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Effect of mobile phase composition, pH and buffer type on the retention of ionizable compounds in reversed-phase liquid chromatography: application to method development

S. Heinisch*, J.L. Rocca

Laboratoire des Sciences Analytiques (CNRS UMR 5180), Université Claude Bernard, 43 Bd du 11 Novembre 1918, 69622 Villeurbanne Cedex, France

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Abstract

Optimizing separation of ionizable compounds in order to find robust conditions has become an important part of method development in liquid chromatography. This work is an attempt to explain the observed variations of retention of acid and basic compounds with the organic modifier content in the mobile phase, according to various factors: the type of modifier, the type of buffer, the temperature and of course the type of solute. This is done by considering the variation of the so-called chromatographic pK_a which refers to the pH measured in the aqueous medium and is determined from retention data. A procedure is described that accurately relates, from nine experiments, retention to solvent composition and pH. The limits of such a procedure are evaluated and two examples of optimized separations of basic compounds are given.

Keywords: Mobile phase composition; Method development; Ionizable compounds; Retention behaviour

1. Introduction

Most pharmaceutical and biological compounds contain ionizable functions such as carboxylic or amino groups. Until now, in most instances, the separation of such compounds is performed either by enhancing ionization with eluents containing anionic or cationic surfactants in hydro organic solvent mixture (ion-pair chromatography) or by suppressing ionization when it is possible according to both solute pK_a and pH range allowed by chromatographic stationary phase. Nowadays, column manufacturers provide silica-based packings [1–4] which resist to high pH. Hence modern bonded silica-based columns can be suitable throughout a 1–10 pH range with carefully chosen non-aggressive buffers. Moreover, the silanol activity has been significantly reduced with the preparation of high purity silica. Other available packings such as polymer-based material or porous graphitic carbon offer these two advantages as well. Then, it has become interesting to investigate chromatographic conditions, without addition of triethylamine or any blocking agents in the mobile phase, in which the solutes are partially ionized. Such conditions are interesting because first they do not require any ionic surfactant and secondly the variation in the degree of ionization of the solute can lead to extreme change in selectivity. In return, however, they are known to be less robust. Then, optimizing separation in order to find robust conditions becomes an important part of method development in ionizable compounds chromatography. pH and mobile phase composition are the two relevant optimization parameters: pH to vary the dissociation rate and mobile phase composition to compensate for the diminution of retention when the solute ionization increases. In fact, the influence of these two parameters to the retention process is somewhat more complex since the variation of the mobile phase composition (type of organic modifier, organic modifier content, type of buffer, ionic strength) induces a variation of the degree of ionization as well. Therefore, this paper examines the role

* Corresponding author. Tel.: +33 4 72448217; fax: +33 4 72431078.
E-mail address: heinisch@univ-lyon1.fr (S. Heinisch).

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of these different parameters, including the type of stationary phase and the temperature, in the variation of the solute dissociation to have a better understanding of the retention process and suggests a general procedure to optimize pH and mobile phase composition.

2. Theoretical section

2.1. pH scales in hydro-organic mixtures used as mobile phases

The pH should be ideally measured in the mixed aqueous organic solution with the pH meter calibrated with two standard buffer solutions of known pH in the identical solvent composition. Provided that the glass electrode responds ideally to hydrogen ion activity, a_H, then it is the sole measure leading to the following relationship [5]:

$$\mathrm{pH} = -\log a_H \tag{1}$$

Different nomenclatures for this measure of pH are found in the literature: pH^* (x) [5], pH_x^* [6] or ${}^{s}_{s}\mathrm{pH}$ [7], this form being recommended by the IUPAC [8].

Because the calibration of a pH-meter with standard of known ${}^{s}_{s}\mathrm{pH}$ values [9] is not easy, some authors make use of ${}^{s}_{w}\mathrm{pH}$ [6,10] which represents the pH measured in the mixed aqueous organic solution using aqueous standards instead of mixed aqueous organic standards in the same solvent composition. In this case, ${}^{s}_{s}\mathrm{pH}$ is related to ${}^{s}_{w}\mathrm{pH}$ by:

$${}^{s}_{s}\mathrm{pH} = {}^{s}_{w}\mathrm{pH} - \delta \tag{2}$$

where δ represents the shift in the pH scale, namely the difference in standard electrode potential in pH units between the two measures. It is given by [5,10]:

$$\delta = -\log({}_{m}\gamma) \tag{3}$$

where ${}_{m}\gamma$ is called the primary medium effect. δ values were determined for various mixed aqueous organic solvent and then correlated to the composition of methanol in aqueous methanol mixtures [10] and to the composition of acetonitrile in aqueous acetonitrile mixtures [11].

The measure of ${}^{s}_{w}\mathrm{pH}$ for a chromatographic purpose presents in our sense four shortcomings. First, Eq. (3) is true only when the residual liquid junction potential (difference in liquid junction potential arising at the junction between the sample solution and the salt bridge) is negligible. Secondly, the primary medium effect is somewhat dependent on the ionic strength which usually decreases when the aqueous buffer is mixed with the organic solvent. Thirdly, the primary medium effect is also dependent on the temperature and its values are usually found at ambient temperature (25 °C) while nowadays, fast chromatographic separations at elevated temperatures (40 °C to 80 °C) are more and more applied to pharmaceutical products. And last, in gradient elution which is widely used in liquid chromatography, it is not possible to fit the retention of ionizable compounds to ${}^{s}_{w}\mathrm{pH}$ because its value change during the elution of the solutes [12].

The most practical way consists certainly in measuring pH into the buffered aqueous medium before mixing it with the organic solvent. Of course, in this case, the electrode is calibrated with aqueous buffers. Then, the obtained measured value, ${}^{w}_{w}\mathrm{pH}$, is related to the activity of hydrogen ions in the buffered aqueous medium according to Eq. (1). It is probably the most practical way to prepare mobile phases. However, the activity of the hydrogen ions changes after dilution of the aqueous buffer with the organic modifier, due to a change in the buffer $\mathrm{p}K_a$. For a buffer prepared in the aqueous medium from a weak acid at concentration c_a and its weak conjugated base at concentration c_b, the following relationships give the buffer $\mathrm{p}K_a$ in the aqueous medium, $\mathrm{p}K_{a,w,buffer}$ and in the hydro-organic medium obtained after mixing the aqueous solution with the organic solvent, $\mathrm{p}K_{a,s,buffer}$:

$$\mathrm{p}K_{a,w,buffer} = {}^{w}_{w}\mathrm{pH} - \log\left(\frac{\gamma_{b,w} \times c_b}{\gamma_{a,w} \times c_a}\right) \tag{4}$$

$$\mathrm{p}K_{a,s,buffer} = {}^{s}_{s}\mathrm{pH} - \log\left(\frac{\gamma_{b,s} \times c_b}{\gamma_{a,s} \times c_a}\right) \tag{5}$$

where $\gamma_{b,w}$ and $\gamma_{a,w}$ are the activity coefficients in the aqueous medium of a and b, respectively and $\gamma_{b,s}$ and $\gamma_{a,s}$ the corresponding activities in the hydro organic medium. The dilution is not taken into account in Eq. (5) since it affects both c_a and c_b in the same way. By assuming that the ratios $\gamma_{b,w}/\gamma_{a,w}$ and $\gamma_{b,s}/\gamma_{a,s}$ are quite identical and using Eqs. (4) and (5), ${}^{s}_{s}\mathrm{pH}$ can be related to ${}^{w}_{w}\mathrm{pH}$ by:

$${}^{s}_{s}\mathrm{pH} = {}^{w}_{w}\mathrm{pH} + (\mathrm{p}K_{a,s} - \mathrm{p}K_{a,w})_{buffer} = {}^{w}_{w}\mathrm{pH} + \Delta\mathrm{p}K_{a,buffer} \tag{6}$$

