electromotive force of a sample solution in an appropriate potentiometric cell in relation to the electromotive force of standard reference solutions of known pH in the same cell (29-41). In analytical practice pH is commonly measured using a glass electrode combined with a reference electrode (very often silver-silver chloride). Usually the reference electrode contains a highly concentrated KCl solution. In this solution the cation and the anion are equitransferent (i.e. they diffuse at nearly the same rate), and thus the liquid junction potential (i.e. a potential difference formed at the boundary between two different compositions) between the reference electrode and the sample or standard calibration solutions is minimized. The temperature of calibration standards and sample solutions should be at least roughly controlled, because of the dependence of the glass electrode potential with the temperature.

Three different procedures are used to measure the pH of hydroorganic mobile phases in HPLC (3, 6-12). A typical one consists on calibrating the electrode systems with commercial aqueous standard buffers, and then measuring the pH of the aqueous buffer before mixing it with the organic modifier. This way the pH value is obtained in the ${}^{w}_{w}pH$ scale (19). In our

opinion this is not the best option because the pH of the solution changes after dilution of the aqueous buffer with the organic modifier. If the electrode system is calibrated with standard buffers prepared in the same solvent composition used as mobile phase and the pH is measured in this particular mobile phase composition, the ${}^{s}_{s}\mathrm{pH}$ value is obtained. Working in the ${}^{s}_{s}\mathrm{pH}$ scale requires a careful preparation and maintenance of the standard buffers and electrodes, and often these standards are not commercially available. Finally, when pH is measured in the hydroorganic mixture, but the electrode system is calibrated with aqueous buffers, the ${}^{s}_{w}\mathrm{pH}$ values are obtained. Notice that here the IUPAC nomenclature (15) has been used: the left hand superscript indicates the medium where the quantity is measured (*w* for water and *s* for hydroorganic mixture), and the subscript indicates the standard state medium (i.e. the solvent where activity coefficients are taken as equal to unity at infinite dilution), which means in practice, the solvent (*w* or *s*) in which electrode systems are calibrated. It has been widely reported that better results are obtained when the pH in the mobile phase is considered instead of the aqueous pH of the buffer (6-8, 12-14, 17, 23-26). ${}^{s}_{s}\mathrm{pH}$ can be easily converted to ${}^{s}_{w}\mathrm{pH}$ by means of δ parameter (9, 36, 37):

$$ {}^{s}_{w}\mathrm{pH} = {}^{s}_{s}\mathrm{pH} + \delta \qquad [3] $$

The δ term is a constant value for each mobile phase composition. It includes the primary medium effect and the difference between the liquid junction potential of the electrode system in the hydroorganic mobile phase and in water. The primary medium effect (related to the standard Gibbs energy change for the transfer of the H^+ ion from water to the non-aqueous or hydroorganic solvent at infinite dilution) depends only on the mobile phase solvent composition, but the liquid junction potential depends also on the particular electrode system, pH standards, and sample composition. 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. In practice, this requirement is fulfilled using a combination electrode containing a reference electrode with a concentrated KCl solution in water as a salt bridge. These δ values for methanol-water mixtures were studied by various authors (7, 9, 42, 43) and they can be estimated from the solvent composition through the empirical equation (9):

$$\delta = \frac{0.09\phi_{MeOH} - 0.11\phi_{MeOH}^2}{1 - 3.15\phi_{MeOH} + 3.51\phi_{MeOH}^2 - 1.35\phi_{MeOH}^3} \quad [4]$$

where ϕ_{MeOH} is the volume fraction of methanol in the hydroorganic mixture. δ values for acetonitrile-water mixtures up to 60% (v/v) of organic modifier can be also estimated from the solvent composition through the equation (4, 8):

$$\delta = \frac{-0.446\phi_{MeCN}^2}{1 - 1.316\phi_{MeCN} + 0.433\phi_{MeCN}^2} \quad [5]$$

The relationship between ${}^{s}_{s}\mathrm{pH}$ and ${}^{s}_{w}\mathrm{pH}$ depends on the organic solvent fraction in the mixture, whereas the difference between ${}^{w}_{w}\mathrm{pH}$ and ${}^{s}_{s}\mathrm{pH}$ (or ${}^{s}_{w}\mathrm{pH}$) depends not only on the mobile phase composition but also on the particular buffering solution employed. δ values are also useful to convert ${}^{s}_{w}\mathrm{p}K_a$ values to ${}^{s}_{s}\mathrm{p}K_a$, and ${}^{s}_{w}\mathrm{p}K_{ap}$ to ${}^{s}_{s}\mathrm{p}K_{ap}$, where p$K_a$ refers to the analyte acid-base constant and pK_{ap} to the autoprotolysis constant of the solvent (organic solvent-water mixture).

Then to obtain precise information about the pH of a particular mobile phase it is convenient to measure pH directly in the hydroorganic mixture, rather than in the aqueous buffer. When the measurement of pH in the mobile phase is not easy, e.g. in the case of highly automated HPLC experiments where independent reservoirs of buffer and organic solvent are pumped into and mixed within the apparatus, it may be very useful to estimate the pH variation for a particular buffer when the organic modifier is added.

3. pH VARIATION OF THE BUFFER WITH THE ADDITION OF ACETONITRILE OR METHANOL

It has been shown that when acetonitrile is added to an aqueous buffer, the pH variation can be considered linearly related to the volume fraction of the organic modifier (ϕ_{MeCN}) (18):

$${}^{s}_{w}\mathrm{pH} - {}^{w}_{w}\mathrm{pH} = m_{pH}\,\phi_{MeCN} \quad [6]$$

where m_{pH} is the proportionality coefficient for the pH change. A similar equation has been proposed to relate the pH variation with the volume fraction of methanol (ϕ_{MeOH}) (44):

$$ {}^{s}_{w}pH - {}^{w}_{w}pH = m_{pH}\,\phi_{MeOH}^{d_{pH}} \qquad [7] $$

The difference between Eqs. 6 and 7 is the d_{pH} parameter. This empirical parameter is assumed to be equal to 1 for cationic buffering acids ($BH^+ \rightleftarrows B + H^+$), and 5/4 for neutral ($HA \rightleftarrows H^+ + A^-$) and anionic buffering acids ($HA^{-z} \rightleftarrows H^+ + A^{-z-1}$). m_{pH} is a proportionality coefficient which depends on the particular buffering system used, and on the aqueous pH value and concentration of the buffer before adding the organic modifier. The variation of m_{pH} with the initial aqueous ${}^{w}_{w}pH$ of the buffer for acetonitrile and methanol-water mixtures can be described by means of Eq. 8 (18, 44):

$$ m_{pH} = \frac{a_0 + \sum_{i=1}^{n} a_i\, 10^{s_i\left(i\,{}^{w}_{w}pH - b_i\right)} + a_{n+1}\, 10^{s_i\left((n+1)\,{}^{w}_{w}pH - b_{n+1}\right)}}{1 + \sum_{i=1}^{n} 10^{s_i\left(i\,{}^{w}_{w}pH - b_i\right)} + 10^{s_i\left((n+1)\,{}^{w}_{w}pH - b_{n+1}\right)}} \qquad [8] $$

where 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. The (n+1) term predominates at very basic pH values (buffers with strong bases). 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. a_i values are associated to the pK_a variation of the buffer when adding the organic modifier and b_i values are related to the pK_a values of the corresponding acid-base pairs of the system. s_i are fitting parameters that account for the sharpness of the transitions (22) between the different pH zones buffered by the different acid-conjugate base pairs of the system. A linear tendency is observed in the graphical representations of the parameters s_i, a_i and b_i value against the logarithm of the aqueous concentration of the buffer (log c_T), before adding the organic modifier. These linear equations for ammonium and acetic, citric and phosphoric acid systems in acetonitrile and methanol-water mixtures are shown in Tables 1 and 2.

Tables 3 and 4 show calculated ${}^{s}_{w}\mathrm{pH}$ values in acetonitrile and methanol-aqueous buffer mixtures for the most commonly used buffering systems in RP-HPLC, in the pH range of good buffer capacity. The m_{pH} values have been calculated by means of Eq. 8, and the ${}^{s}_{w}\mathrm{pH}$ values through Eqs. 6 and 7 for acetonitrile and methanol, respectively.

Table 1. Linear variation of the s_i, a_i and b_i parameters in acetonitrile-water mixtures for some buffering systems depending on the aqueous buffer concentration, c_T ($0.001 < c_T < 0.1$ mol·L^{-1})

Parameter	Acetic acid system	Ammonia system
s_i	0.20 log c_T + 3.56	0.20 log c_T + 3.71
a_0	0.00	0.00
a_1	2.28	-0.60
a_2	1.81	1.81
b_1	-0.52 log c_T + 2.33	-0.45 log c_T + 4.84
b_2	-0.07 log c_T + 11.53	0.06 log c_T + 16.52
Parameter	Phosphoric acid system	Citric acid system
s_i	-0.04 log c_T + 1.99	0.29 log c_T + 2.59
a_0	0.00	0.00
a_1	0.53 log c_T + 2.40	0.14 log c_T + 1.63
a_2	-0.06 log c_T + 1.63	-0.06 log c_T + 1.56
a_3	1.81	-0.16 log c_T + 1.67
a_4	-	1.81
b_1	-0.69 log c_T + 0.93	-0.58 log c_T + 1.47
b_2	-0.97 log c_T + 5.16	-0.79 log c_T + 4.94
b_3	-0.61 log c_T + 15.34	-1.12 log c_T +9.53
b_4	-	-0.75 log c_T + 19.25

4. BUFFER CAPACITY

Buffer capacity (β) is a quantitative measurement of the buffer ability to keep pH constant. It can be calculated by means of the differential equation (36, 37):

$$\beta = \frac{\mathrm{d}\, c_b}{\mathrm{d}(\mathrm{pH})} = -\frac{\mathrm{d}\, c_a}{\mathrm{d}(\mathrm{pH})} \qquad [9]$$

where c_b and c_a are the concentrations of the buffering base and acid, respectively. Buffer capacity is, in rough terms, the strong base or strong acid amount (expressed in equivalents) required to produce one pH unit change in the buffer solution. For a weak acid-weak base

buffer, maximum buffer capacity of a protolyte occurs when the acid species concentration is equal to the concentration of its conjugate base. It means that the apex of buffer capacity is achieved when the pH of the solution is equal to the pK_a' (the pK_a value at the working ionic strength) of the buffering species. The addition of the organic solvent produces a shift of the maximum of buffer capacity towards higher ${}^{s}_{w}pH$ values for neutral or anionic acid buffers (acetic, citric and phosphoric buffering systems), but towards lower ${}^{s}_{w}pH$ values for the cationic acid buffer (ammonia system). These trends have been already explained in terms of electrostatic interactions that contribute to the pK_a values of the buffering species (45, 46). The acid-base constants reported in the literature are normally thermodynamic pK_a values, which are given for zero ionic strength. Table 5 shows calculated aqueous pH values of equimolar mixtures of acid/conjugate base for several buffers at different concentrations and, consequently, ionic strength. Each pH value is related to the maximum buffer capacity achievable in aqueous solutions. It is especially significant the pH variation in case of dihydrogenphosphate/hydrogenphosphate and hydrogencitrate/citrate due to the increase of the ionic strength with the concentration because of the high charge of the buffering species. For the rest of the buffers, no dramatical changes are observed. Fig. 2 shows the buffer capacity of commonly used buffering systems at several methanol-water compositions, and Fig. 3 reproduces the buffer capacity variation for acetonitrile as organic modifier. In both types of mixtures, the buffer capacity presents a similar profile. The buffer capacity decreases when the organic solvent is added to the aqueous buffer, due to the decrease of the buffer concentration on increasing the volume of the solution. The addition of the organic solvent 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-, $H_2Cit^-/HCit^{2-}$, $HCit^{2-}/Cit^{3-}$, $H_3PO_4/H_2PO_4^-$, $H_2PO_4^-/HPO_4^{2-}$…), and towards lower ${}^{s}_{w}pH$ values for cationic acid buffers (NH_4^+/NH_3…). Quantitative values of β are different in both figures, because of the different initial aqueous concentration of the buffers. As a well known rule, the higher the concentration of the buffer, the higher the buffer capacity. It is noteworthy a broad poorly buffered zone between the first and the second pK_a of the phosphoric system, around pH 5. It is also remarkable a wide range of excellent buffer capacity of the citric acid system from pH 3 to pH 7 (18, 22, 44). In this buffering system, the different extent in the variation of the three pK_a values when increasing the organic solvent fraction in the mixture is remarkable too. For example, in pure water the

difference between the first and the third pK_a value is about 3.3 units, whereas for methanol and acetonitrile at 60% this difference increases up to 3.7 pK_a units.

Table 2. Linear variation of the s_i, a_i and b_i parameters in methanol-water mixtures for some buffering systems depending on the aqueous buffer concentration, c_T ($0.001 < c_T < 0.1$ mol·L^{-1})

Parameter	Acetic acid system	Ammonia system
s_1	0.22 log c_T + 3.07	0.05 log c_T + 1.45
s_2	0.13 log c_T + 2.19	0.16 log c_T + 2.18
a_0	1.03	0.91
a_1	-0.03 log c_T + 2.18	0.01 log c_T - 0.67
a_2	0.00	0.00
b_1	-0.51 log c_T + 2.35	-0.45 log c_T + 4.79
b_2	-0.50 log c_T + 8.86	0.53 log c_T + 18.68
Parameter	Phosphoric acid system	Citric acid system
s_1	0.73 log c_T + 3.38	0.03 log c_T + 1.05
s_2	0.02 log c_T + 2.11	0.03 log c_T + 1.05
s_3	0.02 log c_T + 1.73	0.03 log c_T + 1.05
s_4	-	0.03 log c_T + 1.05
a_0	1.03	1.03
a_1	0.57 log c_T + 3.55	0.18 log c_T + 2.52
a_2	-0.00 log c_T + 2.91	-0.10 log c_T + 2.30
a_3	0.00	-0.15 log c_T + 2.57
a_4	-	0.00
b_1	-0.64 log c_T + 0.97	-0.57 log c_T + 1.51
b_2	-1.89 log c_T + 3.32	-0.73 log c_T + 5.05
b_3	-2.12 log c_T + 9.64	-1.02 log c_T + 9.73
b_4	-	-0.76 log c_T + 19.13

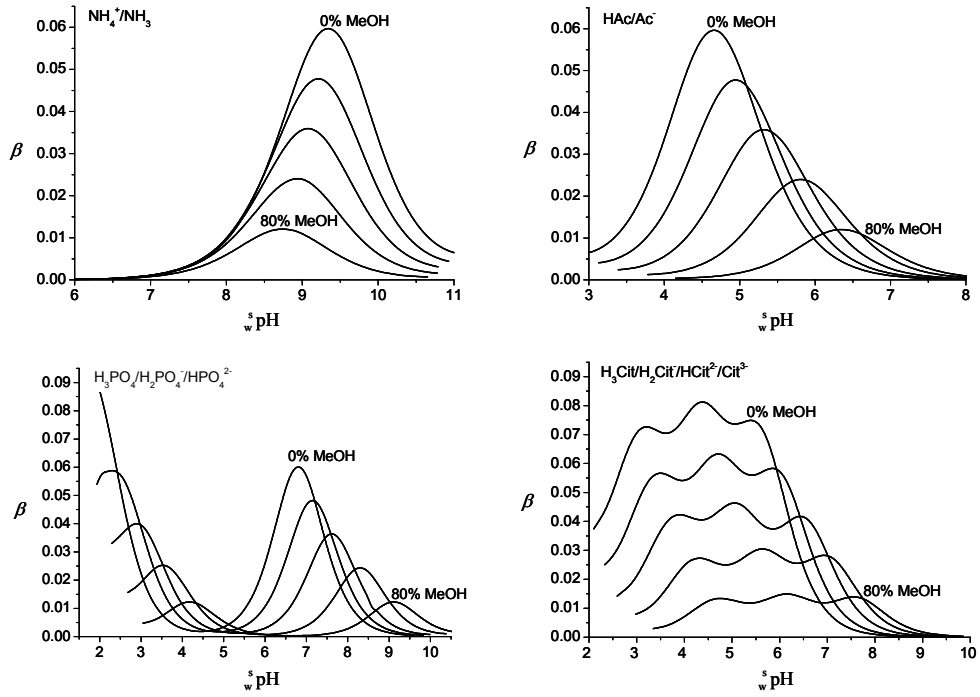


Figure 2. Buffer capacity variation of the ammonia, acetic acid, phosphoric acid and citric acid systems for 0, 20, 40, 60 and 80% (v/v) methanol-water compositions and an initial aqueous buffer concentration of 0.1 mol·L^{-1}. From ref. (44), with permission, © 2007 Elsevier.