2.2. Dependence of the retention on the dissociation rate

Theoretical models describing a sigmoidal dependence of the retention factor k on the pH of the mobile phase for monoacidic or monobasic compounds on reversed phase sorbents have been extensively studied [6,7,13–17]. The retention factor of any ionizable solute as a function of mobile phase pH can be expressed by considering that k is a weighted average of the retention factor of the basic form, k_b and the acidic form, k_a assuming that the distribution of the solute between mobile and stationary phases is just governed by hydrophobic interactions, that is no ionic or hydrogen bonding interactions occurs between the solute and the stationary phase [18]. Then for a solute with a dissociation constant $K_{a,solute}$ k is given by:

$$k = \frac{k_b 10^{\mathrm{pH}-\mathrm{p}K_{a,solute}} + k_a}{1 + 10^{\mathrm{pH}-\mathrm{p}K_{a,solute}}} \tag{7}$$

pH and $\mathrm{p}K_{a,solute}$ refer of course to both given mobile phase and given stationary phases at the operational temperature. This equation is valid provided that the activity coefficients

of both acidic and basic forms can be neglected. In fact, this is possible in liquid chromatography since the molar concentrations are usually very low.

When the pH is measured in the aqueous medium at ambient temperature, replacing Eq. (6) in Eq. (7), k leads to:

$$k = \frac{k_b 10^{{}^{w}_{w}\mathrm{pH}-(\mathrm{p}K_{a,s,solute}-\Delta\mathrm{p}K_{a,buffer})} + k_a}{1 + 10^{{}^{w}_{w}\mathrm{pH}-(\mathrm{p}K_{a,s,solute}-\Delta\mathrm{p}K_{a,buffer})}} \quad (8a)$$

or

$$k = \frac{k_b 10^{{}^{w}_{w}\mathrm{pH}-\mathrm{p}K_{a,chrom}} + k_a}{1 + 10^{{}^{w}_{w}\mathrm{pH}-\mathrm{p}K_{a,chrom}}} \quad (8b)$$

where $\mathrm{p}K_{a,chrom} = (\mathrm{p}K_{a,s,solute} - \Delta\mathrm{p}K_{a,buffer})$ may be called the chromatographic pK_a. The dissociation rate, α, in the aqueous organic mobile phase is related to ${}^{w}_{w}\mathrm{pH} - \mathrm{p}K_{a,chrom}$ by:

$$\alpha = \frac{10^{{}^{w}_{w}\mathrm{pH}-\mathrm{p}K_{a,chrom}}}{1 + 10^{{}^{w}_{w}\mathrm{pH}-\mathrm{p}K_{a,chrom}}} \quad (9)$$

Hence, the variation of α is directly dependent on the variation of ${}^{w}_{w}\mathrm{pH} - \mathrm{p}K_{a,chrom}$. When ${}^{w}_{w}\mathrm{pH}$ is equal to p$K_{a,chrom}$, the concentrations of both neutral and ionized forms are identical during the chromatographic process, namely the solute is half dissociated (α = 0.5) in the mixed mobile phase at the given temperature with both appropriate buffer and stationary phase. The difference for a given solute between p$K_{a,w,solute}$ usually given in the literature at ambient temperature, and p$K_{a,chrom}$, is given by:

$$\mathrm{p}K_{a,w,solute} - \mathrm{p}K_{a,chrom} = \Delta\mathrm{p}K_{a,buffer} - \Delta\mathrm{p}K_{a,solute} \quad (10)$$

and then depends on the influence of several parameters on the dissociation of both buffer and solute. These parameters include the type of organic modifier, the composition of the mobile phase, the type of buffer, the column temperature and even the nature of the stationary phase. In a previous work [16], it was shown that the three coefficients of Eq. (8b) can be calculated with a very good reliability from three retention data collected within a limited pH range (2 or even 3 pH units) provided that the test method proposed by the authors is successful. This test consists in making sure that the $K_{a,chrom}$ value is positive even when the retention data are altered by $a \pm 5\%$ error. Otherwise, a quadratic model is preferred to Eq. (8b) to fit the retention data.

3. Experimental

3.1. Apparatus

The chromatographic system used was a model Alliance from Waters (Waters, Paris) with 0.8 mL dwell volume, a model 996 photodiode array detector (Waters, Paris) equipped with an 8 μL cell. The chromatograms were processed using Waters millennium software. The different columns used were Zorbax SDB-C18150 mm × 4.6 mm i.d. (Interchim, France), Capcell-Pak-C18 150 mm × 4.6 mm i.d. (Interchim, France); RP-Xterra-C18 150 mm × 4.6 mm i.d. (Waters, USA), Nucleodur-C18 70 mm × 4.6 mm (Macherey Nagel, France), PLRP-S 150 mm × 4.6 mm i.d. (Polymer Laboratories, France), Hypercarb 100 mm × 4.6 mm i.d. (Thermo Separations, France). The particle diameters were 5 μm except for the Nucleodur, 3 μm. The dead volumes were estimated by assuming a column porosity of 0.7. The column temperature was controlled at 30 °C by a water bath. Experiments at higher temperatures were performed using a temperature controller system (Varian, France). pH measurements were performed with a glass electrode XG200, a red rod reference electrode (Ag/AgCl) REF201 with a saturated KCl solution in water as salt bridge and a temperature sensor T201 in a pHM210 standard pHmeter with a precision of ±0.02 pH units (all from Radiometer Analytical, France). The electrode was calibrated with pH 4.0, 7.0 and 10.0 standard solutions from IUPAC. The injected volume was 10 μL.

3.2. Chemicals

Dipotassium hydrogenphosphate trihydrate, potassium dihydrogenphophate, Tris(hydroxymethyl)aminomethane (TRIS), 1,3-bis[tris(hydroxymethyl)methylamino]propane (BisTrisPropane), pyrrolidium, trisodium citrate, citric acid were obtained from Sigma (France). All aqueous mobile phases were filtered with Nylon 65 membrane filter. Phosphate, citrate and Tris buffers 30 mM were prepared in 500 mL solution by weighting appropriate KH_2PO_4/K_2HPO_4, trisodium citrate/citric acid, TRIS–acid/TRIS–basic, respectively. The pH was adjusted by adding adequate amount of 6 M sodium hydroxide and measured before the addition of organic modifier. The following compounds, all from Sigma (France) were used: *N*,*N*-dimethylaniline (pK_a = 5.15); benzene, *m*-toluidine (pK_a = 4.73); *p*-toluidine (pK_a = 5.08); *o*-toluidine (pK_a = 4.44); benzoic acid (pK_a = 4.2); *p*-*N*-benzoic acid (pK_a = 3.4); salycilic acid (pK_a = 2.97); *N*-clozapine; clozapine; diphenydramine (pK_a = 9), imipramine (pK_a = 9.5), amitriptylline (pK_a = 9.4), amoxapine (pK_a = 9.5), protriptylline (pK_a = 10), doxepine and phenol. pK_a values are given by ref. [19].

3.3. Software

The algorithms required for the different optimizations reported in this work were first developed in our laboratory and incorporated then into the commercial version 4.0 of OSIRIS (Datalys, Grenoble, France).

4. Results and discussion

4.1. Practical use of the ${}^{w}_{w}$pH scale

In our sense, Eq. (6) leads to three important comments.

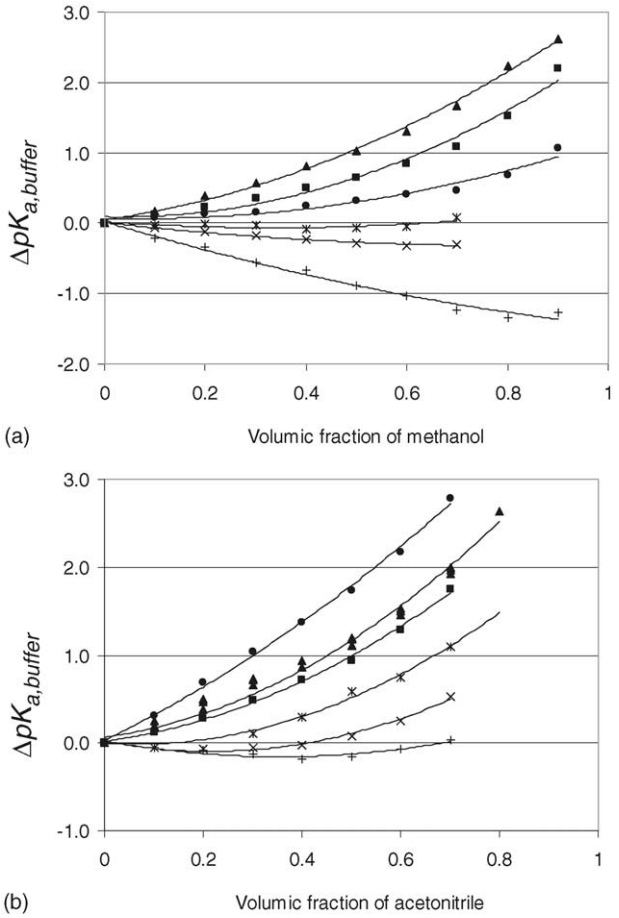

Fig. 1. pK_a variation ($\Delta p K_{a,buffer} = {}^{s}_{s}pH - {}^{w}_{w}pH$) for different buffers in methanol–water (a) and acetonitrile–water (b) mixtures with solvent composition: pyrrolidium (+); TRIS (×); BisTrisPropane (✱); borate (●); phosphate (${}^{w}_{w}$pH 6; ${}^{w}_{w}$pH 7 and ${}^{w}_{w}$pH 8) (▲); citrate (■).

For method development in chromatography, Eq. (6) clearly indicates that it would not be correct to optimize ${}^{w}_{w}$pH in place of ${}^{s}_{s}$pH when different types of buffers are used to vary pH [20] unless these buffers provide the same $\Delta p K_{a,buffer}$. Fig. 1 shows $\Delta p K_{a,buffer}$ variations versus the volumic fraction of organic modifier at 25 °C for several usual buffers that encompasses the whole range of pH from 2 to 12. The deviation values have been determined by measuring both ${}^{w}_{w}$pH and ${}^{s}_{w}$pH and by calculating ${}^{s}_{s}$pH according to Eq. (2) with δ values given by references [10,11]. As shown by Fig. 1, $\Delta p K_{a,buffer}$ is strongly dependent both on the buffer and on the type of organic modifier. The higher the organic content of the mobile phase, the more significant the disparities in $\Delta p K_{a,buffer}$ between the studied buffers. These disparities are particularly significant between acid buffers such as phosphoric or citric acids and basic buffers such as pyrrolidium, BisTrisPropane or TRIS. As a result, optimization of ${}^{w}_{w}$pH must be performed with the same buffer to vary pH and then the investigated ${}^{w}_{w}$pH range is undoubtedly limited to two or three units in order to keep an adequate buffer capacity.

If ${}^{w}_{w}$pH is adjusted to the $p K_{a,w,buffer}$ of the chosen buffer, the aqueous solution is then buffered with a buffering capacity directly proportional to the buffer concentration [5]. Then, any mixtures of this aqueous solution with an organic solvent will also be buffered but at a pH equal to $p K_{a,s,buffer}$ and with a buffering capacity divided by the resulting dilution factor.

Most columns are silica based and so unstable outside the pH range 2 to pH_{maxi}, where pH_{maxi} is the maximum authorized pH value, fixed at 8 for classical columns. However, this pH range is given for aqueous mobile phases and consequently pH_{maxi} refers to aqueous medium. In case of aqueous–organic mobile phases and according to Eq. (6), the authorized pH range will depend on $(p K_{a,s} - p K_{a,w})_{buffer}$. Furthermore, ${}^{s}_{s}$pH maximum depends on the autoprotolysis constant, $K_{ap,s}$ which varies with the mobile phase composition (14 for water at 25 °C). $p K_{ap,s}$ values were given by Roses and coll for different methanol–water, acetontrile–water and tetrahydrofuran–water mixtures [20,21]. pH_{maxi} is related to $p K_{ap,s}$ and pH_{maxi} by:

$$ {}^{s}_{s}pH_{maxi} = p K_{ap,s} - (14 - pH_{maxi}) \quad (11) $$

The difference between the maximum value for pH and pH_{maxi} is then given by

$$ {}^{w}_{w}pH_{maxi} - pH_{maxi} = (p K_{ap,s} - 14) - (p K_{a,s} - p K_{a,w})_{buffer} \quad (12) $$

Two studies have been published about the extension of the pH range for chromatographic columns from water to methanol–water [22] and from water to tetrahydrofuran–water [23] for different buffers. Fig. 2 gives the variation of $({}^{w}_{w}pH_{maxi} - pH_{maxi})$ as a function of the volumic fraction of organic modifier for the same buffers as in Fig. 1. Fig. 2 clearly explains why phosphoric acid buffer may be dangerous for the columns in methanol-aqueous medium if the maximum ${}^{w}_{w}$pH value is exclusively referred to pH_{maxi} value [24]. Citric acid is not as much a problem as its aqueous buffering range is two to six and for most silica-based columns pH_{maxi} value is at least equal to 8. All other studied buffers can be adjusted in the aqueous medium at or over the pH_{maxi} value.

4.2. Determination of $p K_{a,chrom}$

As explained into the theoretical section, it is possible to determine $p K_{a,chrom}$ from three retention data obtained at three different ${}^{w}_{w}$pH within a pH range of 2 units provided that the required test described in reference [16] is successful. The experimental variation of retention as a function of ${}^{w}_{w}$pH is given in Fig. 3 for three basic compounds. Different set of three of these retention data have been used to determine the $p K_{a,chrom}$ according to Eq. (8b). The results listed in Table 1 for different pH ranges show the very good similarity between the obtained $p K_{a,chrom}$ values. It means that the selection of the pH range for the $p K_{a,chrom}$ determination is not very critical. It has just to include or at least to be very