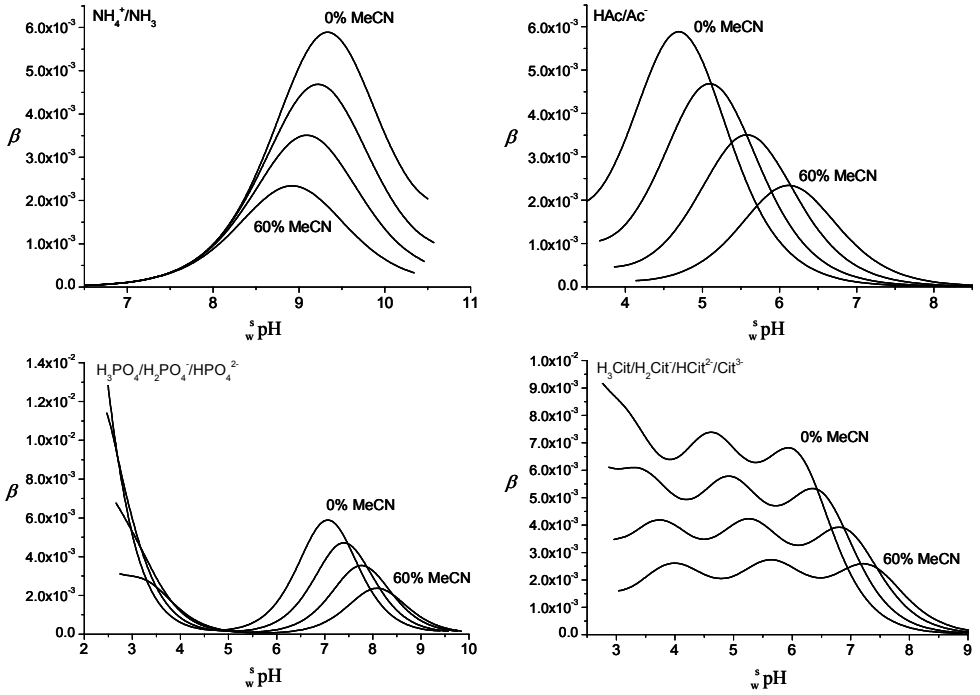


Figure 3. Buffer capacity variation of the ammonia, acetic acid, phosphoric acid and citric acid systems for 0, 20, 40 and 60% (v/v) acetonitrile-water compositions and an initial aqueous buffer concentration of 0.01 mol·L^{-1}. From ref. (22), with permission, © 2004 Elsevier.

Table 3. pH variation of acetonitrile-aqueous buffer mixtures.

$$ {}^{s}_{w}\mathrm{pH} = {}^{w}_{w}\mathrm{pH} + m_{\mathrm{pH}}\,\phi_{\mathrm{MeCN}} $$

Buffering system	Aqueous concentration	${}^{w}_{w}$pH	m_{pH}	${}^{s}_{w}$pH at MeCN volume fraction of 0.1	0.2	0.3	0.4	0.5	0.6
Acetic acid	0.01 mol·L^{-1}	3.50	1.64	3.66	3.83	3.99	4.16	4.32	4.48
		4.00	2.26	4.23	4.45	4.68	4.90	5.13	5.36
		4.50	2.28	4.73	4.96	5.18	5.41	5.64	5.87
		5.00	2.28	5.23	5.46	5.68	5.91	6.14	6.37
		5.50	2.28	5.73	5.96	6.18	6.41	6.64	6.87
		6.00	2.28	6.23	6.46	6.68	6.91	7.14	7.37
	0.05 mol·L^{-1}	3.50	2.23	3.72	3.95	4.17	4.39	4.62	4.84
		4.00	2.28	4.23	4.46	4.68	4.91	5.14	5.37
		4.50	2.28	4.73	4.96	5.18	5.41	5.64	5.87
		5.00	2.28	5.23	5.46	5.68	5.91	6.14	6.37
		5.50	2.28	5.73	5.96	6.18	6.41	6.64	6.87
		6.00	2.28	6.23	6.46	6.68	6.91	7.14	7.37
Citric acid	0.01 mol·L^{-1}	2.50	0.48	2.55	2.60	2.64	2.69	2.74	2.79
		3.00	1.15	3.12	3.23	3.35	3.46	3.58	3.69
		3.50	1.38	3.64	3.78	3.91	4.05	4.19	4.33
		4.00	1.56	4.16	4.31	4.47	4.62	4.78	4.94
		4.50	1.67	4.67	4.83	5.00	5.17	5.34	5.50
		5.00	1.75	5.18	5.35	5.53	5.70	5.88	6.05
		5.50	1.91	5.69	5.88	6.07	6.26	6.46	6.65
		6.00	1.98	6.20	6.40	6.59	6.79	6.99	7.19
		6.50	1.99	6.70	6.90	7.10	7.30	7.50	7.69
		7.00	1.99	7.20	7.40	7.60	7.80	8.00	8.19
		7.50	1.99	7.70	7.90	8.10	8.30	8.50	8.69
	0.05 mol·L^{-1}	2.50	1.16	2.62	2.73	2.85	2.96	3.08	3.20
		3.00	1.43	3.14	3.29	3.43	3.57	3.72	3.86
		3.50	1.49	3.65	3.80	3.95	4.10	4.25	4.39
		4.00	1.60	4.16	4.32	4.48	4.64	4.80	4.96
		4.50	1.65	4.67	4.83	5.00	5.16	5.33	5.49
		5.00	1.75	5.18	5.35	5.53	5.70	5.88	6.05
		5.50	1.86	5.69	5.87	6.06	6.24	6.43	6.62
		6.00	1.88	6.19	6.38	6.56	6.75	6.94	7.13
		6.50	1.88	6.69	6.88	7.06	7.25	7.44	7.63
		7.00	1.88	7.19	7.38	7.56	7.75	7.94	8.13
		7.50	1.88	7.69	7.88	8.06	8.25	8.44	8.63

Table 3 (continued)

Buffering system	Aqueous concentration	${}^{w}_{w}$pH	m_{pH}	${}^{s}_{w}$pH at MeCN volume fraction of 0.1	0.2	0.3	0.4	0.5	0.6
Phosphoric acid	0.01 mol·L^{-1}	2.21	0.51	2.26	2.31	2.36	2.41	2.47	2.52
		3.00	1.29	3.13	3.26	3.39	3.52	3.65	3.77
		3.50	1.34	3.63	3.77	3.90	4.04	4.17	4.30
		6.50	1.75	6.68	6.85	7.03	7.20	7.38	7.55
		7.00	1.75	7.18	7.35	7.53	7.70	7.88	8.05
		7.50	1.75	7.68	7.85	8.03	8.20	8.38	8.55
		8.00	1.75	8.18	8.35	8.53	8.70	8.88	9.05
		8.50	1.75	8.68	8.85	9.03	9.20	9.38	9.55
	0.05 mol·L^{-1}	2.21	1.47	2.36	2.50	2.65	2.80	2.95	3.09
		3.00	1.70	3.17	3.34	3.51	3.68	3.85	4.02
		3.50	1.71	3.67	3.84	4.01	4.18	4.36	4.53
		6.50	1.71	6.67	6.84	7.01	7.18	7.36	7.53
		7.00	1.71	7.17	7.34	7.51	7.68	7.86	8.03
		7.50	1.71	7.67	7.84	8.01	8.18	8.36	8.53
		8.00	1.71	8.17	8.34	8.51	8.68	8.86	9.03
		8.50	1.71	8.67	8.84	9.01	9.18	9.36	9.53
Ammonia	0.01 mol·L^{-1}	8.00	-0.60	7.94	7.88	7.82	7.76	7.70	7.64
		8.50	-0.60	8.44	8.38	8.32	8.26	8.20	8.14
		9.00	-0.60	8.94	8.88	8.82	8.76	8.70	8.64
		9.50	-0.60	9.44	9.38	9.32	9.26	9.20	9.14
		10.00	-0.60	9.94	9.88	9.82	9.76	9.70	9.64
	0.05 mol·L^{-1}	8.00	-0.60	7.94	7.88	7.82	7.76	7.70	7.64
		8.50	-0.60	8.44	8.38	8.32	8.26	8.20	8.14
		9.00	-0.60	8.94	8.88	8.82	8.76	8.70	8.64
		9.50	-0.60	9.44	9.38	9.32	9.26	9.20	9.14
		10.00	-0.60	9.94	9.88	9.82	9.76	9.70	9.64

Table 4. pH variation of methanol-aqueous buffer mixtures.

$$ {}^{s}_{w}\mathrm{pH} = {}^{w}_{w}\mathrm{pH} + m_{\mathrm{pH}}\,\phi_{\mathrm{MeOH}}^{d_{\mathrm{pH}}} $$

Buffering system	Aqueous concentration	${}^{w}_{w}$pH	m_{pH}	${}^{s}_{w}$pH at MeOH volume fraction of 0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
Acetic acid	0.01 mol·L^{-1}	3.50	1.85	3.60	3.75	3.91	4.09	4.28	4.48	4.69	4.90
		4.00	2.22	4.12	4.30	4.49	4.71	4.93	5.17	5.42	5.68
		4.50	2.25	4.63	4.80	5.00	5.22	5.45	5.69	5.94	6.20
		5.00	2.25	5.13	5.30	5.50	5.72	5.95	6.19	6.44	6.70
		5.50	2.25	5.63	5.80	6.00	6.22	6.45	6.69	6.94	7.20
		6.00	2.25	6.13	6.30	6.50	6.71	6.94	7.19	7.44	7.70
	0.05 mol·L^{-1}	3.50	2.17	3.62	3.79	3.98	4.19	4.41	4.65	4.89	5.14
		4.00	2.22	4.13	4.30	4.49	4.71	4.94	5.17	5.42	5.68
		4.50	2.23	4.63	4.80	4.99	5.21	5.44	5.68	5.93	6.18
		5.00	2.23	5.13	5.30	5.49	5.71	5.94	6.18	6.43	6.68
		5.50	2.23	5.63	5.80	5.99	6.21	6.44	6.68	6.93	7.18
		6.00	2.23	6.13	6.30	6.49	6.71	6.94	7.18	7.42	7.68
Citric acid	0.01 mol·L^{-1}	2.50	1.52	2.59	2.70	2.84	2.98	3.14	3.30	3.48	3.65
		3.00	1.88	3.11	3.25	3.42	3.60	3.79	3.99	4.20	4.42
		3.50	2.16	3.62	3.79	3.98	4.19	4.41	4.64	4.88	5.13
		4.00	2.35	4.13	4.31	4.52	4.75	4.99	5.24	5.50	5.78
		4.50	2.49	4.64	4.83	5.05	5.29	5.55	5.81	6.09	6.38
		5.00	2.61	5.15	5.35	5.58	5.83	6.10	6.38	6.67	6.98
		5.50	2.73	5.65	5.87	6.11	6.37	6.65	6.94	7.25	7.57
		6.00	2.81	6.16	6.38	6.62	6.89	7.18	7.49	7.80	8.13
		6.50	2.84	6.66	6.88	7.13	7.40	7.70	8.00	8.32	8.65
		7.00	2.83	7.16	7.38	7.63	7.90	8.19	8.50	8.81	9.14
		7.50	2.76	7.66	7.87	8.11	8.38	8.66	8.96	9.27	9.59
	0.05 mol·L^{-1}	2.50	1.86	2.60	2.75	2.91	3.09	3.28	3.48	3.69	3.91
		3.00	2.15	3.12	3.29	3.48	3.68	3.90	4.13	4.37	4.62
		3.50	2.30	3.63	3.81	4.01	4.23	4.47	4.71	4.97	5.24
		4.00	2.39	4.13	4.32	4.53	4.76	5.01	5.26	5.53	5.81
		4.50	2.48	4.64	4.83	5.05	5.29	5.54	5.81	6.09	6.37
		5.00	2.58	5.15	5.35	5.57	5.82	6.08	6.36	6.65	6.95
		5.50	2.68	5.65	5.86	6.09	6.35	6.63	6.91	7.21	7.53
		6.00	2.73	6.15	6.37	6.61	6.87	7.15	7.44	7.75	8.07
		6.50	2.75	6.65	6.87	7.11	7.37	7.66	7.95	8.26	8.58
		7.00	2.74	7.15	7.37	7.61	7.87	8.15	8.45	8.76	9.07
		7.50	2.70	7.65	7.86	8.10	8.36	8.63	8.92	9.23	9.54

d_{pH} = 5/4 for acetic, citric and phosphoric acid systems.
d_{pH} = 1 for ammonia system.

Table 4 (continued)

Buffering system	Aqueous concentration	${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{pH}$	m_{pH}	${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{pH}$ at MeOH volume fraction of 0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
Phosphoric acid	0.01 mol·L^{-1}	2.11	1.51	2.19	2.31	2.44	2.59	2.74	2.91	3.08	3.25
		3.00	2.36	3.13	3.32	3.52	3.75	3.99	4.25	4.51	4.79
		3.50	2.40	3.64	3.82	4.03	4.27	4.51	4.77	5.04	5.32
		6.50	2.99	6.67	6.90	7.16	7.45	7.76	8.08	8.41	8.76
		7.00	2.98	7.17	7.40	7.66	7.95	8.25	8.57	8.90	9.25
		7.50	2.94	7.67	7.89	8.15	8.44	8.74	9.05	9.38	9.73
		8.00	2.85	8.16	8.38	8.63	8.91	9.20	9.51	9.83	10.16
		8.50	2.62	8.65	8.85	9.08	9.33	9.60	9.88	10.18	10.48
	0.05 mol·L^{-1}	2.21	2.54	2.25	2.45	2.67	2.92	3.18	3.45	3.73	4.03
		3.00	2.81	3.16	3.38	3.62	3.89	4.18	4.48	4.80	5.12
		3.50	2.81	3.66	3.88	4.12	4.39	4.68	4.99	5.30	5.63
		6.50	2.96	6.67	6.90	7.16	7.44	7.74	8.06	8.40	8.74
		7.00	2.95	7.17	7.40	7.66	7.94	8.24	8.56	8.89	9.24
		7.50	2.94	7.67	7.89	8.15	8.43	8.73	9.05	9.38	9.72
		8.00	2.88	8.16	8.39	8.64	8.92	9.21	9.52	9.84	10.18
		8.50	2.73	8.65	8.87	9.11	9.37	9.65	9.94	10.25	10.57
Ammonia	0.01 mol·L^{-1}	8.00	-0.69	7.93	7.86	7.79	7.73	7.66	7.59	7.52	7.45
		8.50	-0.69	8.43	8.36	8.29	8.22	8.16	8.09	8.02	7.95
		9.00	-0.69	8.93	8.86	8.79	8.72	8.66	8.59	8.52	8.45
		9.50	-0.69	9.43	9.36	9.29	9.23	9.16	9.09	9.02	8.95
		10.00	-0.66	9.93	9.87	9.80	9.73	9.67	9.60	9.53	9.47
	0.05 mol·L^{-1}	8.00	-0.68	7.93	7.86	7.80	7.73	7.66	7.59	7.52	7.45
		8.50	-0.68	8.43	8.36	8.30	8.23	8.16	8.09	8.02	7.95
		9.00	-0.68	8.93	8.86	8.80	8.73	8.66	8.59	8.52	8.45
		9.50	-0.68	9.43	9.36	9.30	9.23	9.16	9.09	9.02	8.95
		10.00	-0.68	9.93	9.86	9.80	9.73	9.66	9.59	9.52	9.46

$d_{\mathrm{pH}} = 5/4$ for acetic, citric and phosphoric acid systems.
$d_{\mathrm{pH}} = 1$ for ammonia system.

Table 5. pH values at different buffer concentrations corresponding to maximum buffer capacity in aqueous solutions, calculated from de ${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ (54) of the buffering species.

Buffer	${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$	Equimolar concentration (mol·L^{-1}) 0.001	0.01	0.05	0.1
Acetic acid/acetate	4.76	4.74	4.72	4.69	4.67
Ammonium/ammonia	9.25	9.26	9.28	9.32	9.34
Phosphoric acid/dihydrogenphosphate	2.16	2.15	2.13	2.09	2.07
Dihydrogenphosphate/hydrogenphosphate	7.21	7.14	7.01	6.85	6.76
Citric acid/dihydrogencitrate	3.13	3.12	3.10	3.06	3.04
Dihydrogencitrate/hydrogencitrate	4.76	4.69	4.56	4.40	4.31
Hydrogencitrate/citrate	6.40	6.21	5.91	5.59	5.44

5. pK_a VARIATION OF THE ANALYTES WITH THE ADDITION OF ACETONITRILE OR METHANOL

For the most common families of analytes, linear relations have been established for pK_a values in the hydroorganic mobile phases in relation to their aqueous pK_a. Rived *et al.* (46-48) and Espinosa *et al.* (22, 49) developed equations to estimate ${}^{s}_{s}\mathrm{p}K_a$ from ${}^{w}_{w}\mathrm{p}K_a$ values of pyridines, amines, carboxylic aromatic acids, carboxylic aliphatic acids and phenols in methanol-water and acetonitrile-water, respectively. They proposed the same general equations:

$${}^{s}_{s}\mathrm{p}K_a = a_s\, {}^{w}_{w}\mathrm{pK}_a + b_s \qquad [10]$$

with

$$a_s = \frac{1 + a_{s1}\,\phi_{Org} + a_{s2}\,\phi_{Org}^2}{1 + a_{s3}\,\phi_{Org} + a_{s4}\,\phi_{Org}^2} \qquad [11]$$

$$b_s = \frac{b_{s1}\,\phi_{Org} + b_{s2}\,\phi_{Org}^2}{1 + b_{s3}\,\phi_{Org} + b_{s4}\,\phi_{Org}^2} \qquad [12]$$

where ϕ_{Org} is the volume fraction of organic solvent (acetonitrile or methanol) in the hydroorganic mixture, and a_{s1}, a_{s2}, a_{s3}, a_{s4}, b_{s1}, b_{s2}, b_{s3} and b_{s4} are fitting constants for all acids of the same family at any organic solvent-water composition. These a_{si} and b_{si} values are shown for methanol in Table 6 and for acetonitrile in Table 7. The analyte pK_a in the hydroorganic mobile phase can be expressed in the ${}^{s}_{w}\mathrm{p}K_a$ scale, instead of the ${}^{s}_{s}\mathrm{p}K_a$, through the already known δ parameter (Eqs. 4 or 5). Therefore Eq. 10 is converted to the following expression:

$${}^{s}_{w}\mathrm{p}K_a = a_s\, {}^{w}_{w}\mathrm{pK}_a + b_s + \delta \qquad [13]$$

Tables 8 and 9 show several examples of calculated ${}^{s}_{w}\mathrm{p}K_a$ values for families of compounds when increasing the acetonitrile or the methanol fraction in the hydroorganic mixture. ${}^{s}_{w}\mathrm{p}K_a$

of neutral acids or anionic acids (aliphatic and aromatic carboxylic acids and phenols) increase when acetonitrile or methanol is added, whereas the ${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ of cationic acids (amines and pyridines) decreases, mainly due to electrostatic interactions that contribute to the pK_{a} value (36, 45).