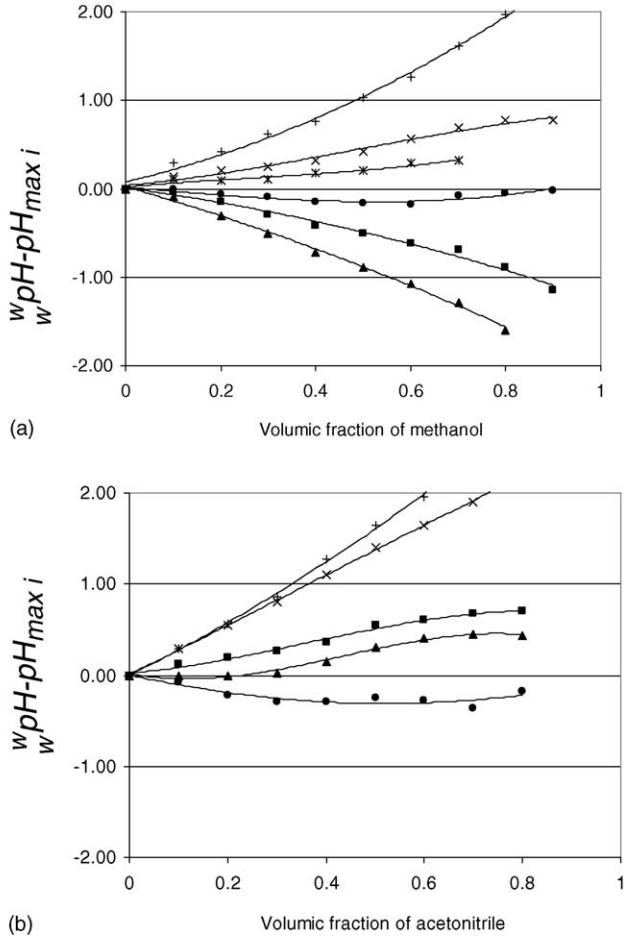

Fig. 2. Comparison of the variation of ($^{w}_{w}pH_{maxi} - pH_{maxi}$) in methanol–water (a) and acetonitrile–water (b) mixtures with solvent composition for different buffers: pyrrolidium (+); TRIS (×); BisTrisPropane (✱); borate (●); phosphate (▲); citrate (■).

close to the p$K_{a,chrom}$; actually, in one case only, it has not been possible to determine p$K_{a,chrom}$. In all other cases the p$K_{a,chrom}$ values are estimated with a very good reliability (±0.2 pH unit) as shown by the standard deviation.

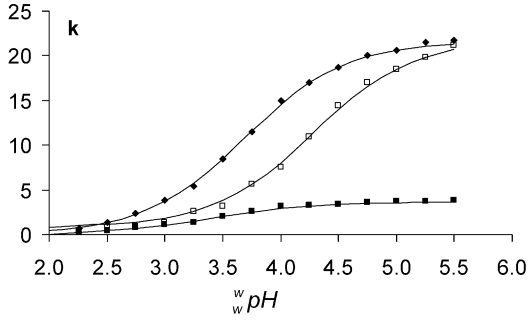

Fig. 3. Variation of the experimental retention factor vs. $^{w}_{w}$pH for *N*,*N*-dimethylaniline in 40% of acetonitrile (♦); *m*-toluidine in 40% of acetonitrile (■); *m*-toluidine in 20% of acetonitrile (□). Stationary phase: PLRP-S; mobile phase: acetonitrile-sodium citrate buffer 30 mM.

Table 1
Comparison of the different p$K_{a,chrom}$ values calculated with Eq. (8b) from three experimental retention data corresponding to different sets of three $^{w}_{w}$pH (see Fig. 3 for details)

$^{w}_{w}$pH sets	*N*,*N*-dimethylaniline (40% ACN)	*m*-Toluidine (40% ACN)	*m*-Toluidine (20% ACN)
2.25; 3.25; 4.25	3.74	3.52	4.06
2.5; 3.5; 4.5	3.69	3.42	4.44
2.75; 3.75; 4.75	3.71	3.47	4.23
3; 4; 5	3.63	3.22	4.31
3.25; 4.25; 5.25	3.72	3.51	4.28
3.5; 4.5; 5.5	3.80	–	4.23
Average value	3.71	3.42	4.25
Standard deviation	0.06	0.12	0.12

En-dash (–) denotes failure of the method test.

4.3. Variation of solute p$K_{a,chrom}$ with the mobile phase composition according to various parameters

Using the above method, we have studied the influence of various parameters on the evolution of p$K_{a,chrom}$ for different ionizable solutes (acid and basic) with the organic content of the mobile phase. These parameters include the type of organic modifier, the type of buffer and the temperature. The results are given in Figs. 4–6. These figures show the variation of p$K_{a,w,solute}$ − p$K_{a,chrom}$ as a function of the organic modifier content, p$K_{a,w,solute}$ representing the solute pK_a value found in the literature [19] for solutes in aqueous medium at 25 °C. The values are well fitted using a quadratic model. These results give rise to some relevant comments concerning the observed shifts in pK_a.

First, the extrapolations of the different curves at 0% of organic content, except of course those corresponding to high temperature (Fig. 6), are close to zero and confirms the good reliability of this method in finding, for ionizable compounds, not only p$K_{a,chrom}$ values but if necessary p$K_{a,w,solute}$ values when these are not available in the literature. While these shifts are not really important for acidic compounds such

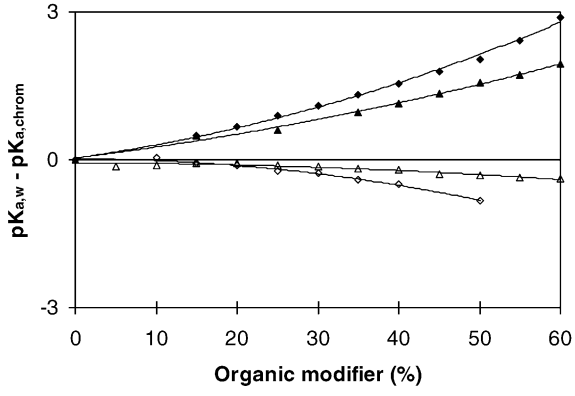

Fig. 4. Effect of the type organic modifier on the variation of p$K_{a,w,solute}$ − p$K_{a,chrom}$ with the percentage of methanol for *N*,*N*-dimethylaniline (▲) and benzoic acid (♦) and with the percentage of acetonitrile for *N*,*N*-dimethylaniline (△) and benzoic acid (◊). Other conditions: sodium citrate buffer 30 mM; 30 °C; Zorbax SDB C8 column.

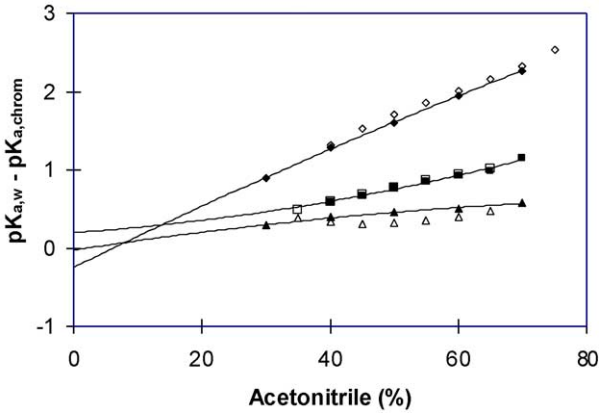

Fig. 5. Effect of the type of buffer on the variation of $pK_{a,w,solute} - pK_{a,chrom}$ with the percentage of acetonitrile for amitryptilline: TRIS buffer 30 mM (▲); BisTrisPropane buffer 30 mM (■); phosphate buffer 30 mM (♦). Other conditions: 30 °C; RP-Xterra-C18 column. Comparison with the $pK_{a,w,solute} - pK_{a,chrom}$ values calculated from nine experiments (see text for explanations) with TRIS buffer 30 mM (△); BisTrisPropane buffer 30 mM (□); phosphate buffer 30 mM (◊).

as benzoic acid (Fig. 4), they are significant for basic compounds, particularly when acetonitrile is used as organic modifier. The shift can reach up to three unit of pH in case of *N*,*N*-dimethylaniline with citric acid as buffer and 60% of acetonitrile. Such a shift means that with an aqueous buffer adjusted at ${}^{w}_{w}pH = pK_{a,w,solute}$ this basic compound is completely neutral in 60% of acetonitrile.