Table 6. Parameters for the prediction of the slope a_{s} (Eq. 11) and the intercept b_{s} (Eq. 12) of the linear correlation between ${}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_{\mathrm{a}}$ values in methanol-water and ${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ in water (Eq. 10) (46)

Family of compounds	a_{s1}	a_{s2}	a_{s3}	a_{s4}
Phenols	-0.656	-0.030	-0.844	0.133
Aliphatic carboxylic acids	-1.406	0.680	-1.551	0.827
Aromatic carboxylic acids				
with *ortho*-substituents	-1.189	0.190	-1.424	0.425
without *ortho*-substituents	-1.101	0.103	-1.516	0.518
Amines	-0.476	0.209	-0.400	0.158
Pyridines	2.617	0.000	2.809	0.000
Family of compounds	b_{s1}	b_{s2}	b_{s3}	b_{s4}
Phenols	-0.454	0.866	-0.017	-0.865
Aliphatic carboxylic acids	1.034	-0.898	-1.250	0.277
Aromatic carboxylic acids				
with *ortho*-substituents	0.449	-0.429	-1.674	0.677
without *ortho*-substituents	-0.178	0.187	-1.699	0.702
Amines	-0.458	0.477	-1.674	0.690
Pyridines	-1.733	1.763	-1.214	0.272

Valid equations up to 100% (v/v) of methanol.

Table 7. Parameters for the prediction of the slope a_{s} (Eq. 11) and the intercept b_{s} (Eq. 12) of the linear correlation between ${}^{\mathrm{s}}_{\mathrm{s}}\mathrm{p}K_{\mathrm{a}}$ values in acetonitrile-water and ${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ in water (Eq. 10) (22, 49)

Family of compounds	a_{s1}	a_{s2}	a_{s3}	a_{s4}
Aliphatic carboxylic acids	9.97	-8.59	8.83	-8.72
Aromatic carboxylic acids	52.04	-10.93	49.33	-32.69
Phenols	10.05	-10.04	7.97	-8.37
Amines	-0.73	-0.27	-0.87	-0.12
Pyridines	-1.67	0.67	-1.66	0.67
Family of compounds	b_{s1}	b_{s2}	b_{s3}	b_{s4}
Aliphatic carboxylic acids	-0.68	9.94	8.45	-8.59
Aromatic carboxylic acids	-5.32	8.99	22.56	-23.21
Phenols	-5.33	9.95	0.19	-0.70
Amines	-1.82	2.25	-1.75	0.90
Pyridines	-1.78	1.89	-0.58	-0.40

Valid equations up to 60% (v/v) of acetonitrile (100% for pyridines).

Table 8. pK_a variation of analytes in acetonitrile-water mixtures.

Family of analytes	${}^{w}_{w}\mathrm{p}K_a$	${}^{s}_{w}\mathrm{p}K_a$ at MeCN volume fraction of 0.1	0.2	0.3	0.4	0.5	0.6
Aliphatic carboxylic acids	2.00	2.14	2.28	2.43	2.61	2.82	3.09
	2.50	2.67	2.83	3.00	3.19	3.41	3.70
	3.00	3.21	3.38	3.56	3.76	4.01	4.32
	3.50	3.74	3.93	4.12	4.34	4.60	4.94
	4.00	4.27	4.47	4.68	4.92	5.19	5.55
	4.50	4.80	5.02	5.24	5.49	5.79	6.17
	5.00	5.33	5.57	5.81	6.07	6.38	6.78
Aromatic carboxylic acids	2.00	2.02	2.12	2.23	2.35	2.47	2.57
	2.50	2.57	2.69	2.84	3.00	3.16	3.32
	3.00	3.11	3.27	3.45	3.64	3.85	4.08
	3.50	3.65	3.84	4.05	4.29	4.55	4.83
	4.00	4.20	4.41	4.66	4.94	5.24	5.58
	4.50	4.74	4.99	5.27	5.58	5.94	6.33
	5.00	5.28	5.56	5.88	6.23	6.63	7.08
Phenols	7.00	7.35	7.40	7.49	7.70	8.07	8.64
	7.50	7.90	7.97	8.08	8.30	8.67	9.26
	8.00	8.46	8.55	8.67	8.89	9.28	9.88
	8.50	9.02	9.13	9.26	9.49	9.89	10.49
	9.00	9.57	9.71	9.85	10.09	10.50	11.11
	9.50	10.13	10.28	10.44	10.69	11.10	11.73
	10.00	10.68	10.86	11.03	11.29	11.71	12.34
	10.50	11.24	11.44	11.62	11.89	12.32	12.96
	11.00	11.79	12.02	12.21	12.49	12.93	13.58
Amines	7.00	6.90	6.76	6.59	6.39	6.18	6.02
	7.50	7.41	7.28	7.11	6.92	6.72	6.55
	8.00	7.91	7.79	7.63	7.44	7.25	7.08
	8.50	8.42	8.30	8.15	7.97	7.78	7.62
	9.00	8.93	8.82	8.67	8.49	8.31	8.15
	9.50	9.43	9.33	9.19	9.02	8.84	8.69
	10.00	9.94	9.84	9.71	9.55	9.37	9.22
	10.50	10.45	10.36	10.23	10.07	9.90	9.76
	11.00	10.95	10.87	10.75	10.60	10.43	10.29
Pyridines	4.00	3.82	3.64	3.46	3.25	3.01	2.70
	4.50	4.32	4.14	3.95	3.75	3.50	3.19
	5.00	4.82	4.64	4.45	4.24	3.99	3.68
	5.50	5.32	5.14	4.95	4.74	4.49	4.16
	6.00	5.82	5.64	5.45	5.23	4.98	4.65
	6.50	6.32	6.13	5.94	5.73	5.47	5.14
	7.00	6.82	6.63	6.44	6.22	5.96	5.63
	7.50	7.32	7.13	6.94	6.72	6.46	6.12
	8.00	7.82	7.63	7.43	7.21	6.95	6.60

Table 9. pK_a variation of analytes in methanol-water mixtures.

Family of analytes	${}^{w}_{w}\mathrm{p}K_a$	${}^{s}_{w}\mathrm{p}K_a$ at MeOH volume fraction of 0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
Aliphatic carboxylic acids	2.00	2.15	2.32	2.50	2.72	2.96	3.21	3.45	3.62
	2.50	2.66	2.83	3.03	3.25	3.50	3.76	3.99	4.16
	3.00	3.16	3.35	3.55	3.78	4.04	4.30	4.54	4.70
	3.50	3.67	3.86	4.08	4.32	4.58	4.85	5.09	5.24
	4.00	4.18	4.38	4.60	4.85	5.12	5.40	5.63	5.77
	4.50	4.69	4.90	5.13	5.38	5.66	5.94	6.18	6.31
	5.00	5.19	5.41	5.65	5.92	6.20	6.49	6.73	6.85
Aromatic carboxylic acids with *ortho*-substituents	2.00	2.11	2.23	2.38	2.56	2.78	3.03	3.29	3.51
	2.50	2.62	2.76	2.93	3.12	3.36	3.63	3.91	4.15
	3.00	3.13	3.29	3.47	3.68	3.93	4.22	4.53	4.79
	3.50	3.65	3.81	4.01	4.23	4.51	4.82	5.14	5.44
	4.00	4.16	4.34	4.55	4.79	5.08	5.41	5.76	6.08
	4.50	4.67	4.86	5.09	5.35	5.65	6.01	6.38	6.72
	5.00	5.18	5.39	5.63	5.90	6.23	6.60	6.99	7.36
without *ortho*-substituents	2.00	2.08	2.17	2.28	2.41	2.56	2.73	2.87	2.91
	2.50	2.60	2.72	2.85	3.01	3.20	3.41	3.59	3.69
	3.00	3.12	3.26	3.43	3.62	3.84	4.09	4.32	4.47
	3.50	3.65	3.81	4.00	4.22	4.48	4.77	5.05	5.25
	4.00	4.17	4.36	4.57	4.83	5.12	5.45	5.77	6.03
	4.50	4.69	4.90	5.15	5.43	5.76	6.13	6.50	6.81
	5.00	5.21	5.45	5.72	6.04	6.40	6.81	7.22	7.59
Phenols	7.00	7.10	7.23	7.37	7.54	7.73	7.93	8.13	8.27
	7.50	7.61	7.75	7.90	8.07	8.27	8.48	8.68	8.83
	8.00	8.12	8.27	8.43	8.61	8.81	9.03	9.24	9.39
	8.50	8.63	8.78	8.96	9.15	9.36	9.58	9.79	9.94
	9.00	9.14	9.30	9.48	9.68	9.90	10.13	10.35	10.50
	9.50	9.65	9.82	10.01	10.22	10.44	10.68	10.90	11.05
	10.00	10.16	10.34	10.54	10.75	10.99	11.23	11.45	11.61
	10.50	10.67	10.86	11.07	11.29	11.53	11.78	12.01	12.17
	11.00	11.18	11.38	11.59	11.83	12.08	12.33	12.56	12.72
Amines	7.00	6.91	6.82	6.74	6.66	6.59	6.52	6.41	6.20
	7.50	7.41	7.32	7.23	7.15	7.08	7.00	6.89	6.68
	8.00	7.90	7.81	7.72	7.64	7.56	7.49	7.37	7.16
	8.50	8.40	8.30	8.21	8.12	8.05	7.97	7.85	7.64
	9.00	8.90	8.79	8.70	8.61	8.53	8.45	8.33	8.12
	9.50	9.39	9.29	9.19	9.10	9.02	8.94	8.82	8.61
	10.00	9.89	9.78	9.68	9.59	9.50	9.42	9.30	9.09
	10.50	10.38	10.27	10.17	10.07	9.99	9.90	9.78	9.57
	11.00	10.88	10.77	10.66	10.56	10.47	10.39	10.26	10.05

Table 9 (*continued*)

Family of analytes	${}^{w}_{w}\mathrm{p}K_a$	${}^{s}_{w}\mathrm{p}K_a$ at MeOH volume fraction of 0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
Pyridines	4.00	3.77	3.57	3.38	3.20	3.05	2.91	2.76	2.58
	4.50	4.27	4.06	3.86	3.69	3.53	3.39	3.24	3.06
	5.00	4.76	4.54	4.35	4.17	4.01	3.86	3.72	3.54
	5.50	5.25	5.03	4.83	4.65	4.49	4.34	4.19	4.01
	6.00	5.74	5.52	5.32	5.13	4.97	4.82	4.67	4.49
	6.50	6.24	6.01	5.80	5.61	5.45	5.30	5.15	4.97
	7.00	6.73	6.50	6.29	6.10	5.93	5.78	5.63	5.44
	7.50	7.22	6.98	6.77	6.58	6.41	6.26	6.10	5.92
	8.00	7.71	7.47	7.25	7.06	6.89	6.74	6.58	6.39

6. ESTIMATION OF THE DEGREE OF IONISATION AND VARIATION ON CHROMATOGRAPHIC RETENTION OF ANALYTES

The retention of acid-base analytes in RP-HPLC mainly depends on their hydrophobicity and ionisation degree (1-3, 6, 7, 9, 14, 50-53). Whereas the hydrophobicity of a substance is a property inherent to the own nature of the analyte, the degree of ionisation depends on both, analyte dissociation constant and mobile phase pH. As a general rule for analytes of similar hydrophobicity, the higher the degree of ionisation, the lower the retention.

For a compound that has a unique acid-base equilibrium (HA^z-A^{z-1}), ruled by an acidity constant (K_a), its ionisation degree (α), i.e. the mole fraction of the ionised species, can be calculated by:

$$\alpha_A = \frac{[A^{z-1}]}{[HA^z]+[A^{z-1}]} = \frac{1}{1+10^{\mathrm{p}K_a-\mathrm{pH}}} \qquad [14]$$

or

$$\alpha_{HA} = \frac{[HA^z]}{[HA^z]+[A^{z-1}]} = \frac{1}{1+10^{\mathrm{pH}-\mathrm{p}K_a}} \qquad [15]$$

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 should be ${}^{s}_{s}\mathrm{pH}$ and ${}^{s}_{s}\mathrm{p}K_a$. However, we can use ${}^{s}_{w}\mathrm{pH}$ and ${}^{s}_{w}\mathrm{p}K_a$ values because ${}^{s}_{s}\mathrm{pH} - {}^{s}_{s}\mathrm{p}K_a = {}^{s}_{w}\mathrm{pH} - {}^{s}_{w}\mathrm{p}K_a$ since ${}^{s}_{w}\mathrm{pH} - {}^{s}_{s}\mathrm{pH} = {}^{s}_{w}\mathrm{p}K_a - {}^{s}_{s}\mathrm{p}K_a = \delta$.

Inserting the estimated values of both the analyte pK_a and the mobile phase pH in Eq. 14 or 15 we are able to predict the ionisation degree of an analyte in a particular mobile phase. Now we are capable of explaining the retention changes observed in the chromatograms of Fig. 1, in which two different buffering systems of initial aqueous concentration of 0.01 mol·L^{-1} and ${}^{w}_{w}\mathrm{pH}$ 8.00 prepared from dihydrogenphosphate/ /hydrogenphosphate and ammonium/ammonia were considered. The pK_a values of the chromatographied acid-base analytes were relatively close to 8 (with their corresponding ${}^{w}_{w}\mathrm{p}K_a$ values in brackets (11, 54)): 4-nitrophenol (7.15), 2-nitrophenol (7.23), 2,4,6-trimethylpyridine (7.43), 3-nitrophenol (8.36), 2-chlorophenol (8.56), *N,N*-dimethylbenzylamine (8.91), and 3-bromophenol (9.03). The hydrophobicities of these compounds were quite similar. Figure 4 shows the calculated ionisation degrees (Eqs. 14 or 15) for the analytes from their estimated pK_a (Eq. 13) and mobile phase pH (Eq. 6 or 7) at several fractions of methanol. At 60% (v/v) of methanol the ${}^{s}_{w}\mathrm{pH}$ of the dhydrogenphosphate/hydrogenphosphate and ammonium/ammonia mobile phases were 9.51 and 7.59, respectively, and the ${}^{s}_{w}\mathrm{p}K_a$ of the analytes in both mobile phases were, 8.10, 8.19, 6.19, 9.43, 9.65, 8.37, and 10.17, respectively. In case of pyridines and amines the ionisation degree is high when the pH of the mobile phase is lower than the analyte pK_a ($\mathrm{BH^+} \rightleftarrows \mathrm{B} + \mathrm{H^+}$), and in the rest of the cases (aromatic and aliphatic carboxylic acids and phenols) the ionisation is high when the pH is higher than the pK_a ($\mathrm{HA} \rightleftarrows \mathrm{H^+} + \mathrm{A^-}$).

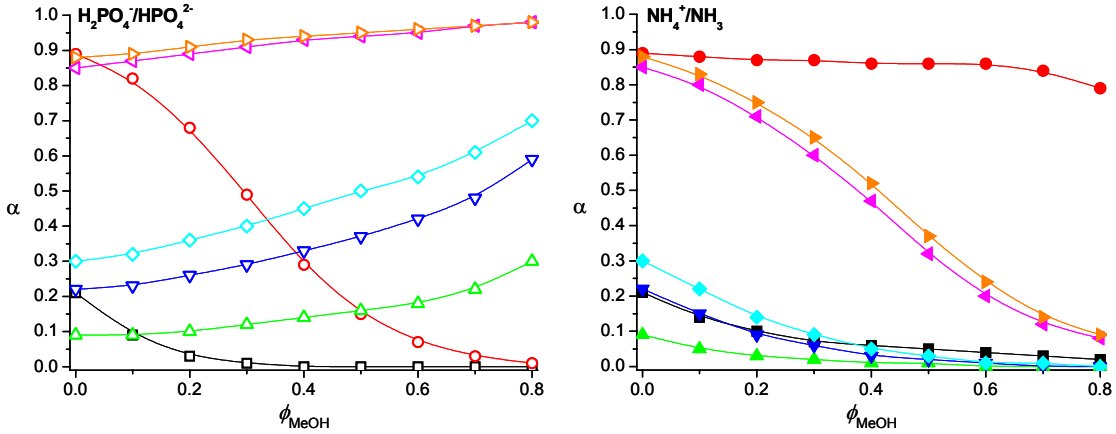


Figure 4. Variation of the ionisation degree of acid-base compounds with the addition of methanol to $H_2PO_4^-$-HPO_4^{2-} and NH_4^+-NH_3 aqueous buffers of ${}^{w}_{w}$pH 8.00 and concentration 0.01 mol·L^{-1}. Legend: (■,□) 2,4,6-trimethylpyridine, (●,○) *N,N*-dimethylbenzylamine, (▲,△) 3-bromophenol, (▼,▽) 2-chlorophenol, (◀,◁) 2-nitrophenol, (◆,◇) 3-nitrophenol, and (▶,▷) 4-nitrophenol. From ref. (44), with permission, © 2007 Elsevier.