Fig. 5 shows that the shifts are very dependent on the type of buffer and as a result that $pK_{a,chrom}$ depends not only of the measured compounds but also of the particular buffer employed. As expected, the shifts are significant for acidic buffers such as phosphoric acid. On the other hand, it is notable that these shifts are also large and quite different for basic buffers depending on the type of basic buffers (TRIS and BisTrisPropane in this example). It confirms that any optimization of ${}^{w}_{w}pH$ has be to be realized with the same buffer

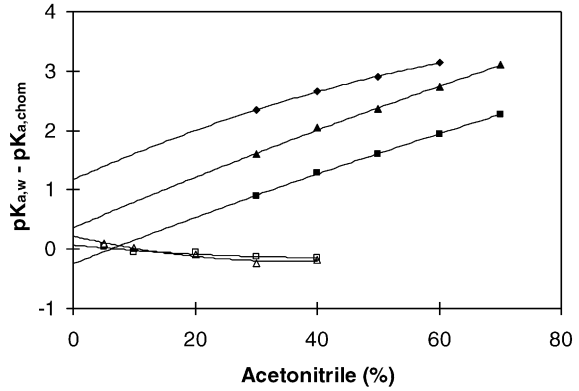

Fig. 6. Effect of the temperature on the variation of $pK_{a,w,solute} - pK_{a,chrom}$ with the percentage of acetonitrile for amitryptilline with phosphate buffer 30 mM and RP-Xterra-C18 column: 30 °C (■); 60 °C (▲); 90 °C (♦). For benzoic acid with citrate buffer 30 mM and Nucleodur column: 30 °C (□); 60 °C (△).

to explore the pH range, and moreover that the optimum ${}^{w}_{w}pH$ must be related to a unique buffer in the description of the chromatographic method. In addition, the distance between two curves of Fig. 5, for a given percentage of acetonitrile, corresponds to the difference in $pK_{a,chrom}$, namely the difference in $pK_{a,buffer}$. These distances are quite similar to those shown on Fig. 2b which actually represents the differences in $pK_{a,s,buffer}$.

Last, it appears that the shifts increase significantly for basic compounds when the temperature increases (Fig. 6) that is not the case for acidic compounds. This increase is directly related to a decrease in $pK_{a,s,buffer}$ with temperature. The resulting variation of the solute dissociation with the temperature is very attractive as it offers an additional parameter to vary the selectivity of ionizable compounds. Effect of temperature on the ionization of solutes will be more extensively discussed in a future work.

4.4. Variation of solute retention with mobile phase composition

It is well known that the dependence of log(*k*) on the percentage of organic modifier in reversed liquid chromatography is well described either by a linear model [25] within a small range of *k* values (1–15) or by a quadratic model [26] within a wider range (0.5–30). For ionizable compounds, the problem is more complex since the solute is present in the mobile phase under the two neutral and ionized forms. First, the ionized form may lead to non-hydrophobic interactions with the stationary phase and secondly, the dissociation rate varies with the mobile phase composition. The above results have shown that the determination of $pK_{a,chrom}$ for a given percentage of organic modifier, a given buffer and a given temperature is easy and is very useful to predict the variation of the dissociation rate with ${}^{w}_{w}pH$. The $pK_{a,s}$ of an acidic compound increases with the organic modifier content and the $pK_{a,s}$ of a basic one somewhat decreases. As a result, for acidic solutes, the $pK_{a,chrom}$ increases or decreases with the organic modifier content depending on the pK_a variation of the buffer but most times, for basic solutes the $pK_{a,chrom}$ decreases. In case of basic solutes, the dissociation rate will then decrease when the content of organic solvent increases. This phenomenon will induce a competition between on the one hand, a diminution of retention due to lower hydrophobic interactions and, on the other hand an increase in retention due to a decrease of the dissociation rate and as a result a curvature of plots of log(*k*) versus the percentage of organic modifier. This is illustrated by the curves of Figs. 7–9 where important deviations to linearity are observed even for *k* values ranging from 1 to 10. In the past, curvatures of log(*k*) for basic compounds have already been noted and were often attributed to interactions of the basic solute with accessible silanols of the stationary phase via a normal-phase process [27]. In the present work, the studied silica-based stationary phase (MS-Xterra-C18, Capcell-C18 or Zorbax SDB-C18)

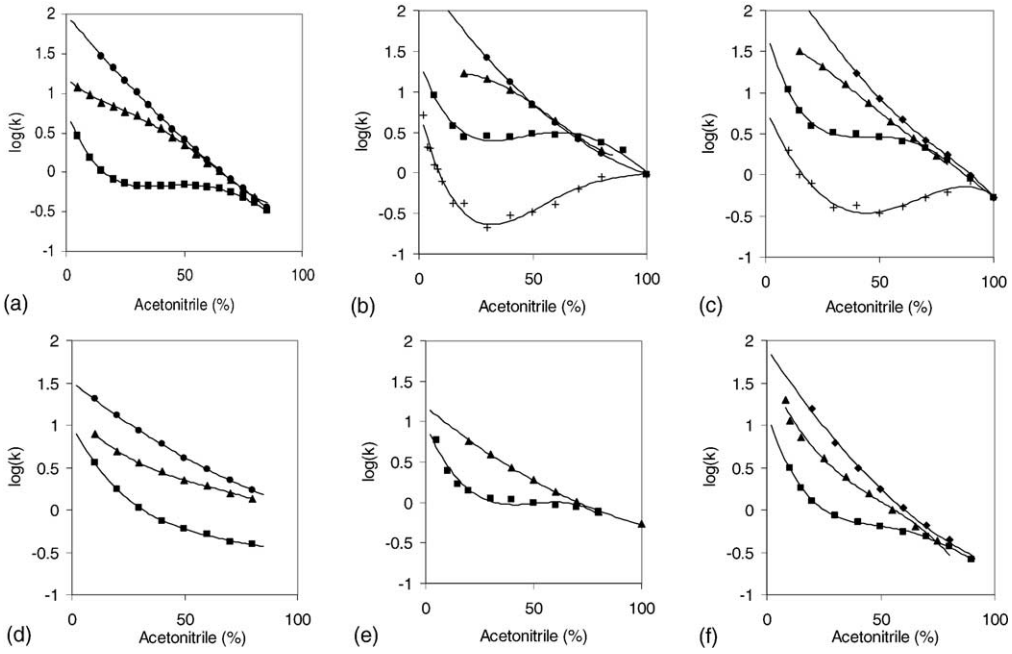

Fig. 7. Variation of the experimental log(*k*) with the percentage of acetonitrile for *N*,*N*-dimethylaniline ($pK_{a,w}$ = 5.15) (a, b and c) and *m*-toluidine ($pK_{a,w}$ = 4.7) (d, e and f) with different stationary phases: Zorbax SDB-C18 (a); Capcell-C18 (d); Hypercarb (b, e) and PLRP-S (c, f) and different values of ${}^{w}_{w}$pH: ${}^{w}_{w}$pH 2.2 (+); ${}^{w}_{w}$pH3 (■); ${}^{w}_{w}$pH 4 (▲) and ${}^{w}_{w}$pH 5 (♦). Other conditions: 30 °C; citrate buffer 30 mM.