7. ESTIMATION OF CHROMATOGRAPHIC RETENTION OF IONISABLE ANALYTES

The pH and pK_a models exposed above can be used to achieve quantitative information about the retention of weak acid-base analytes (Eq. 1). It is possible to predict the retention from both, the estimated buffer pH and solute pK_a, and from the retentions of the pure acidic and basic forms of the analyte. These retention times can be measured in mobile phases with a pH at least two or three units lower and higher than the pK_a of the analyte. In a recent paper (26), several drugs with known aqueous pK_a were studied to test this retention time estimation model in acetonitrile-aqueous buffer mobile phases: diclofenac, ibuprofen and naproxen (nonsteroidal anti-inflammatory drugs), codeine (narcotic analgesic), trazodone, imipramine, nortriptyline and maprotiline (antidepressants). Fig. 5 shows the differences between the experimental and the estimated retention times at several measured aqueous pH. Generally, there is a very good correspondence between the estimated and the experimental retention times. Except for ibuprofen and imipramine, the average of the absolute error for all the analytes and studied pH values is less than 5%. These differences in retention times for imipramine and ibuprofen can be attributed to a mismatch between the chromatographically

obtained ${}^{s}_{w}\mathrm{p}K_a$ values and the estimated ones. We must take into account that when the difference in retention times of the neutral and fully ionised species is large, this pK_a mismatch has a significant effect on retention estimation. When no experimental aqueous pK_a value is available in the literature for a particular analyte, it is possible to resort to computational programs, e.g. SPARC (55) and ACD/Labs (56). The former is freely accessed through Internet, and the latter is embedded in the SciFinder Scholar™ 2006 data base research tool.

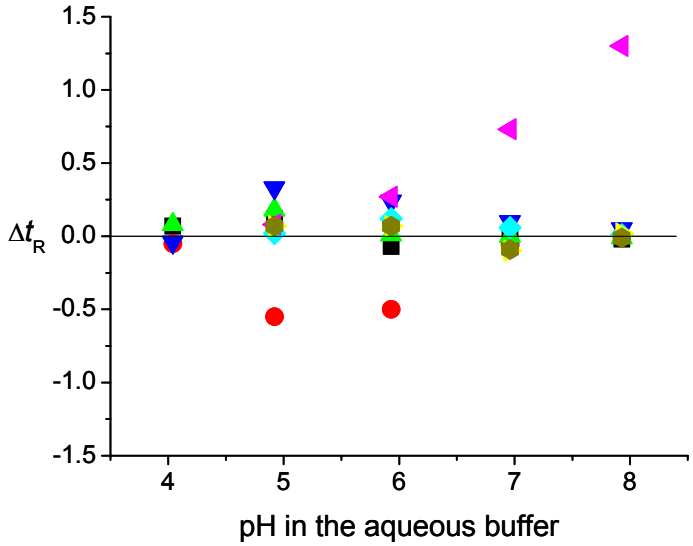


Figure 5. Differences between the experimental and the estimated retention times at several measured ${}^{w}_{w}\mathrm{pH}$ ($\Delta t_R = t_R^{est} - t_R$). Estimated retention times were calculated through Eq. 1, where ${}^{s}_{w}\mathrm{p}K_a$ were estimated from the literature ${}^{w}_{w}\mathrm{p}K_a$ values, and ${}^{s}_{w}\mathrm{pH}$ were estimated from measured aqueous ${}^{w}_{w}\mathrm{pH}$. Buffer aqueous concentration was, in all cases, 0.01 mol·L^{-1}. Legend: (▼) trazodone, (■) diclofenac, (◆) codeine, (▲) naproxen, (●) ibuprofen, (◀) imipramine, (★) maprotiline, (▶) nortriptyline. From ref. (26), with permission, © 2006 Elsevier.

Sometimes it is not possible to measure both of the pure acidic and basic forms of the analyte, either because the required pH value is not recommended for the column (e.g. high pH values in silica based columns) or because the *k* value is too high and the solute can not be detected in a reasonable analysis time. In these cases it is recommended to resort to models able to infer the chromatographic behaviour of the analytes upon changes in the experimental factors. Once the models are built with data obtained from sets of experiments, molecular modelling or other approaches, they can be applied to predict the performance of new conditions (57).

8. CONCLUSIONS

When adding acetonitrile or methanol to an aqueous buffer to prepare a mobile phase, the pH of the hydroorganic mixture depends on the nature of the buffering species, the organic solvent content, and the aqueous pH and concentration of the buffer. Models have been developed to allow and accurate prediction of this pH change for several commonly used buffers in RP-HPLC (acetic, citric and phosphoric acid and ammonia systems) in acetonitrile-water and methanol-water mobile phases. Both models cover initial aqueous concentrations between 0.001 and 0.1 $mol \cdot L^{-1}$, and organic solvent contents up to 60% in volume for acetonitrile and 80% for methanol.

The buffer capacity decreases when the organic solvent is added, due to the dilution effect of the mixture, and their maximum values shift together with the pK_a variation of the buffer species.

Linear relationships have been also modelled between the pK_a values of acid-base analytes in methanol-water and acetonitrile-water and their corresponding pK_a values in water. The pK_a variation depends on the nature of family of compounds, the aqueous pK_a and the organic solvent content in the mixture. These linear relations have been established for the most common families of acid-base analytes: aromatic and aliphatic carboxylic acids, phenols, amines and pyridines. In acetonitrile-water these relations are applicable up to 60% in volume of organic modifier (100% for pyridines).

From both the analyte pK_a and the mobile phase pH, the analyte ionisation degree, which plays an important role in the chromatographic retention of acid-base compounds, can be easily calculated. Moreover, with the measured retention times of neutral and fully ionised species this approach is able to estimate the retention times of weak acids and bases at any hydroorganic pH.

ACKNOWLEDGEMENTS

We thank financial support from the MCYT of the Spanish Government and FEDER of EU (projects CTQ2004-00633/BQU and CTQ2004-00965/BQU).

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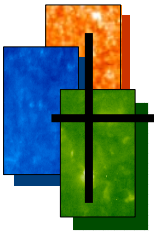
ARTICLE V



Nitromethane as solvent in capillary electrophoresis

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J. Chromatogr. A 1079 (2005) 246

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Nitromethane as solvent in capillary electrophoresis

Xavier Subirats [a,b], Simo P. Porras [c,*], Martí Rosés [b], Ernst Kenndler [a,*]

[a] *Institute for Analytical Chemistry, University of Vienna, Währingerstrasse 38, A-1090 Vienna, Austria*
[b] *Departament de Química Analítica, Universitat de Barcelona, Diagonal 647, E-08028 Barcelona, Spain*
[c] *Laboratory of Analytical Chemistry, Department of Chemistry, P.O. Box 55, FIN-00014, University of Helsinki, Finland*

Available online 16 March 2005

Abstract

Nitromethane has several properties that make it an interesting solvent for capillary electrophoresis especially for lipophilic analytes that are not sufficiently soluble in water: freezing and boiling points are suitable for laboratory conditions, low viscosity leads to favourable electrophoretic mobilities, or an intermediate dielectric constant enables dissolution of electrolytes. In the present work we investigate the change of electrophoretically relevant analyte properties – mobilities and pK_a values – in nitromethane in dependence on the most important experimental conditions determined by the background electrolyte: the ionic strength, I, and the pH. It was found that the mobility decreases with increasing ionic strength (by, e.g. up to 30% from $I = 0$ to 50 mmol/L) according to theory. An appropriate pH scale is established by the aid of applying different concentration ratios of a buffer acid with known pK_a and its conjugate base. The mobility of the anionic analytes (from weak neutral acids) depends on the pH with the typical sigmoidal curve in accordance with theory. The pK_a of neutral acids derived from these curves is shifted by as much as 14 pK units in nitromethane compared to water. Both findings confirm the agreement of the electrophoretic behaviour of the analytes with theories of electrolyte solutions. Separation of several neutral analytes was demonstrated upon formation of charged complexes due to heteroconjugation with chloride as ionic constituent of the background electrolyte.

Keywords: Capillary electrophoresis; Nitromethane; Organic solvent; Contactless conductivity detection; Non-aqueous; UV detection; Heteroconjugation; pK_a shift; Mobility

1. Introduction

Although water is by far the most common solvent in capillary electrophoresis (CE), it has the disadvantage that lipophilic compounds may exhibit a low solubility in it, and the amount of analytes dissolved often does not reach the limit of detection. In such cases it is favourable to substitute water by aqueous–organic mixtures or organic solvents. It is obvious that in some cases these solvent systems might also improve the separation selectivity (though the case can be vice versa as well). Most probably the effect of organic solvents on separation efficiency is overestimated, as has been discussed in detail in a previous paper [1]. Methanol and acetonitrile are certainly the most common members of the class of organic solvents for solutions of analytes and of the constituents of the background electrolyte (BGE). However, not only are these two solvents used in CE, but also a number of other protic or dipolar aprotic solvents (see, e.g. ref. [2]).

Nitromethane (NM) has not been applied as solvent to CE so far; only one recent application dealt with the separation of chlorophenols in binary mixtures of water with NM [3]. However, NM is widely used, e.g. as extraction solvent or as a reaction medium. It has a broad application range in organic synthesis (e.g. pharmaceuticals, pesticides, fibres, etc.) and as stabilisation agent, e.g. for halogenated hydrocarbons. It is also used as a fuel for high performance engines (e.g. in drag racing) because of the low amount of air it needs to burn. In addition, NM is also used for cleaning electronic circuit boards and in explosive industry. It should be noted, however, that NM itself is not classified as an explosive, but an explosive is formed only when it is mixed together with inorganic nitrite [4].

* Corresponding authors. Tel.: +43 1 4277 52305; fax: +43 1 4277 9523 (E. Kenndler)/+358 9 19150 253 (S.P. Porras).
E-mail addresses: porras@cc.helsinki.fi (S.P. Porras), ernst.kenndler@univie.ac.at (E. Kenndler).

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doi:10.1016/j.chroma.2005.02.072

For potential use in CE it has a number of interesting properties, e.g. its freezing (−28.6 °C [5]) and boiling (101.2 °C [5]) points, its moderate relative permittivity (36.3 at 25 °C [5]), low dynamic viscosity (0.614 cP at 25 °C [5]), and wide pH range of at least 24 pH units (autoprotolysis constant, $pK_{auto} \geq 24$ [6]). It should also be noted that NM is a solvent where hetero- and homoconjugation [6–9] as well as ion pair formation [10,11] might be present.

It is therefore the goal of the present work to investigate the suitability of NM for CE. This investigation will not be directed to a special application, from which often no general conclusions can be drawn. This work rather deals with fundamental physicochemical parameters, which describe the main electrolyte properties CE is based on. Therefore, we consider the influence of the solvent on the electrophoretic mobility and the acid–base properties of electrolytes, being either analytes or constituents of the BGE. We treat the influence of the solvent on the mobility as function of the ionic strength by the extended Debye–Hückel–Onsager (DHO) conductivity theory. We further prove the applicability of the Henderson–Hasselbalch relation to nitromethane as solvent, describing the dependence of the effective mobility on the pH and the pK_a via the degree of dissociation. Such dependence could give the tool to establish a pH scale in nitromethane without the use of an electrode for the pH measurement, avoiding in this way the experimental bias introduced by liquid junction potentials.

2. Experimental

2.1. Instrumentation

Capillary electrophoresis was carried out with an HP ^{3D}CE instrument (Hewlett-Packard, Waldbronn, Germany) using photometric diode-array detection (DAD) and a dual cell contactless conductivity detector (CCD) mounted in the capillary cassette as described elsewhere [12]. DAD signals were recorded at 240, 254 and 340 nm.

CCD signals were processed by a Hewlett-Packard 35900E dual channel A/D converter. Data collection from detectors was performed with ChemStation software. Uncoated fused-silica capillaries (Composite Metal Services, Ilkley, UK) of 25 μm I.D. × 375 μm O.D. were used. Total capillary length was 58.5 cm, and effective capillary lengths for normal/short end-injection were 50.0/8.5 cm (DAD), 43.4/15.1 cm (CCD, cell 1) and 44.9/13.6 cm (CCD, cell 2). The capillary cassette was thermostatted at 25 °C with forced air-cooling. Samples were hydrodynamically injected at 50 mbar for 4 s. The applied positive voltage was 29,880 kV (set at 30 kV), as averaged from the recorded voltage signal.

The water content of pure nitromethane and BGEs was measured with a coulometric Karl Fischer titrator (756 KF Coulometer, Metrohm, Herisau, Switzerland).

2.2. Reagents

Nitromethane (HPLC grade 96+%; the purity of the lots used was 99.43%) was from Aldrich (Steinheim, Germany). Analytes injected were *p*-nitrophenol (98%), pyrene (99%, both Aldrich), *p*-toluic acid (4-methylbenzoic acid, 98%), *p*-chlorobenzoic acid (both EGA-Chemie, Steinheim, Germany), phenylacetic acid (99%, Fluka, Steinheim, Germany), tetramethylammonium (TMA) chloride (97%, Aldrich), tetraethylammonium (TEA) chloride (98%, Sigma, Steinheim, Germany) and tetrabutylammonium (TBA) chloride (>97%, Fluka). BGEs were prepared with benzoic acid (>99%, Fluka), tetramethylammonium benzoate (>98%, Fluka) and tetrapropylammonium (TPA) perchlorate (>98%, Fluka). Hydranal-Coulomat AD Karl Fischer reagent from Riedel-de Haën (Seelze, Germany) was used for coulometric titrations of the water content. All chemicals were used as received. Water was double distilled from a quartz apparatus.

2.3. Procedures

Water uptake experiments were performed in two open vessels containing 20 mL of pure nitromethane each, both exposed to atmospheric moisture. One of these vessels was subjected to gentle stirring with a magnetic bar. Relative humidity during the measurements was 44 ± 2%, temperature was 21.7 ± 0.3 °C. These parameters were measured with a Rotronic Hygroskop DV-2 meter (Bassersdorf, Switzerland).

The BGEs used to measure the mobilities of several analytes at different pH were prepared by mixing the required amount of benzoic acid (pK_a of 19.5 at 25.0 ± 0.3 °C [6]) with tetramethylammonium benzoate in NM. The BGEs used to determine the mobility of the analytes at varying ionic strength were prepared from tetrapropylammonium perchlorate. All BGEs were degassed after preparation in an ultrasonic bath.

Several neutral solutes were tested as electroosmotic flow markers (pyrene, phenanthrene, naphthalene, benzene, aniline and pyridine) and they all exhibited the same behaviour. Pyrene was finally selected due to its high molar extinction coefficient in a range of wavelengths used for UV detection of the analytes.

3. Results and discussion

Despite nearly not used so far, NM can be considered as a suitable solvent for CE due to its favourable thermal properties (its melting and boiling point allow its use under laboratory conditions), its relative permittivity (which is high enough to dissociate electrolytes in solutions), and its low viscosity favourable for fast analysis and comfortable manipulation of the solutions. One possibly restrictive aspect that has to be considered as well is related to its optical properties, because it absorbs light in the UV range. According to the literature the UV cut-off is 380 nm [5] (the cut-off is defined as

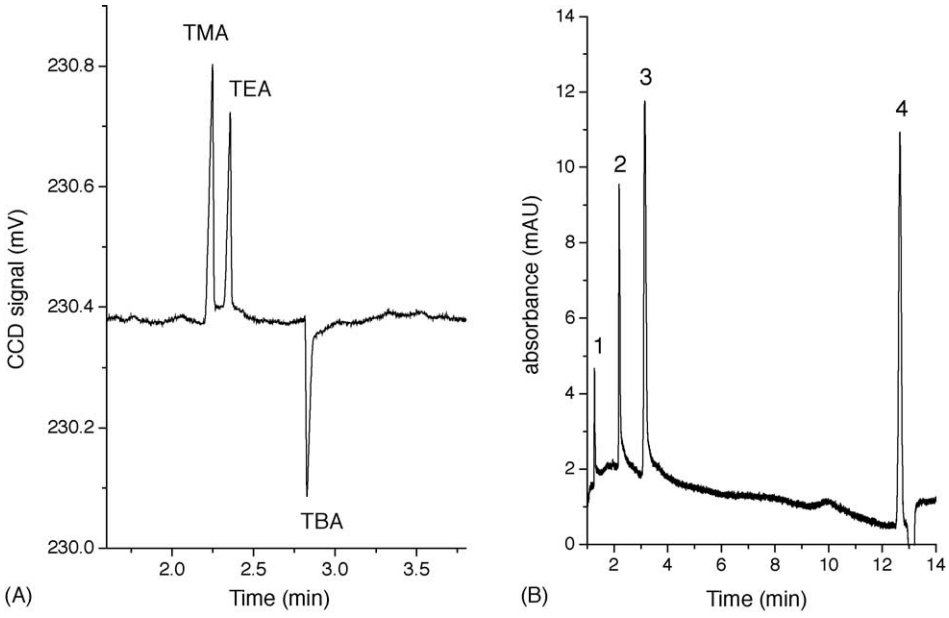

Fig. 1. Electropherogram of (A) tetraalkylammonium cations: TMA, TEA and TBA obtained with contactless conductivity detector (CCD). BGE: unbuffered, 30 mmol/L tetrapropylammonium perchlorate. (B) Neutral analytes: (1) *p*-nitrophenol; (2) *p*-chlorobenzoic acid; (3) *p*-toluic acid; (4) pyrene; obtained with UV absorbance detection at 240 nm. BGE: unbuffered, 20 mmol/L TMA chloride. Sample components 1, 2 and 3 are injected from the short end, sample 4 from the normal end of the capillary. Experimental conditions: uncoated fused silica capillary, I.D./O.D. 25/375 μm, total length 58.5 cm, effective lengths for normal/short-end injection 50.0/8.5 cm (DAD), 43.4/15.1 cm (CCD). Temperature 25 °C. Voltage +30 kV.

the point where the absorbance in a 1.0 cm light-path-length cell against dry air is 1.0). However, this absorptivity does not exclude its use as solvent in CE (as will be shown below) and because conductivity detection is not interfered by the optical behaviour of the solvent. Note that the use of the conductivity detector extends not only the applicability range of the solvents, but also that of the potential components for the background electrolyte: UV absorbing BGE components can be applied without any restriction. Note also that the conductivity detector has a further advantage being able to detect non-UV absorbing analytes. Its disadvantage in many cases is related to the tendency for triangulating peaks, which has the cause in the relatively high analyte concentrations often needed for detection (the same holds, by the way, for indirect UV detection).

An example for an electropherogram obtained in NM with such separands is given in Fig. 1A. It shows the separation of tetraalkyammonium ions in a non-buffered BGE consisting of tetrapropylammonium (TPA) perchlorate. Tetramethylammonium (TMA) and tetraethylammonium (TEA), both exhibiting a higher mobility than TPA, give positive peaks, whereas tetrabutylammonium (TBA) gives a negative one due to its lower mobility. A second example (Fig. 1B) demonstrates the possibility of UV detection at 240 nm even with this light-absorbing solvent. Separation was carried out in a non-buffered salt solution (20 mmol/L TMA^+Cl^-), in which the analytes should not be ionised by protolysis (the analytes are very weak acids). However, although being not dissociated, they are migrating as anions (note that the sample is injected from the short end of the capillary). Their anionic form indicate that complex formation with the chloride ion of the BGE due to heteroconjugation is the cause for their charge (for detailed discussion, see Ref. [13]). This phenomenon will be discussed below in more detail.

3.1. Mobility, ionic strength, viscosity

The actual mobility of the ions (i.e. the mobility of the fully charged ion at a certain ionic strength) depends on the ionic strength. According to the extended DHO theory this dependence is formulated for a monovalent 1:1 electrolyte as

$$\mu_{\mathrm{act},i} = \mu_{0,i} - \left[\frac{8.204\times10^5}{(\varepsilon T)^{3/2}}\mu_{0,i} + \frac{4.275}{\eta(\varepsilon T)^{1/2}}\right] \times \frac{\sqrt{I}}{1+50.29a(\varepsilon T)^{-1/2}\sqrt{I}} \quad (1)$$

where $\mu_{\mathrm{act},i}$ is the actual mobility, $\mu_{0,i}$ the absolute mobility (i.e. the mobility of fully charged ion at zero ionic strength), ε the relative permittivity, T the absolute temperature, η the dynamic viscosity (in Pa s or cP) and a is the ion size parameter or distance of closest approach (in Å). All mobilities are in $10^{-9}\ \mathrm{m^2\ V^{-1}\ s^{-1}}$.

It can be seen from Eq. (1) that the decisive solvent-related physical properties are the relative permittivity and dynamic viscosity. In order to obtain an insight into the extent of the influence of the ionic strength on the mobility in nitromethane, and to compare it with other solvents, we have calculated

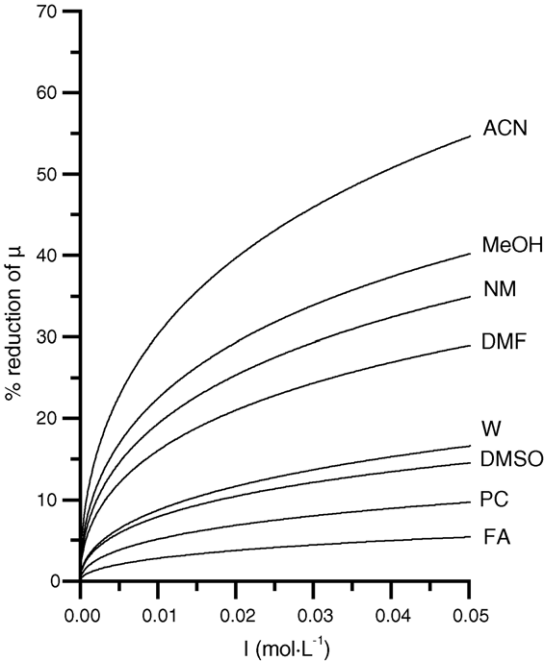

Fig. 2. Theoretical reduction of the mobility, μ, as a function of ionic strength, I, in several solvents according to the extended DHO theory (Eq. (1)). For all solvents a hypothetical absolute ion mobility of 40×10^{-9} m^2 V^{-1} s^{-1} and a distance of closest approach of 5 Å was taken. Temperature 25 °C. Abbreviations: ACN, acetonitrile; MeOH, methanol, NM; nitromethane, DMF; *N*,*N*-dimethylformamide; W, water; DMSO, dimethylsulfoxide; PC, propylene carbonate; FA, formamide.

the decrease in mobility according to Eq. (1) with solvent data taken from ref. [5]. For simplicity the calculation was carried out for hypothetical ions with an absolute mobility of 40×10^{-9} m^2 V^{-1} s^{-1} and 5 Å as distance of closest approach (we neglect at this stage that a particular ion will exhibit different absolute mobilities in the different solvents).

The relative reduction of the absolute mobility increases with ionic strength (Fig. 2) as predictable by Eq. (1). Only considering monocharged 1:1 electrolytes (for higher charged ions Eq. (1) has to be extended; moreover, the effect is much larger) the following conclusions can be drawn from the plots. The decrease in water is not very pronounced as it reaches, e.g. at $I = 50$ mmol/L, not more than 15% compared to infinite dilution. This is in fair agreement with practical daily experience. The influence is less than for water in case of formamide (FA), dimethyl sulfoxide (DMSO) and propylene carbonate (PC), but a much higher effect is predicted for acetonitrile (ACN), methanol (MeOH) and *N*,*N*-dimethylformamide (DMF). NM behaves rather similar to MeOH, and thus the reduction of the mobility with ionic strength is much more pronounced here than in water.

The theoretical dependence of μ on I was examined experimentally for three permanent monovalent ions, TMA, TEA and TBA. TPA could not be used as analyte because it was a constituent of the BGE (TPA perchlorate). The resulting mobilities decrease nonlinearly for ionic strength between 2.5 and 75 mmol/L (see Fig. 3). The data match to Eq. (1),

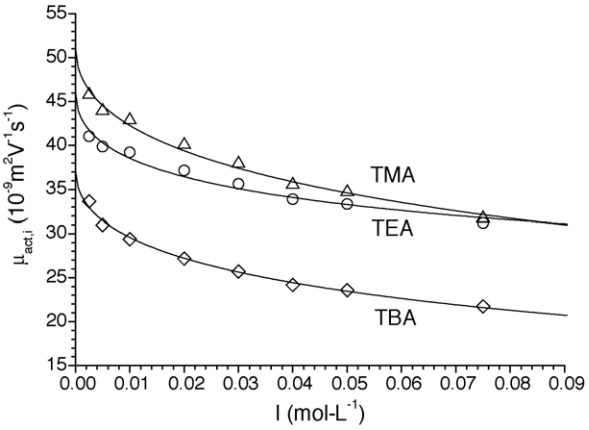

Fig. 3. Experimental actual mobilities, $\mu_{act,i}$, of tetramethylammonium (TMA), tetraethylammonium (TEA) and tetrabutylammonium (TBA) as a function of ionic strength. Temperature 25 °C. Relative standard deviation for the measurement of the mobilities ($n = 3$) was typically 0.4%. Solid lines are fitted curves according to Eq. (1).

as seen from the fitted curves in the figure. We can therefore conclude that the mobility dependence on I follows quite well the extended DHO theory.

Extrapolation of the curves to $I = 0$ leads to the absolute mobilities, which are given in Table 1. They range between 37.2 and 51.1×10^{-9} m^2 V^{-1} s^{-1}. Although the indeterminate error for measurement of the mobility is only in the range of about 1%, the absolute mobility is possibly more biased due to the asymptotic nature of the extrapolation to zero I. Parameter a, the distance of closest approach, can also be derived from the curve fitting. The resulted values are between 4 and 7 Å in the case of the selected analytes, which are realistic concerning the sizes of the ions.

Moreover, the obtained absolute mobilities match well to the ones expected from Walden's rule (Table 2). This rule states that the product of dynamic viscosity and absolute mobility is constant ($\eta\mu_{0,i} = \text{const}$) at a certain temperature, independent of the solvent. Although not well obeyed by the smallest ion (TMA), the rule is followed very well especially by the largest ion (with the lowest charge density): for TBA Walden's products agree in such different solvents as NM, ACN, MeOH and FA within 5% relative, despite the viscosities vary nearly by a factor of 10.

In the measurement of the mobilities, the capillary cassette was thermostatted at 25 °C. Temperature increase due to Joule heating does not play a role under the working conditions (at 30 kV, the typical current for 10 mmol/L TMA

Table 1
Absolute mobilities, $\mu_{0,i}$, of tetraalkylammonium ions obtained by extrapolation of the fitted curve according to Eq. (1) to zero ionic strength

Solute	$\mu_{0,i}$ (10^{-9} m^2 V^{-1} s^{-1})
Tetramethylammonium (TMA)	51.1 ± 0.4
Tetraethylammonium (TEA)	46.1 ± 0.5
Tetrabutylammonium (TBA)	37.2 ± 0.3

$T = 25$ °C.

Table 2
Product of absolute ionic mobility, $\mu_{0,i}$, and dynamic viscosity, η, of the solvent (Walden product) of the tetraalkylammonium ions in different solvents

Solute	$\mu_{0,i}\eta$ (10^{-12} N V^{-1})					
	NM[a]	NM[b]	ACN[b]	MeOH[b]	FA[b]	Water[b]
Tetramethylammonium	31.4	34.69	33.44	39.31	43.94	40.94
Tetraethylammonium	28.3	30.32	30.14	34.56	35.73	29.66
Tetrabutylammonium	22.8	21.67	21.81	22.28	22.38	17.84

T = 25 °C. For error see Table 1.
[a] This work; viscosity 0.614 cP [5].
[b] Calculated from conductivities taken from [19] and viscosities from [5]. Viscosities: NM, 0.614; ACN, 0.341; MeOH, 0.551; FA, 3.302; water, 0.8903 cP.

benzoate BGE was 2.5 μA; for 75 mmol/L TPAClO$_4$ it was 17.5 μA), because no significant changes of the mobilities were observed when repeating the measurements with lower applied voltages (20 and 10 kV).

It should be mentioned that a strong functional effect of I on μ has the consequence that the separation efficiency, expressed by the ultimate plate number, N^{ult}, is also strongly affected by the ionic strength, and is always decreased. N^{ult} considers the limiting case that only longitudinal diffusion is the source of peak broadening. It depends on the ratio of mobility and diffusion coefficient of the analyte. Both parameters are correlated at zero ionic strength according to the Nernst–Einstein relation, resulting in the well-known equation $N^{\text{ult}} = 19.46zU$, where z is the charge number of the ion, and U is the voltage. Note that at infinite dilution all solvents should have the same ultimate plate number (for a given voltage). However, the ionic strength dependence of the diffusion coefficient and mobility is not the same. As at finite electrolyte concentrations the mobility is decreased by both, the relaxation effect and the electrophoretic effect, but the diffusion coefficient only by the relaxation effect, the former parameter is more reduced with increasing ionic strength than the latter. As a consequence plate number is always lost at finite ionic strength compared to the case with $I = 0$, and it can be predicted that the reduction will be as more pronounced as stronger a functional dependence of μ on I is. This aspect is discussed in detail for various solvents in previous papers [1,2,14]. In practice (at ionic strengths of several tenths of mmol/L) NM should be a less favourable solvent than water when the separation efficiency is considered.

3.2. pH scale and effective mobilities

By the aid of the commonly used glass electrode the pH is derived from the difference in the electrochemical response of pH-sensible electrodes, e.g. according to $\text{pH} = \text{pH}_{\text{st}} + (E - E_{\text{st}})F/RT \ln 10$, where E is the cell potential of the sample of a certain pH, E_{st} the standard cell potential of a buffer of known pH_{st}, F the Faraday constant and R is the gas constant.

In a normal glass electrode, the electric contact between the glass sensitive membrane electrode and the reference electrode is produced through the external solution by means of a porous material, normally a frit. A low current is established when the ions of the internal reference filling solution and the external solution migrate across the frit. If these ions have different mobilities a charge separation is produced and a liquid junction potential is generated. This potential is strongly affected by the nature of the solvent at both sides of the membrane. If the solvent is the same in the filling reference solution and in the external solution this contribution is ideally cancelled, because then it has the same magnitude in calibration and in pH measurement. Consequently, the same solvent as in the sample should be used in the reference solution and in the calibration solutions in order to minimize the liquid junction potential effect. This means that, in the present case, the glass electrode had to be adapted with a reliable NM reference filling solution, and the preparation of trustworthy pH standard solutions with an accurately known and stable pH. However, such special glass electrodes are hard to maintain, and the solutions might not be stable under the habitual working conditions in a chemistry laboratory, especially under atmospheric moisture.

An alternative for the problematic use and maintenance of the glass electrode is the application of a buffer composed from an acid with known pK_a in the given solvent, and its conjugated base, mixed at defined ratios. The Henderson–Hasselbalch equation (with activity correction) allows then calculating the pH of a solution from the composition of the buffer according to

$$\text{pH} = \text{p}K_a - \log\left(\frac{a_{\text{HA}}}{a_{\text{A}}}\right) \quad (2)$$

where pH is the pH value in the organic solvent scale, pK_a the dissociation constant of the buffering acid–base pair, a_{HA} the activity of the acid and a_{A} is the activity of the conjugate base of the acid. Strictly speaking, Eq. (2) considers only the acid–base equilibria, not parallel or side equilibria like ion pairing or homo- and heteroconjugation. It is obvious that the requirement for applying a pH scale with this concept is the knowledge of the accurate pK_a of the acid in the given solvent.

Using this concept, we established a pH scale with benzoic acid (with the known pK_a of 19.5 [6]) and its tetramethylammonium salt in different proportions. Keeping constant the salt concentration at 10 mmol/L led to the same constant ionic strength in all electrolytes. The activity of the anion, a_{A}, was calculated from the product of the anion concentration, c_{A}, and the activity coefficient, f_i. The latter was calculated from $-\log f_i = (Az_i^2 I^{1/2})/(1 + aBI^{1/2})$ according to the extended theory of Debye and Hückel, taking the mean

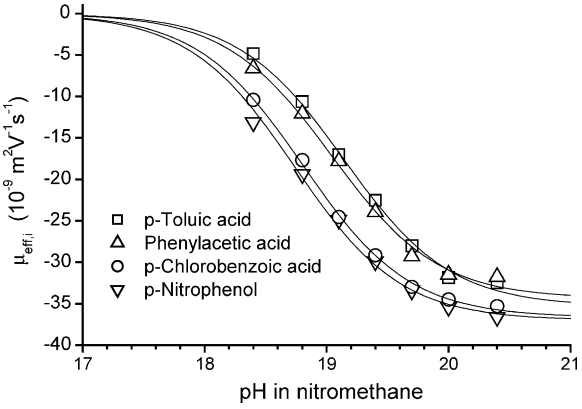

Fig. 4. Measured effective mobilities, $\mu_{\mathrm{eff},i}$, vs. the pH of the BGE. The BGE consisted of tetramethylammonium benzoate (10 mmol/L) and benzoic acid at different proportions. The pH was calculated by means of the Henderson–Hasselbalch equation (Eq. (2)), corrected for the activity of the buffer anion. Relative standard deviation for the measurement of the mobilities ($n \geq 3$) was typically 0.7%. Temperature 25 °C. Solid lines are the fitted curves according to Eq. (3).

distance of closest approach as 5 Å. *A* and *B* are the appropriate parameters for NM. The activity coefficient for the ionic strength of 0.010 mol/L was then 0.737. The activity of the molecular acid, HA, was taken equal to its concentration.

Considering this activity correction, the pH scale was shifted by −0.13 units compared to the concentration-based scale. Agreement with the Henderson–Hasselbalch conditions in this solvent system was proved for four different compounds, for which the effective mobilities were determined as a function of the pH.

These effective mobilities, $\mu_{\mathrm{eff},i}$, are depicted in Fig. 4. In the same figure the curves are obtained by fitting the data to

$$\mu_{\mathrm{eff},i} = \frac{\mu_{\mathrm{act},i}}{1 + 10^{\mathrm{p}K_{\mathrm{a}} - \mathrm{pH}}} \quad (3)$$

which gives the effective mobility as a function of the pK_a of the analyte and the pH of the solution. Fittings were carried out taking a value of zero for the analyte mobility at pH lower than 14 (which is more than three pH units smaller than the pK_a of the samples, see below). It can be concluded that the fitted mobility versus pH curve is well followed by the measured data points for all analytes.

This agreement is rather surprising when taking into account that strong homoconjugation occurs in NM (see Ref. [6]; for a detailed discussion, see e.g. [15]). In fact, a rather high homoconjugation constant, K^{f}, of 5620 L/mol ($\log K^{\mathrm{f}} = 3.75$) [6] has been reported for the present reference buffer acid, benzoic acid (water content of NM 5 mmol/L). It is known that an increase in water content decreases homoconjugation due the competition between the water and the acid molecules in hydrogen bonding. We can thus expect that under the present conditions with a water content being higher than 5 mmol/L the effect of homoconjugation is most probably less pronounced, whereas we cannot quantify it due to the lack in supporting data in the literature. Anyway, for a given initial concentration ratio of HA and A^-, homoconjugation would change the pH. Consequently, the mobilities determined in a buffer with given initial $c_{\mathrm{HA}}/c_{\mathrm{A}}$ should not match to Eq. (3). It is seen in Fig. 4 that in fact they do, which leads us to conclude that for the BGE under discussion homoconjugation does not play the role it should according to literature data.