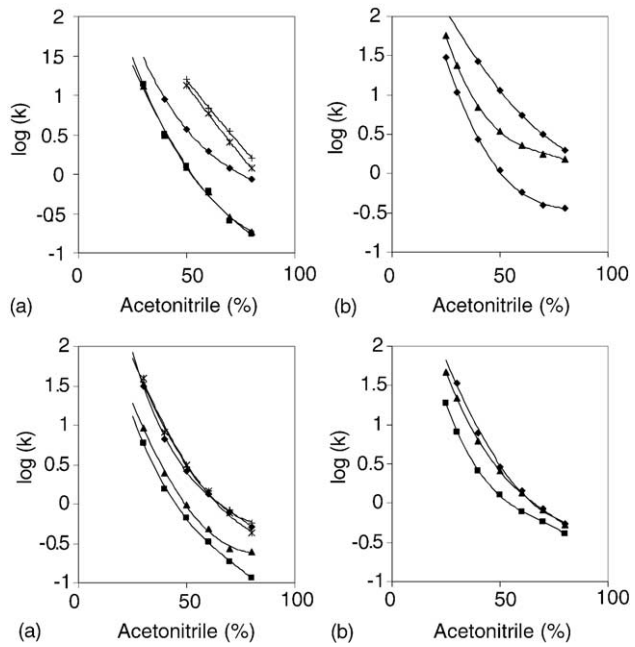

Fig. 8. Variation of the experimental log(*k*) with the percentage of acetonitrile for amitriptylline ($pK_{a,w}$ = 9.4) with BisTrisPropane 30 mM buffer (a) and phosphate buffer 30 mM (b) and for clozapine ($pK_{a,w}$ = 7.5) with BisTrisPropane 30 mM buffer (c) and phosphate buffer 30 mM (d) and different values of ${}^{w}_{w}$pH: ${}^{w}_{w}$pH 6 (■); ${}^{w}_{w}$pH 7 (▲); ${}^{w}_{w}$pH 8 (♦); ${}^{w}_{w}$pH 9 (✱) and ${}^{w}_{w}$pH 10 (+). Other conditions: 30 °C; MS-Xterra stationary phase.

are poor in accessible silanols and obviously the other studied stationary phases (Hypercarb and PLRP-S) have no silanol groups at all. Whatever the stationary phase, the same form of curve is observed for a given solute at a given ${}^{w}_{w}$pH. These results show that these important deviations to linearity are not due to some secondary interactions but fully explained by the existence of the competition described above.

In fact, for a basic solute if ${}^{w}_{w}$pHis one to two units lower than $pK_{a,w,solute}$, the variation of log(*k*) versus the organic modifier content is neither linear nor quadratic but sigmoidal with an inflection point corresponding to a composition for which $pK_{a,chrom}$ = ${}^{w}_{w}$pH. For *N*,*N*-dimethylaniline ($pK_{a,w}$ = 5.15), the inflection point is indeed for nearly 50% of acetonitrile at ${}^{w}_{w}$pH = 3, and nearly 60% of acetonitrile at ${}^{w}_{w}$pH = 2.2 (Fig. 7a–c). These values are in good accordance with the results of Fig. 4 showing that the difference $pK_{a,w,solute} - pK_{a,chrom}$ is nearly equal to 2 at 50% and to 3 at 70%. The sigmoidal form of the plots log(*k*) versus percentage of organic modifier is all the more obvious as the deviation $pK_{a,w,solute} - pK_{a,chrom}$ is important. This gives the reason why this sigmoidal form is less evident in case of amitriptylline or clozapine (Fig. 8) than in case of *N*,*N*-dimethylaniline or *m*-toluidine (Fig. 7). Hence, it appears from the comparison of Figs. 4 and 5 that the increase in $pK_{a,w,solute} - pK_{a,chrom}$ with the percentage of acetonitrile is particularly significant for *N*,*N*-dimethylaniline. According to Eq. (10) and since the variation of $\Delta pK_{a,buffer}$ is nearly the same for phosphate and citrate buffers (Fig. 1), this difference in behaviour for these basic solutes is then essentially due to the solutes themselves, namely $\Delta pK_{a,solute}$ is probably more important for *N*,*N*-dimethylaniline or *m*-toluidine than for amitriptylline or clozapine. For acidic solutes such as benzoic acid, the decrease in $pK_{a,w,solute} - pK_{a,chrom}$ with percentage of acetonitrile is less significant and consequently the competition of both phenomenon evocated above is less important. As a

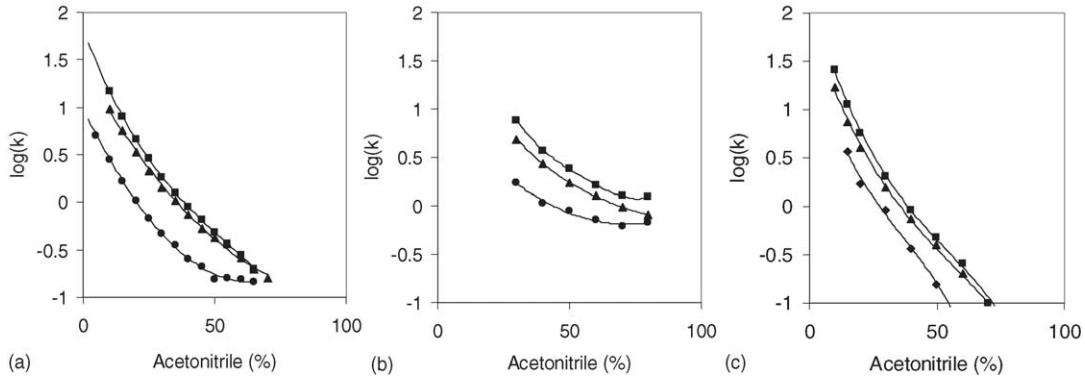

Fig. 9. Variation of the experimental log(*k*) with the percentage of acetonitrile for benzoic acid (p$K_{a,w}$ = 4.2) with different stationary phases: Zorbax SDB-C18 (a); hypercarb (b) and PLRP-S (c) and different values of $^{w}_{w}$pH: $^{w}_{w}$pH 2.2 (+); $^{w}_{w}$pH 3 (■); $^{w}_{w}$pH4 (▲) and $^{w}_{w}$pH5 (♦). Other conditions: 30 °C; citrate buffer 30 mM.

conclusion of this study, it appears clearly that a linear model must never be used for ionizable compounds (as usually done for neutral solutes), to fit the experimental log(*k*) versus the percentage of organic modifier. In most cases, a quadratic model provides a good fitting of the experimental data but for some basic solutes when $^{w}_{w}$pH is lower than p$K_{a,w,solute}$, a quadratic model is not valid within the whole range of compositions but only within a small range depending on the curvature. Fortunately, in most cases (i.e. Figs. 7–9 except Fig. 7b and c) a sigmoidal model is only essential for large range of compositions including those leading to very low *k* values and then incompatible with good chromatographic conditions. Then in most cases, the solute retention is well approximated by the following quadratic model:

$$\log k = a\varphi^2 + b\varphi + c \quad (13)$$

Here, φ is the percentage of organic solvent; *a*, *b* and *c* are constants for a given solute, a given $^{w}_{w}$pH and a given reversed phase system (buffer, stationary phase, temperature).