Heteroconjugation data are not available from the literature for the analytes and buffer constituents depicted in the plots in Fig. 4. We think that we can exclude heteroconjugation between these specific analytes and benzoate, because the mobilities match very well to the curves when values of zero are taken for μ at low pH. If heteroconjugation would play a role, finite (negative) values for the mobilities at low pH would otherwise lead to a better match.

It is, however, evident that in our system heteroconjugation takes place under other conditions, which follows from the electropherogram shown in Fig. 1B. In this case, chloride as constituent of the BGE forms anionic heteroconjugation complexes with the neutral analytes, which are thus electrophoretically separated.

Note that neither homo- nor heteroconjugation influences the ionic strength of the BGE.

3.3. pK_a values

Although the analyte mobilities are well following the fitted curves (Fig. 4), only for *p*-chlorobenzoic acid the pK_a derived from curve fitting is identical with the literature data (Table 3). For the other analytes the deviation between our values and those given in the literature (determined by potentiometric method using a glass electrode [6]) is between 0.8 and 1.3 units. This is noticeable because the literature pK_a values for both, the reference acid and the analytes, are reported by the same authors (see citations given in ref. [6]), and it is assumed that the data is measured under identical experimental conditions. An explanation for the pK_a difference might be the occurrence of secondary equilibrium, which shifts the measured mobilities of some analytes and thus the pK_a values. Again, this is hard to confirm due to lack of the literature data for such equilibria. However, the difference between our pK_a values and published data is even much smaller than the pK_a discrepancy found in some cases for an individual acid reported in the literature (see the compilation of Izutsu [6]).

Table 3
pK_a values of neutral acids in NM derived from the measured mobilities as function of the pH

Solute	pK_a	Literature pK_a [6][a]	pK_a in water [20]
p-Nitrophenol	18.74 ± 0.03	20.1	7.14
Phenylacetic acid	19.04 ± 0.03	20.1	4.31
p-Toluic acid	19.14 ± 0.02	19.95	4.37
p-Chlorobenzoic acid	18.81 ± 0.01	18.8	4.00

Literature values for NM and water are given for comparison. $T = 25$ °C.
[a] Water content 5 mmol/L.

The difference in pK_a values, $\Delta pK_a^{NM-W} = pK_a^{NM} - pK_a^{W}$, of the carboxylic acids in Table 3 between NM and water is 14 units (for phenol, it is 11.6 units only). This total shift is much larger than for methanol, and slightly smaller than for acetonitrile. ΔpK_a^{NM-W} is connected to the stabilisation of the acid–base equilibrium, in particular to the stabilisation of the individual particles involved in the equilibrium in the two solvents (we have discussed about these concepts in a number of previous works (see, e.g. [2,16,17]) and readers are referred to these papers and to the literature cited therein). For the present analytes and buffer acid, which are of type HA, the particles are the proton, the anion and the molecular acid. The standard free energy of transfer, ΔG_t^0, is related to ΔpK_a^{NM-W} by basic thermodynamics. ΔG_t^0 for the proton is 95 kJ mol^{-1} from water to NM [18]. Positive value means that NM is less basic than water (it is even less basic than ACN, which has a ΔG_t^0 for the proton of 46.4 kJ mol^{-1} [18]). The contribution of the proton destabilization in NM to the pK_a shift is accordingly 4.0 pK_a units. Unfortunately, data for ΔG_t^0 of molecular acids is scant; only for acetic acid data could be found, not for the analytes. However, for acetic acid the pK_a shift is similar (from 4.76 in water to 20.5 in NM [6]). For acetate ΔG_t^0 is positive (56 kJ mol^{-1} [18]), which allows the conclusion that anionic carboxylates are destabilised in NM. The contribution of acetate on the pK_a shift is accordingly 3.2 pK_a units. Both effects contribute to the reduction of the acidity in NM compared to water, but they cannot fully explain the total shift. The remaining contribution must come from the better stabilisation of the molecular acid in NM compared to water. It is expressed by the higher solubility of the relatively lipophilic organic acids in NM.

3.4. Water uptake

Organic solvents have an intrinsic trend to be hygroscopic. This has to be taken into account because under normal analytical laboratory conditions there is no protective anhydrous atmosphere to prevent from the uptake of water. As the water content can change the properties of organic solvents, it is important to control at least roughly how much water the solvent takes up from the laboratory atmosphere. For this purpose the water concentration of NM stored in an open vessel was measured as a function of time of exposure under conditions of a typical laboratory environment.

It was found that the water content of freshly opened NM was lower than 0.03%, in agreement with the specifications of the manufacturer. As seen in Fig. 5, the water content increases when the solvent is exposed to laboratory air; it is clear that the increase is stronger in the stirred than in the non-stirred vessel. However, the content was less than 0.5% (all water concentrations are given in % w/w) within about 2.5 h, independently whether the solvent was stirred or not. After 5 h of exposure the content reached 1%, after 10 h it was less than 2%, and after 24 h the curves flattened at contents lower than 3%.

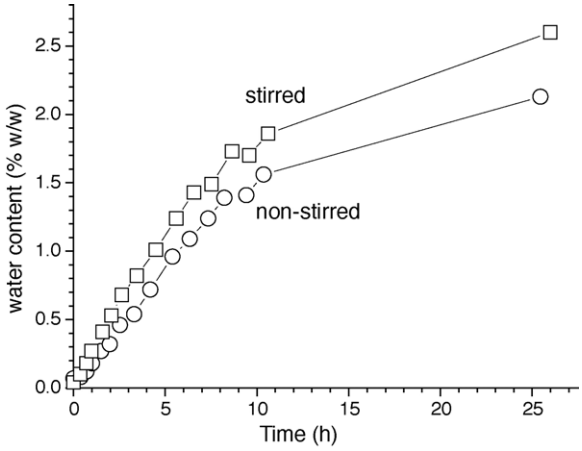

Fig. 5. Water content of pure nitromethane exposed to atmospheric moisture as a function of the time of exposure. Relative humidity 44 ± 2%, temperature 21.7 ± 0.3 °C.

It is clear that the content itself does mean anything if not related to the possible extent of changes in the properties of the analytes and the BGE constituents. One of the most important influences is on the pK_a values. The prediction of the detailed pK_a shift with water content is not easy to make because the pK_a values do not linearly depend on the composition of the mixed aqueous–organic media. However, it can be assumed that the influence of small amounts of water will be smaller when ΔpK_a^{NM-W} is small. For the carboxylic acids in Table 3, ΔpK_a^{NM-W} is about 14 units. Under the (unrealistic) assumption that the pK_a shift is a linear function of the water content, the decrease of the pK_a values in NM would be about 0.14 pK_a units per % water. It is expected that in reality the decrease is much more pronounced at the side of low water concentrations, because there the slope of the pK_a versus % water curve is normally steeper. However, when the analytes and the reference acid taken for the adjustment of the pH are both neutral acids (of type HA), their behaviour is similar. This means that the change will result in an only parallel shift of the data. For accuracy reasons the change of the pK_a values should be taken into account even for an uptake of say few tenth % water in NM, which might not be exceeded when some care is taken on the protection of the solvent. Although in this case the shift of few tenths pK_a units is perhaps lower than the difference in pK_a data taken from different sources (see, e.g. [6]), its fluctuation with varying water concentrations can lead to a low reproducibility of the electrophoretic behaviour.

4. Conclusions

Nitromethane as solvent in capillary electrophoresis has certainly the advantage over water that it dissolves lipophilic compounds much better. The behaviour of electrolytes in this

solvent can be well described by the models developed for electrochemistry and solution chemistry. Consequently, the decrease of the actual mobility with increasing ionic strength follows the dependence formulated by the extended theory of DHO. The establishment of a pH scale using BGEs with different ratios of an acid and its salt leads to a good agreement between effective mobility and pH according to the Henderson–Hasselbalch equation for the selected experimental conditions. This is surprising because the expected homoconjugation should alter the pH scale.

Conjugation clearly takes place in NM. This is documented by the high values of the homoconjugation constants published in the literature. Also, it is demonstrated in the present work by the separation of neutral analytes upon complex formation with chloride from the BGE.

The pK_a values of the analytes are shifted by about 11–15 units compared to water. Interestingly they are even levelled in NM: whereas the maximum difference in pK_a between the phenolic analyte and the carboxylic acids is more than +3 units in water, it is −0.4 units in NM.

Although the optical properties of NM may restrict its use in CE, it is still possible to measure analytes with UV detection. With the conductivity detector no such restrictions exist.

Acknowledgements

X.S. thanks the MCYT of the Spanish Government (project BQU2001-3226) for financial support. S.P. thanks the Academy of Finland for financial support (grant No. 204829). The authors acknowledge the loan of a KF coulometer from Professor M.-L. Riekkola (University of Helsinki, Finland), and the gift of the CCD from Professor B. Gaš (Charles University, Prague, Czech Republic).

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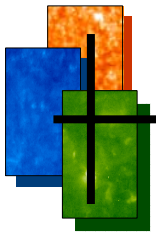
ARTICLE VI

Comparison of methanol and acetonitrile as solvents for the separation of sertindole and its major metabolites by capillary zone electrophoresis



X. Subirats, S. Reinstadler, S.P. Porras, M.A. Raggi and E. Kenndler

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Xavier Subirats[1, 2]
Sigrun Reinstadler[1, 3]
Simo P. Porras[4]
Maria Augusta Raggi[3]
Ernst Kenndler[1]

[1]Institute for Analytical Chemistry, University of Vienna, Vienna, Austria
[2]Department of Analytical Chemistry, University of Barcelona, Barcelona, Spain
[3]Faculty of Pharmaceutical Sciences, University of Bologna, Bologna, Italy
[4]Laboratory of Analytical Chemistry, Department of Chemistry, University of Helsinki, Helsinki, Finland

Comparison of methanol and acetonitrile as solvents for the separation of sertindole and its major metabolites by capillary zone electrophoresis

Sertindole (1-[2-[4-[5-chloro-1-(4-fluorophenyl)-1*H*-indol-3-yl]-1-piperidinyl]ethyl]-2-imidazolidinone), an atypical antipsychotic drug, was separated by capillary electrophoresis from its two main metabolites norsertindole and dehydrosertindole. The low solubility of the analytes in water (octanol-water partition coefficient is about 10^5) is overcome by the use of methanol (MeOH) and acetonitrile (ACN) as solvents for the background electrolyte (BGE). Mobilities were measured in BGEs with defined pH in a broad range. It was found that in MeOH the mobility of the analytes is mainly governed by acid–base equilibria, whereas in ACN other reactions like ion pairing and homoconjugation play a pronounced role and lead to a complex pattern of the mobility as function of the pH. However, separation can be obtained in less than 10 min in both solvent systems.

Keywords: Capillary zone electrophoresis / Homoconjugation / Metabolites / Mobility / Non-aqueous solvents / Sertindole DOI 10.1002/elps.200500056

1 Introduction

Sertindole (Serlect®, 1-[2-[4-[5-chloro-1-(4-fluorophenyl)-1*H*-indol-3-yl]-1-piperidinyl]ethyl]-2-imidazolidinone, SRT) is a new nonsedating atypical antipsychotic drug with high selectivity for dopaminergic neurons in the mesolimbic system [1], which has shown efficacy against both positive and negative symptoms of schizophrenia [2]. Sertindole also has affinity for adrenergic [3] and serotonergic receptors [4]; in particular, it is a serotonin 5-HT_{2C} receptor inverse agonist [5] like clozapine (the parent drug of the atypical antipsychotic class), and this can contribute to therapeutic and side effects [6].

Sertindole was withdrawn from the market during 1999 because of QT interval prolongation observed in some patients [7]. Subsequently, however, no association with an excess of cardiac or all-cause mortality [8] was found during sertindole treatment. Thus, the European Agency for the Evaluation of Medicinal Products (EMEA) has re-evaluated the drug in September 2002 and concluded that it could be re-introduced, provided that the maximum dose is reduced to 20 mg/day, extensive ECG monitoring is carried out before and during treatment, and extensive contraindications and warnings for patients at risk of cardiac dysrhythmias are added to the patient information sheets.

Apart from cardiac side effects, sertindole appears to be well tolerated and to be associated with a very low incidence of extrapyramidal side effects [9], which are the most worrisome side effects of classical antipsychotics (such as chlorpromazine and butyrophenones). Other side effects of sertindole include weight gain, rhinitis and possibly male sexual dysfunction [2].

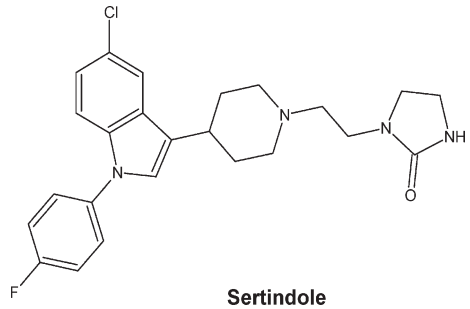
Sertindole is metabolized by hepatic cytochrome P450 (CYP) enzymes, namely by CYP2D6 and CYP3A4 isoforms [10], resulting in the formation of dehydrosertindole and norsertindole [11], which are the main plasma metabolites. The structures of the compounds are shown in Fig. 1.

Only a few analytical methods have been reported for the determination of sertindole and metabolites in plasma or serum, based on HPLC in combination with spectrophotometric detection [12, 13] or with different mass spectrometric techniques [13–15] mainly for screening purposes [16–18].

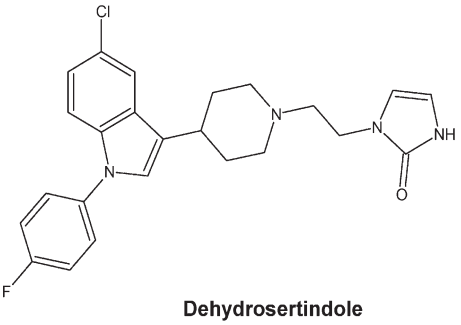
From Fig. 1 it can be seen that the analytes comprise several nitrogen atoms in their molecules, one of them, that in the piperidine ring, forming a secondary

Correspondence: Prof. Ernst Kenndler, Institute for Analytical Chemistry, University of Vienna, Währingerstr. 38, A-1090 Vienna, Austria
E-mail: ernst.kenndler@univie.ac.at
Fax: +43-1-4277-9523

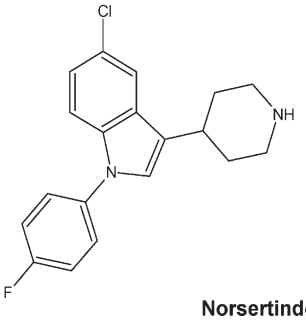
Abbreviations: MeOH, methanol; $\mathbf{\Phi_4P^+}$, tetraphenylphosphonium



Sertindole



Dehydrosertindole



Norsertindole

Figure 1. Structures of sertindole, dehydrosertindole and norsertindole.

(norsertindole) or a tertiary amino group (sertindole and dehydrosertindole). These groups are moderately basic, and can thus be protonated at not too high pH. This property makes the analytes accessible to electrophoretic separation. However, the compounds possess low aqueous solubility due to their high lipophilicity – sertindole has a log P in the range of 5 [19] – and thus the detection limit by UV absorbance detection is hardly reached. This restriction can be overcome by applying organic liquids as solvents for the background electrolyte (BGE). From the several candidate solvents, methanol (MeOH) and acetonitrile (ACN) have, besides other advantages, the advantage of being not UV-absorbing in the wavelength range of interest; both have a UV cutoff at about 200 nm. They were therefore used in the present work. Although these solvents have a similar dielectric constant (MeOH 32.66, ACN 35.94) [20], they behave very differently concerning their solvation properties. MeOH is a protic solvent with pronounced hydrogen bonding ability, whereas ACN is a dipolar, aprotic solvent. MeOH is able to solvate both cations and anions (although less than water) and ACN has a very poor solvation ability for both types of ions (especially for anions). MeOH is slightly less basic than water, ACN is many orders of magnitude less basic (see, *e.g.* [21]). Their autoprotolysis constants are 16.91 (MeOH) and 32.2 (ACN) [20]. It is the goal of the present work to investigate the electrophoretic behavior of the analytes in these two different solvents. The paper does not deal with the applicability of the results to the bioanalysis of the compounds in body fluids; this will be the topic of future work.

2 Materials and methods

2.1 Chemicals

MeOH was from Fisher Scientific (Springfield, NJ, USA) and ACN from J.T. Baker (both HPLC grade; Phillipsburg, NJ, USA). BGEs were prepared from trichloroacetic acid (TCA, puriss. p.a., Riedel-de Haën, Seelze, Germany), TCA sodium salt (97%, Aldrich, Milwaukee, WI, USA), dichloroacetic acid (>99%, Riedel-de Haën), potassium dichloroacetate (98%, Aldrich), chloroacetic acid (>99%, Fluka, Buchs, Switzerland), sodium dichloroacetate (98%, Aldrich), glacial acetic acid (p.a., Merck), sodium acetate (>99%, anhydrous, Fluka), perchloric acid (70%, aqueous solution, Fluka), salicylic acid (99.7%, Sigma, St. Louis, MO, USA), salicylic acid sodium salt (>99.5%, Sigma), phthalic acid (puriss. p.a., Fluka) and oxalic acid (p.a., Merck, Darmstadt, Germany). Tetramethylammonium chloride (>98%, Fluka) was used to adjust the ionic strength. Tetraphenylphosphonium tetraphenylborate (Selectophore®, Fluka) was used as internal standard for the determination of the electrophoretic mobility, and dimethyl sulfoxide (DMSO, >99%, Aldrich) as neutral marker. Tetraethylammonium hydroxide (40%) was from Fluka. The analytes used were sertindole (Lu 23–174), norsertindole fumarate (Lu 25–073-F) and dehydrosertindole (Lu 28–092) in 1000 ppm methanolic stock solutions; they were stored in the freezer. All were provided by H. Lundbeck A/S (Copenhagen, Denmark).

2.2 Apparatus

Capillary zone electrophoresis (CZE) was carried out in an HP 3DCE instrument (Hewlett-Packard, Waldbronn, Germany) using a photometric diode-array detector (DAD). Uncoated fused-silica capillaries (Composite Metal Ser-

vices, Ilkley, UK) of ID/OD 50/375 μm were used, with a total length of 58.5 cm and effective length of 50.0 cm. The capillary cassette was thermostated at 25°C with forced air-cooling. Samples were hydrodynamically injected at 50 mbar for 1.5 s. The applied positive voltage was 19 930 V, which was the average of the recorded voltage signal when set at 20 kV. The typical currents in the methanolic BGEs with 10 mM ionic strength were about 5 μA, and in the ACN solutions with 5 mM ionic strength they were between 2 and 5 μA depending on the nature of the buffer.

2.3 Procedures

BGEs were prepared by mixing the required amount of acid and conjugate base (the salt). When using MeOH as a solvent for the BGE, the salt concentration of the buffer was always 10 mM, keeping the ionic strength constant. Due to the low solubility of salts in ACN their concentrations had to be selected lower, namely at 5 mM. When it was not possible to dissolve this amount of base (*e.g.*, in the oxalic acid buffer system), tetramethylammonium chloride was added to the BGE to adjust the ionic strength to 5 mM. To obtain a soluble conjugate base of phthalic acid, tetraethylammonium hydrogenphthalate was prepared by direct titration of a methanolic solution of phthalic acid to the equivalent point with tetraethylammonium hydroxide in water, followed by the evaporation of the solvent in a rotary evaporator. All BGEs were degassed after preparation in an ultrasonic bath, and the running buffers were changed before each run. Mobilities were measured at least in triplicate. To overcome the problem of measuring a very small electroosmotic mobility at low pH for the determination of the effective mobility of the analytes, tetraphenylphosphonium (Φ_4P^+) was injected as an internal standard together with the drugs. Φ_4P^+ has a permanent positive charge, and its mobility is independent of pH at a certain ionic strength. DMSO (0.05% v/v) was used as neutral marker to determine the electroosmotic flow (EOF) in the methanolic BGEs. Analytes were dissolved in methanolic solutions, and prior to injection the samples were diluted to the desired concentration with the corresponding BGE.

3 Results and discussion

The electrophoretic mobility is the primary analytical feature that determines separation selectivity and thus resolution in CZE. Accordingly, independent of the solvent used, the first parameter to be selected in CZE of weak acids or bases is the pH of the BGE. In organic solvents, however, this is a less trivial task than in water because of the problem in defining an appropriate pH scale. In the present work we have used an approach, which was successfully applied in previous works, namely using BGEs consisting of buffer acids with known pK_a values in the particular solvent (see [21] and the literature cited therein). Buffer solutions are composed from different ratios of the acid and its conjugated base (its salt), and the pH is given according to the well-known Henderson–Hasselbalch buffer equation

$$\mathrm{pH} = \mathrm{p}K_a - \log(a_{HA}/a_A) \quad (1)$$

where pH and pK_a refer to their values in the aqueous or the organic solvent scale, and a_{HA} and a_A are the activities of the acid and the conjugate base, respectively. As usual, the activity is defined as the product of the concentration of the acid (c_{HA}) or the conjugate base (c_A), and its activity coefficient (γ_{HA} or γ_A). The activity coefficient of the neutral species of the buffer system was considered equal to unity ($\gamma_{HA} = 1$). Those of the ionic substances were calculated from the extended Debye–Hückel theory as $-\log \gamma_A = (Az_i^2\sqrt{I})/(1 + aB\sqrt{I})$, where A and B are the Debye–Hückel constants in the respective solvents and z_i is the valency of the ion; for the ion size parameter a, the value of 5 Å was taken. When the activity correction is taken into account, the whole pH scale is shifted to slightly more acidic values for the present type of buffers. It should be noted that even under these well-defined conditions the proper adjustment of the pH is hampered in solvents with low or moderate relative permittivity, and/or in solvents where the solvent molecule has low ability for hydrogen bonding. The former solvent leads to an increased tendency for ion pair formation, and the latter one is prone to interactions called homoconjugation and heteroconjugation. Ion pair formation has been reported to take place in both ACN and MeOH, but conjugation effects are much more likely in ACN than in MeOH (see, *e.g.* [21]).

It is reasonable to cover an as wide as possible pH range in the given solvent especially when absolute or actual mobilities and the ionisation behavior of the analytes of interest are not known. Even when pK_a values of the analytes are known (or can be approximated), the pH range investigated should preferably cover also the pH values where the analyte ions are fully dissociated or protonated, *i.e.*, where the actual mobility is reached. However, often the limiting factor for the selected pH range in organic solvents is the availability of suitable buffer chemicals. Unfortunately no data were found about the dissociation constants of the present analytes, even in water. All analytes (Fig. 1) contain an aliphatic nitrogen in the piperidine ring (the other nitrogens are less basic). Thus, we estimated their pK_a with regards to this group in aqueous solution by means of the SPARC On-Line Calculator [22,

23]: the calculated pK_a values are 7.96 for sertindole, 7.87 for dehydrosertindole and 10.39 for norsertindole. The pK_a values in MeOH and ACN were then estimated from solvation parameters (see below).

Before going into a detailed discussion about the mobility *versus* pH behavior in MeOH and ACN, problems associated with the measurement of the EOF mobility should be mentioned. It is clear that for the determination of the electrophoretic mobility of the analyte the EOF mobility has to be subtracted from the total mobility. It is relatively easy to measure a large EOF mobility (at high pH), because then a neutral EOF marker can be detected within an acceptably short time. However, at low pH where the capillary surface has only low charge, the time required to detect the EOF marker might be unacceptably long. This can especially be a problem in organic solvents in which the dissociation behaviour of the silanols of the capillary wall is affected. In both MeOH and ACN, the pK_a of silanol – it is a weak acid – is shifted to higher values, which means that a higher pH is needed for the ionization of the capillary wall. Accordingly, at low or moderate pH the EOF mobility might be rather low in these solvents; at very low pH it can even be reversed (see, *e.g.* [24]). In such cases alternative methods to measure the EOF mobility can be applied, *e.g.*, pressure-induced mobilisation as introduced elsewhere [25, 26]. We have taken an internal standard with known mobility at given ionic strength in the particular solvent, namely Φ_4P^+. We have controlled the literature values in MeOH by determining the actual mobilities of Φ_4P^+ at high pH, where the EOF mobility was measurable. In the methanolic system at ionic strength of 0.010 M, an average of the measured actual mobilities of Φ_4P^+ at pH values of 7.6, 8.6, 9.5 and 10.5 was $(31.8 \pm 0.2)10^{-9}$ m^2/V $\times$ s, which is relatively close to the literature value of 32.8×10^{-9} m^2/V $\times$ s [27]. In the case of ACN, the literature value of 47.78×10^{-9} m^2/V $\times$ s for Φ_4P^+ [27] was taken to determine the mobility of the analytes.

3.1 MeOH as solvent

Assuming constant solvation parameters, the pK_a values of the analytes in pure MeOH are estimated to be between 8.7 and 11.2 (see Table 1). This estimation is based on the equation ${}^{s}_{s}pK_a = 0.968\,{}^{w}_{w}pK_a + 1.171$ published in [28]. ${}^{s}_{s}pK_a$ and ${}^{w}_{w}pK_a$ are the acid dissociation constants of the analyte in solvent S and water W. The used methanolic BGEs with the final (activity corrected) pH values, which should cover the pK_a range of the analytes, are listed in Table 2, together with the pK_a values of the buffer acids. The activity coefficient at 10 mM ionic strength in MeOH is 0.706, which shifts the pH scale by ~0.2 units to more acidic values.

Table 1. pK_a values of the analytes

Analyte	pK_a				
	Water calculated	MeOH calculated	MeOH from mobility	ACN calculated	ACN from mobility
Sertindole	7.96	8.9	9.4	14.6	15.7
Dehydrosertindole	7.87	8.7	8.9	14.5	16.5
Norsertindole	10.39	11.2	11.2	18.2	13.5

Data in water were calculated by computer software, in MeOH and ACN they were calculated from solvation parameters with the data in water as basis. pK_a values in MeOH and ACN were alternatively determined from the effective mobilities by CE. For details, see text.

Table 2. Composition and pH of the buffers used in methanolic solutions

pH	Acid	pK_a [33]	Base
4.7	10 mM TCA	4.9	10 mM sodium trichloroacetate
6.1	10 mM dichloroacetic acid	6.3	10 mM potassium dichloroacetate
7.6	10 mM chloroacetic acid	7.8	10 mM sodium chloroacetate
8.6	1 mM chloroacetic acid		10 mM sodium chloroacetate
9.5	10 mM acetic acid	9.7	10 mM sodium acetate
10.5	1 mM acetic acid		10 mM sodium acetate

pK_a values are for the buffer acids in MeOH. pH values are corrected by means of the activity coefficient.

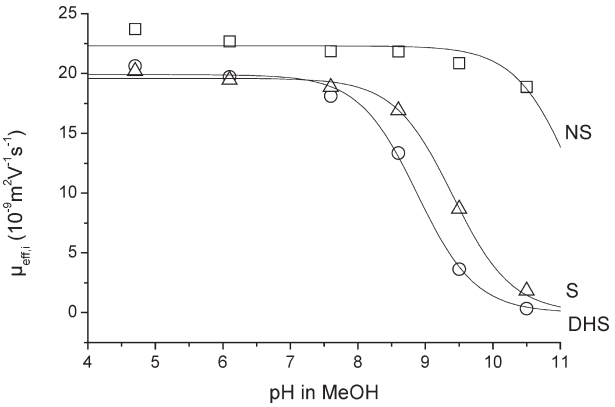


Figure 2. pH dependence of mobility in MeOH for sertindole (S), dehydrosertindole (DHS) and norsertindole (NS). BGEs: see Table 2. Temperature, 25°C.

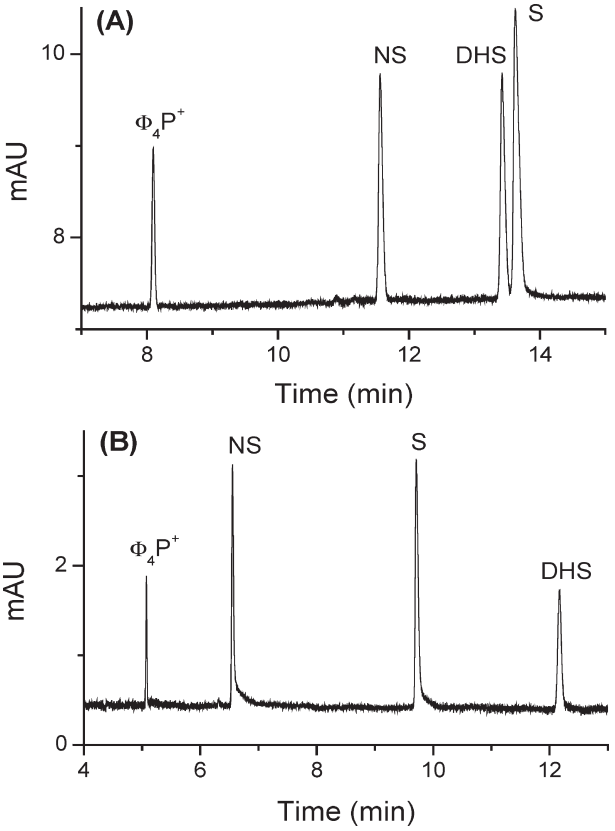


Figure 3. Electropherograms of sertindole (S), dehydrosertindole (DHS) and norsertindole (NS) in methanolic BGEs with 10 mM dichloroacetic acid/10 mM potassium dichloroacetate, pH 6.1 (A) and with 10 mM acetic acid/10 mM sodium acetate, pH 9.5 (B). Tetramethylphosphonium (Φ_4P^+) was used as internal standard to determine the effective mobilities of the analytes. Experimental conditions: +20 kV, 50/375 μm ID/OD uncoated fused-silica capillary, 50.0 cm effective length and 58.5 cm total length, 25°C, wavelength of 224 nm and injection of analytes (0.20 μg/g) at 50 mbar for 1.5 s.

In Fig. 2, the effective mobilities, $\mu_{eff,i}$, of the three analytes are depicted, measured at different pH of the BGEs. They are fitted as function of the pH to the equation

$$\mu_{eff,i} = \frac{\mu_{act,i}}{1 + 10^{pH - pK_a}} \quad (2)$$

where $\mu_{eff,i}$ is the mobility of a weak cationic acid at a certain ionic strength corrected by its ionization degree, and $\mu_{act,i}$ is the actual mobility, *i.e.* mobility of the fully charged analyte at working ionic strength. The pH is referred to the BGE in the methanolic scale and the pK_a is the acid dissociation constant of the analyte in MeOH. In the denominator of Eq. (2) the exponent is (pH - pK_a), because the drugs are considered as cation acids ($BH^+ = B + H^+$) under the present conditions.

It can be seen that the data match rather well to the fitted curves, which means that mainly acid–base equilibria determine the mobility. Some small deviations are, however, visible and this will be discussed later in this section. The result of the fitting to Eq. (2) leads to two parameters, the actual mobilities and the pK_a values. The actual mobilities obtained for dehydrosertindole, sertindole and norsertindole were 19.9 (±0.4), 19.6 (±0.2) and 22.3 (±0.4), all in 10^{-9} $m^2/V \times s$, respectively. The pK_a values were 8.88 (±0.05), 9.41 (±0.04) and 11.2 (±0.2). Obviously, the confidence for the pK_a value of norsertindole is not as high as for the other two analytes because of the lack of experimental points at pH higher than 11. However, the aim of the present work is not the determination of the accurate pK_a. In such a case it would be preferable to have more data points in the range where the degree of dissociation varies. In case of norsertindole, this could be done, *e.g.*, by using some diprotic carboxylic acid as buffering component (see [29]). Despite this restriction, the agreement between the pK_a values calculated from solvation parameters and those derived by CZE is remarkable; note that the computer calculations of the pK_a values in the organic solvents are even based on their approximation in water. For sertindole the deviation is 0.5 units, for dehydrosertindole it is only 0.2 pK_a units; for norsertindole the values are even identical.

It was found that at all applied pH values the analytes were resolvable; their mobilities were different enough, even at low pH. At pH 6.1 separation takes place according to the actual mobilities, and dehydrosertindole migrates close to sertindole (Fig. 3A), which is not surprising due to the almost same size and the very similar structure: the two compounds differ only by one double bond in the five-ring (see Fig. 1). It is even more surprising that this small difference leads to a different actual mobility. At pH 9.5 separation is much better (Fig. 3B) as here

the analytes are separated according to their effective mobilities. Under all conditions analysis times were larger than 10 min.

It is mentioned above that some small deviations of the mobilities of the analytes from the theoretical pH curves are seen (Fig. 2). This is especially visible for norsertindole, which is, according to these data, the weakest cation acid (the strongest base) of the analytes. Norsertindole is protonated over a wider pH range than the other two analytes, *i.e.* most of the measured mobilities are actual mobilities; only at highest pH (>8.7) it seems to be partially uncharged. A closer look on the data at lower pH shows that the mobility values are not constant but are actually slightly scattering. However, the scatter is larger than the experimental error (the relative standard deviations in all buffers in MeOH were typically less than 1%). This might be due to equilibria other than protolysis of the analyte, *e.g.*, due to ion pairing between the analyte cation and the BGE counterions. Obviously, when different counterions are applied in the different buffers (like in the present work), the degree of ion pairing at each pH might be different. Unfortunately, there are no supporting data available in the literature for ion association of the present analytes and the BGE anions. However, the change in mobility in Fig. 2 seems to be caused by ion pairing even though the ionic strength of the BGE is the same at every pH. This assumption is supported by the finding that, *e.g.*, the actual mobilities of norsertindole are almost the same at pH values 7.6 and 8.6 whereby in both cases the BGE counterion is the same (chloroacetate). At the two lowest pH values, with dichloroacetate (pH 6.1) and trichloroacetate (pH 4.7) as counterions, the actual mobilities of norsertindole are clearly different from those with chloroacetate as BGE anion. Ion pair formation between the analyte cations and the acetate ion might influence the mobilities at the two highest pH as well.

Ion pair formation gives an explanation for the mobility deviations in MeOH. This assumption is supported by data published in previous works. There the BGE counterions were acetate and perchlorate [30]. In that investigation the cationic analytes were different. In more recent work the effect of different counterions on the actual mobilities of anionic analytes in MeOH was demonstrated [31]. It was further observed that better agreement of the measured mobilities and the theoretical mobility behaviour in MeOH is found when the BGE counterion is the same at each pH value of the BGE [32]. However, with the present experimental set-up this was not possible, but it was already pointed out that the primary aim of the present work is to find suitable separation conditions for the analytes. For such purpose the data shown in Fig. 2 suffice by far.