4.5. Simultaneous optimization of $^{w}_{w}$pH and mobile phase composition

It has been shown by many authors [28–30] that preliminary gradient elution experiments are far more appropriate to mobile phase optimization in liquid chromatography than isocratic ones. This is due to the a priori lack of knowledge about the range of compositions to be investigated and furthermore about the elution mode to be used. In case of the simultaneous optimization of pH and mobile phase composition, this is more crucial because the interesting composition range is a priori unknown and moreover dependent on $^{w}_{w}$pH. The proposed procedure makes use of nine experiments to calculate the retention models of ionizable solutes: three experiments (two gradients runs with two different slopes and one isocratic run) performed at three different $^{w}_{w}$pH. For each $^{w}_{w}$pH, the three coefficients of Eq. (13) are calculated by two steps. The first step consists in calculating from the two gradient retention data the two coefficients of a linear model and then to evaluate the higher possible composition leading to *k* values higher than 0.2 and lower than 30 for all solutes. If such a composition exists, it is selected for the third experiment; if this should not be the case, it means that the elution mode cannot be isocratic and then a third experiment under gradient elution with a higher slope is performed. The second step consists in calculating the three coefficient of Eq. (13) from three retention data. The first one is provided by the isocratic run; the two others are calculated from the linear model and correspond, for each gradient run, to the *k* value and the composition just as the solute has travelled all over half column length [31]. Once the three coefficients at the three $^{w}_{w}$pH have been calculated, the published method [16] for modelling retention as a function of pH is applied to each possible composition (i.e. leading to acceptable *k* values). In order to prove its validity, we have applied this procedure to a mixture of six solutes, three basic compounds (*p*-toluidine, *m*-toluidine and *o*-toluidine) and three acidic compounds (benzoic acid, salycilic acid and *p*-nitrobenzoic acid) at three $^{w}_{w}$pH (3, 4 and 5) close to their p$K_{a,w}$ values. Calculated retention times have been calculated from nine experiments, namely two gradient runs (5% to 50% of acetonitrile in 45 min and 15 min, respectively) and one 30% acetonitrile isocratic run, these three experiments being performed at each $^{w}_{w}$pH, Experimental retention times at $^{w}_{w}$pH ranging from three to five by step of 0.5 unit and with acetonitrile compositions ranging from 5% to 35% by step of 5% have been collected and compared to the calculated values. The average difference between calculated and experimental values listed in Table 2 is 0.2% with a standard deviation equal to 3.3% that is quite sufficient for an optimization purpose. This procedure was implemented into Osiris software.

Modelling the solute behaviour allows calculation of chromatograms for any values of the parameter space. The aim of optimizing is evaluation and comparison of computerized simulated chromatograms. This is performed by using a suitable response function in order to fulfil the objectives of the chromatographer. This optimization procedure, described elsewhere [16] takes into account three relevant criteria by means of a response function based on a desirability function: the quality of separation, the analysis time and the robustness

Table 2
Experimental and calculated retention times (min) for six ionizable solutes (see text for explanations)

	pH	3.0		3.5		4.0		4.5		5.0	
	Percentage ACN	Experimental	Calculated	Experimental	Calculated	Experimental	Calculated	Experimental	Calculated	Experimental	Calculated
p-Toluidine	5	5.99	6.12	7.31	6.87	9.61	9.08	14.58	14.64	24.22	24.64
	10	3.70	3.65	4.41	4.35	6.20	6.27	9.94	10.51	16.22	16.50
	15	2.75	2.67	3.40	3.25	4.77	4.79	7.69	7.88	11.70	11.64
	20	2.30	2.24	2.84	2.74	4.10	3.99	6.34	6.26	8.95	8.65
	25	2.10	2.05	2.61	2.50	3.60	3.57	5.22	5.26	6.84	6.76
	30	1.97	1.97	2.51	2.43	3.39	3.39	4.60	4.64	5.54	5.54
o-Toluidine	5	6.19	6.51	9.40	8.99	15.59	15.04	24.77	25.25	33.10	35.05
	10	4.19	3.96	6.44	6.13	10.85	10.78	16.49	17.05	20.93	21.75
	15	3.34	3.13	5.29	5.00	8.41	8.43	11.99	12.09	14.25	14.33
	20	2.95	2.81	4.54	4.41	7.00	6.87	9.19	8.98	10.40	10.07
	25	2.79	2.68	4.16	4.04	5.75	5.78	6.99	7.02	7.71	7.58
	30	2.65	2.63	3.87	3.80	5.04	5.04	5.80	5.79	6.09	6.11
m-Toluidine	5	6.89	7.08	8.93	8.40	13.41	12.15	21.23	21.00	31.90	35.05
	10	4.19	3.97	5.54	5.40	8.78	8.97	14.34	15.27	20.93	21.66
	15	3.09	2.85	4.34	4.13	6.85	7.00	10.70	11.07	14.25	14.31
	20	2.60	2.40	3.64	3.53	5.74	5.74	8.44	8.36	10.40	10.10
	25	2.40	2.25	3.34	3.23	4.82	4.93	6.55	6.63	7.71	7.61
	30	2.27	2.28	3.14	3.11	4.41	4.41	5.54	5.52	6.09	6.09
Salicylic acid	5	–	45.08	22.06	23.01	13.19	13.44	9.69	10.04	8.74	8.92
	10	21.74	22.84	11.74	11.94	7.28	7.22	5.52	5.54	5.02	5.00
	15	12.50	12.71	7.20	6.97	4.51	4.49	3.62	3.61	3.32	3.32
	20	7.54	7.81	4.67	4.60	3.24	3.20	2.69	2.71	2.54	2.55
	25	5.35	5.31	3.56	3.40	2.52	2.56	2.17	2.27	2.11	2.17
	30	3.97	3.97	2.94	2.76	2.22	2.22	2.00	2.03	1.97	1.97
Benzoic acid	5	–	59.70	–	53.03		39.36	20.42	22.21	10.27	10.21
	10	26.85	28.82	23.49	25.67	18.67	19.28	11.05	11.38	5.94	5.93
	15	15.00	15.36	13.69	13.85	10.69	10.73	6.87	6.76	3.99	3.96
	20	8.77	9.10	8.19	8.37	7.00	6.79	4.82	4.64	3.07	2.99
	25	6.04	6.01	5.76	5.66	4.82	4.86	3.55	3.60	2.52	2.49
	30	4.39	4.39	4.39	4.24	3.86	3.86	3.02	3.10	2.22	2.22
p-Nitrobenzoic acid	5	–	70.34	–	44.02	22.01	23.04	12.55	12.79	9.02	8.97
	10	32.22	34.70	20.26	21.69	11.72	11.76	7.05	7.06	5.39	5.33
	15	18.10	18.61	11.99	11.94	6.94	6.87	4.54	4.47	3.62	3.58
	20	10.40	10.92	7.29	7.38	4.72	4.57	3.27	3.21	2.75	2.70
	25	7.00	7.02	5.19	5.09	3.39	3.42	2.52	2.56	2.26	2.23
	30	4.92	4.91	3.97	3.85	2.81	2.80	2.22	2.21	1.97	1.97
Mean error (%)		0.2									
Standard deviation (%)		3.3									

Capcell-pak column (dead volume = 1.5 mL); citrate buffer 30 mM; 1 mL min^{-1}.