3.2 ACN as solvent

Although ACN is also a suitable solvent in CE, *e.g.*, due to its compatibility with the UV absorbance detector, it is rather rarely used as pure liquid for the BGE. It is often applied as a mixture with other solvents, *e.g.*, with water, MeOH, ethanol, propanol, acetic acid. One of the reasons for this is that many potential BGE components are not well soluble in ACN. This problem is especially pronounced due to the fact that ACN poorly stabilizes anions, in contrast to water, where all ions are stabilized by hydrogen bonding. ACN molecules, however, are very poor hydrogen bond donors [33]. The anion stability may be enhanced by an additive capable of hydrogen bond donation. A typical example of such a "solubilization agent" is acetic acid, which is used occasionally at concentration as high as 1 mol/L (~6%) in CE. Note that such a high concentration means that the solvent system has to be considered as a binary system (ACN–acetic acid) rather than a single solvent.

One problem upon application of ACN might lie in the wide pH scale in this solvent, which covers more than 30 pH units compared to about 14 U in water. Due to the low number of potential buffer acids with known pK_a values in ACN, it is very difficult to cover the entire pH range here. It is therefore helpful to get at least an idea about the magnitude of the pK_a values of the analytes of interest. This was done using the equation ${}^{s}_{s}pK_a = 1.479\,{}^{w}_{w}pK_a + 2.842$ for the pK_a estimation in ACN found in the literature [28]. The resulting acid dissociation constants are 14.6, 14.5 and 18.2 for sertindole, dehydrosertindole and norsertindole, respectively. For this estimation procedure it was supposed that all amines (primary, secondary and tertiary) have more or less the same pK_a shift in relation to water. This is a pragmatic approximation (not based, *e.g.*, on QSAR).

The suitable BGEs, which cover the pH range where the dissociation degree of the analytes varies, are given in Table 3, together with the pK_a values of the buffer acids; note that perchloric acid is considered to be a strong acid in ACN [34]. The pH of the BGEs was calculated by the aid of the Henderson–Hasselbalch equation. Solubility problems were indeed observed for a number of compounds, and for this reason we had to use ionic strengths of as low as 5 mM. In case of oxalate even this concentration was too high for complete dissolution, and hydrogenoxalate at a concentration of 0.5 mM had to be applied. For this BGE the ionic concentration was adjusted to 5 mM with tetramethylammonium chloride. It is clear that the rather low acid concentration limits the buffer capacity. It can also result in peak triangulation by electromigration dispersion, because this concentration

Table 3. Composition and pH of the buffers used in solutions of ACN

pH	Acid	pK_a [38, 39]	Base
2.4	5 mM perchloric acid	a)	–
13.4	5 mM oxalic acid	14.5	0.5 mM hydrogenoxalate[b)]
14.2	5 mM phthalic acid	14.3	5 mM tetraethylammonium hydrogenphthalate
14.7	50 mM dichloroacetic acid	15.8	5 mM potassium dichloroacetate
15.2	0.5 mM phthalic acid	14.3	5 mM tetraethylammonium hydrogenphthalate
15.7	5 mM dichloroacetic acid	15.8	5 mM potassium dichloroacetate
15.7	50 mM salicylic acid	16.8	5 mM salicylate
16.7	5 mM salicylic acid		5 mM salicylate
17.6	0.6 mM salicylic acid		5 mM salicylate

a) Assumed to be a strong acid
b) Contains 4.5 mmol/L tetramethylammonium chloride
pK_a values are for the buffer acids in this solvent. pH values are corrected by means of the activity coefficient.

Table 4. Actual mobilities and their products with dynamic viscosity of the solvent, for sertindole, dehydrosertindole and norsertindole, and the internal standard in MeOH and ACN

	MeOH		ACN	
	μ (10^{-9} $m^2/V \times s$)	$\mu \times \eta$ (10^{-12} N/V)	μ (10^{-9} $m^2/V \times s$)	$\mu \times \eta$ (10^{-12} N/V)
Dehydrosertindole	19.9	11.0	32.7	11.2
Sertindole	19.6	10.8	33.5	11.4
Norsertindole	22.3	12.3	40.9	13.9
Tetraphenylphosphonium	31.8	17.5	47.8	16.3

Dynamic viscosity (cp or mPa $\times$ s): MeOH 0.551, ACN 0.341 [20]

is sometimes only about 50 times higher than that of the analytes. The latter could not be reduced further due to the necessary detectability.

The analyte mobilities measured with the BGEs given in Table 3 are shown in Fig. 4. A totally different picture is obtained compared to the MeOH systems (Fig. 2): the mobilities fluctuate strongly. Severe deviations from the expected sigmoid curve are found. Several explanations for the deviations can be suggested, most of them being related to hydrogen bonding interactions (homoconjugation or heteroconjugation) and to ion pair formation reactions in which either buffer components or analytes (or both) are involved [35, 36].

Problems of the deviation of the pH from that derived by the Henderson–Hasselbalch equation can be related to effects connected to the poor ability of ACN to stabilise anions. As the present analytes are cations, anion stabilisation plays a role only for the buffer components. When

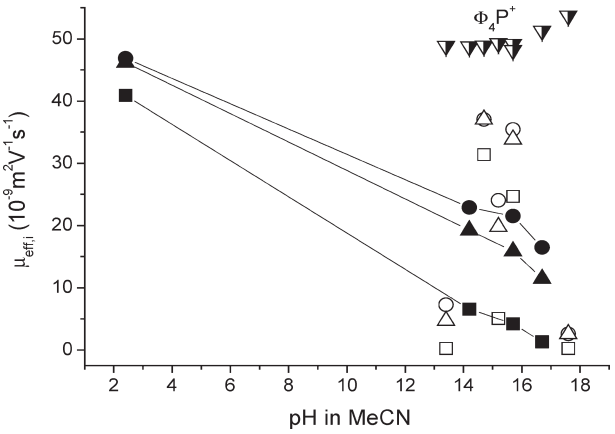


Figure 4. pH dependence of mobility in ACN for sertindole (▲, △), dehydrosertindole (● ○) and norsertindole (■, □), and tetraphenylphosphonium (Φ_4P^+) as internal standard. BGEs consisting of either equimolar (full symbols) or nonequimolar (open symbols) concentrations of acid and salt (see Table 3). Temperature, 25°C.

the buffer anion (A^-) is poorly stabilized by solvent molecules, it tends to pair with other hydrogen bond donors in the solution. In the present case A^- could interact by hydrogen bonding with the neutral form of the acid (HA) giving the product HA_2^- (this reaction is called homoconjugation). As a consequence, the concentrations of both A^- and HA are decreased by the same amount. This reduction of the initial concentrations may lead to a shift in pH due to the fact that the ratio HA/A^- in Eq. 1 is affected. Three cases can be differentiated:

(i) The initial concentrations of A^- and HA are identical. Theoretically the pH is then not affected, even when homoconjugation is present. However, buffer capacity of the BGE is decreased.

(ii) The initial concentration of A^- is smaller than that of HA. When A^- complexes with HA *via* hydrogen bond, both concentrations are reduced, but the ratio HA/A^- increases and thus the pH of the solution is decreased.

(iii) The initial concentration of A^- is higher than that of HA. Upon complexation of A^- with HA the ratio HA/A^- decreases and thus the pH of the solution is increased.

In cases (ii) and (iii) the pH of the BGE cannot be correctly calculated from the Henderson–Hasselbalch equation unless a correction for the degree of homoconjugation is undertaken. As only very few homoconjugation constants are available in the literature (see, *e.g.*, [37]), we do not take this complicated matter into consideration here (for further discussion, see [35]), but we exclude those BGEs which do not consist of equimolar concentrations of the acid and the anion. When the mobilities are plotted *versus* pH considering only these BGEs – indicated by the full symbols in Fig. 4 – μ *versus* pH approaches the sigmoid shape better, especially at higher pH values. However, the mobilities determined at low pH with perchloric acid as BGE component seem to be too high when compared to the other mobilities. One cause for this deviation could be the second amino group of the analyte molecule, which is partially protonated at low pH as well. This is plausible due to the larger pK_a shifts in ACN when compared to solvents like MeOH. Therefore, a cation acid with very low pK_a (close to 0) in MeOH can be partially protonated at pH around 2–3 in ACN.

Other reasons for the mobility deviations in Fig. 4 than homoconjugation of the buffer acid are difficult to confirm without supporting data. Thus, we make an only rough approximation of the pK_a values and the actual mobilities of the analytes from the curves fitted to the full symbols in Fig. 4. The resulting pK_a values for dehydrosertindole, sertindole and norsertindole are 16.5 ($\pm$0.7), 15.7 ($\pm$0.8) and 13.5 ($\pm$0.2), respectively; the actual mobilities are 32.7 ($\pm$8.7), 33.5 ($\pm$10.5) and 40.9 ($\pm$2.9), respectively. It is obvious that the reliability of these data is much lower than that in MeOH.

We can see from Table 1 that the pK_a values measured by CZE deviate considerably from those estimated from literature [28], the latter being based on the approximation of the aqueous pK_a values obtained by the computer software, followed by an estimation from solvation parameters. In the case of sertindole and dehydrosertindole the pK_a values are 1.1 and 2 units higher, and for norsertindole as much as 4.7 units lower than the calculated ones. Taking into account the very approximate kind of the measured pK_a values, it is likely from the plots shown in Fig. 4 that the present CE data give at least the correct sequence of the pK_a values.

It should be pointed out that the BGEs, which are seemingly less feasible for an appropriate mobility *versus* pH determination, are nevertheless well suited for the separation of the analytes. Indeed successful separations were achieved in perchloric acid solution, in 10 mM dichloroacetic acid/dichloroacetate, in 5.5 mM oxalic acid/hydrogenoxalate (with 4.5 mM tetramethylammonium chloride), in 5.5 and 10 mM phthalic acid/hydrogenphthalate and finally in 10 and 55 mM salicylic acid/hydrogensalicylate. In 55 mM dichloroacetic acid/dichloroacetate and 5.6 mM salicylic acid/salicylate, separations were not obtained; this was a BGE in which the mobilities seem to follow reasonably well the Henderson–Hasselbalch relation.

Two examples for the electropherograms of the analytes in the ACN systems are given in Fig. 5, the one showing separation according to the actual mobilities at low pH and the other at high pH (in the range of their pK_a values). In the latter case a BGE (salicylic acid/salicylate) was used, which is itself UV-absorbing. It can be seen that analysis could be carried out in less than 6 min.

3.3 Comparison of the mobilities in the two solvents

The simplest approach to compare the mobilities of a certain ion in different solvents is based on Stokes law and takes the difference in the frictional resistance into consideration, which acts on an ion during electrophoretic motion. It considers the ions as spherical particles moving in a continuum and leads to the following relation known as Walden's rule

$$\mu_{0,i}\eta = \text{const.} \quad (3)$$

where $\mu_{0,i}$ is the mobility of the fully charged ion at infinite dilution (*i.e.* null ionic strength) and η is the dynamic viscosity of the pure solvent.

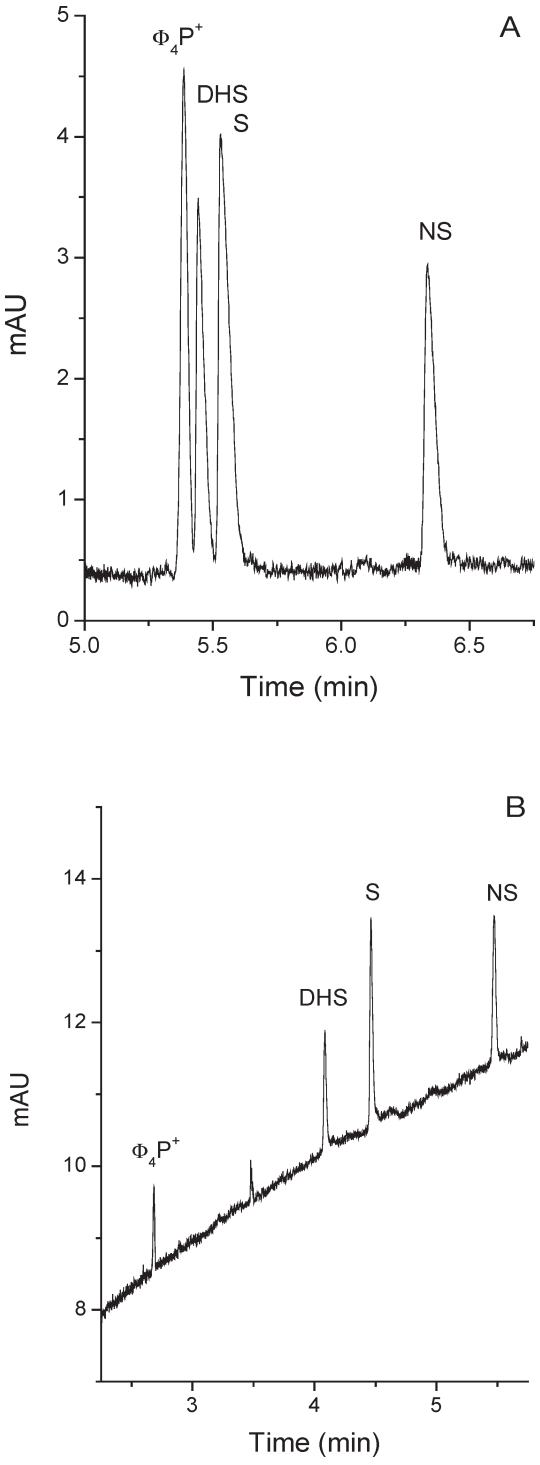


Figure 5. Electropherograms of sertindole (S), dehydrosertindole (DHS) and norsertindole (NS) in BGEs with ACN as solvent with 5 mM perchloric acid, pH 2.4 (A) and with 5 mM salicylic acid/5 mM potassium salicylate, pH 16.7 (B). Tetramethylphosphonium (Φ_4P^+) was used as internal standard to determine the effective mobilities of the analytes. Experimental conditions as in Fig. 3. Wavelength for detection: 200 nm (A); 224 nm (B).

Although this rule is formulated for limiting conditions, namely for zero ionic strength, we apply it in the present case to the actual mobility resulting from the fitting of Eq. (2). This is done because the reduction of mobility in an MeOH solution of 10 mM ionic strength and in a 5 mM ACN solution in relation to the absolute mobility is about the same. In both solvents the actual mobilities are *ca.* 22% lower than the absolute ones. This follows from the extended Debye–Hückel–Onsager relation between mobility and ionic strength (see, *e.g.*, [36]). It can be seen from Tab. 4 that the actual mobilities differ in the two solvents by up to 90%, but the Walden products (Eq. 3), in contrary, change by not more than 2–11%. This is a strong indication that the movement is at least in main parts governed by the frictional resistance of the solvent. This result is not unexpected for the present large organic ions with their low charge density.

4 Concluding remarks

It was demonstrated that MeOH or ACN are favorable solvents for the analysis of lipophilic compounds that are only very sparingly soluble in water. In the present case, the limit of detection (at three times the standard deviation of the baseline noise) of the analytes was about 0.3 μg/mL, a concentration that is below the solubility in water. Due to the low optical cut-off of the two organic solvents, UV detection can be applied without problem.

It was tried to use BGEs with well-defined pH values by applying buffers composed from an acid with known pK_a in the organic solvent and its salt at certain concentration ratios. The pH of the electrolyte solution can then be calculated according to the Henderson–Hasselbalch equation. The effective mobility of the analyte should follow the pH of the BGE by the typical sigmoid function. In fact we have found two extreme results.

In MeOH the mobility fitted very well to the theoretical curve, indicating that the pH scale was established in an appropriate way, and that the effective mobility is indeed governed mainly by the acid–base equilibrium. In remarkable contrast was the behaviour in ACN as solvent, where a zigzag curve was obtained for the effective mobility *versus* the pH. The situation is complicated here by several potential sources of the deviations: (i) the low solubility of some buffer constituents, leading to a low ionic strength and a low buffer capacity of the BGE; (ii) the more pronounced homoconjugation tendency of the buffer constituents and the analytes; (iii) perhaps a lower reliability of the pK_a values; (iv) the buffer acids, which had to be taken due to the limited number of buffer candidates in ACN, are probably less suited due to structural reasons.

When the data of the questionable BGEs in ACN were deleted, the interpretation of mobility *versus* pH became more straightforward.

The mobility *versus* pH curves enabled the derivation of the pK_a values (from the inflection points) and the actual mobilities (at low pH) of the analytes. Agreement of the pK_a values with those calculated by computer programs was good for MeOH, although the calculation by computer software was based on the pK_a in water derived from structural features, followed by solvation parameters in the organic solvents. In ACN the agreement between calculated pK_a values and those derived by CE was worse, but we assume that the latter CE data are more reliable.

The sequence of the mobilities of the analytes, and therefore the separation selectivity is different in MeOH and ACN. In MeOH norsertindole is the analyte with the highest mobility in all systems, which is expected due to its smallest size and largest pK_a value among all analytes. In ACN, on the other hand, norsertindole exhibits lower mobility than sertindole and dehydrosertindole at all applied pH values. It has, by the way, also the smallest pK_a in ACN. However, a straightforward interpretation of this finding will not be carried out due to the complex structures of the analytes.

Separation of the analytes was obtained even in the pH range where they exhibit their actual mobilities, although the structural difference between sertindole and dehydrosertindole is marginal. At pH values in the range of the pK_a values of the analytes, separation is increased, and a huge resolution in MeOH is possible within 10 min and in ACN within 4 min.

Received January 24, 2005

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