Table 3
Experimental conditions for the examples of optimization given in Figs. 10 and 11

	First example (Figs. 10 and 11)	Second example (Figs. 12 and 13)
Solutes	(1) Phenol; (2) protriptylline; (3) amoxapine; (4) diphenydramine; (5) clozapine; (6) doxepine; (7) imipramine; (8) amitriptylline	(1) Phenol; (2) *N*-clozapine; (3) benzene; (4) amoxapine; (5) clozapine; (6) imipramine; (7) amitriptylline
Experimental conditions	Column: RP-Xterra C18100 mm × 4.6 mm (dead volume = 1.5 mL)	
	BisTrisPropane buffer; 30 °C; 1 mL min^{-1}	Phosphate buffer; 70 °C; 3 mL min^{-1}
Experimental design	$^{w}_{w}$pH 8 $^{w}_{w}$pH 9 $^{w}_{w}$pH 10	$^{w}_{w}$pH 6 $^{w}_{w}$pH 7 $^{w}_{w}$pH 8
Gradient runs	30% to 70% in 40 min	10% to 52% in 14 min
Isocratic run	30% to 70% in 13 min	10% to 61% in 5 min
	60% 65% 70%	50% 60% 70%
Optimum conditions	$^{w}_{w}$pH 8.7 and 50% acetonitrile (Fig. 11)	$^{w}_{w}$pH 6.9 and 45% acetonitrile (Fig. 13)

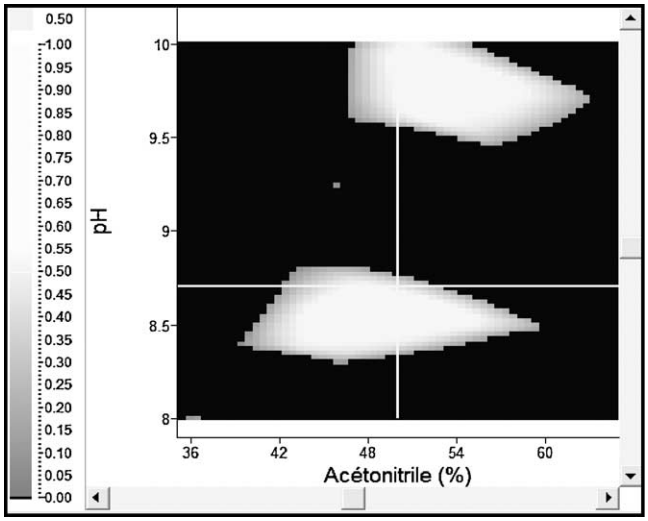

Fig. 10. Response surface vs. $^{w}_{w}$pH and percentage of acetonitrile for the first example of optimization. Conditions are given in Table 3.

of the method. The response function varies from zero to one with a zero value when one at least of the three criteria has not reached the threshold value fixed by the chromatographer. Two examples of robust analysis conditions research are discussed below. Both deal with the simultaneous optimization of $^{w}_{w}$pH and acetonitrile composition for a mixture of basic and neutral compounds. The first example concerns the separation of eight solutes at 30 °C with BisTrisPropane as buffer and the second example, the separation of seven solutes at 70 °C with phosphate as buffer. As discussed above, it is interesting to separate compounds at elevated temperatures since it allows faster separations without loss of efficiency (here the flow-rate is three times higher at 70 °C than at 30 °C). The conditions of the preliminary experiments are listed in Table 3. Both response surfaces computed by Osiris software are given in Figs. 10 and 12, respectively. They are determined from the following threshold values: resolution higher than 1.5 for the less separated pair of peaks ($R_{s_{min}}$), retention factors within 0.5 and 15; 0.1 unit of pH and 1% of acetonitrile for the dimensions of the robustness window. As shown by the chromatograms of Figs. 11 and 13, experimental results are in very good agreement with calculated ones and that confirms the reliability of the proposed procedure in optimizing both $^{w}_{w}$pH and mobile phase composition. In addition, this procedure which requires nine experiments only can provide more rapidly the p$K_{a,chrom}$ of all the solutes over a wide range of organic modifier composition as it can be seen on Fig. 5 where the empty characters represent the p$K_{a,chrom}$ calculated from the set of nine experiments which can be successfully compared to those calculated at each acetonitrile composition from a set of three retention data obtained at three different $^{w}_{w}$pH.

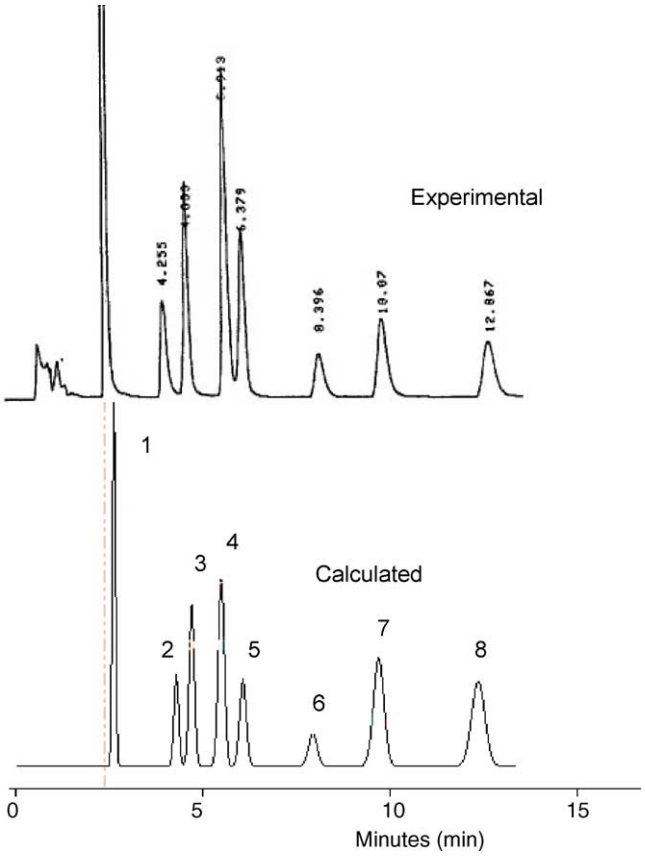

Fig. 11. Experimental and calculated separations for the optimum conditions of Fig. 10. Conditions and solutes are given in Table 3.

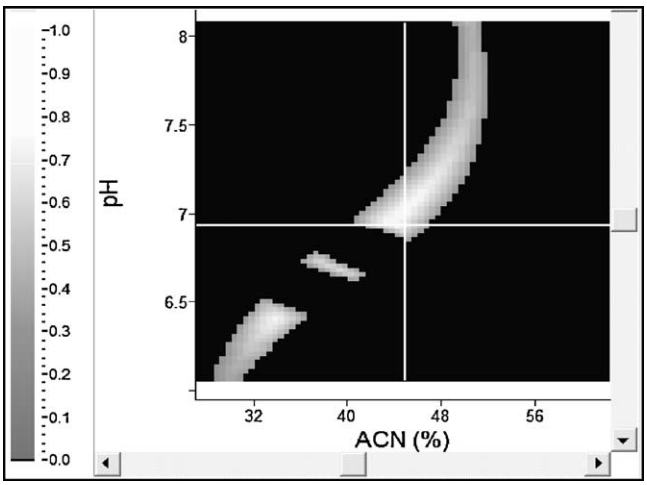

Fig. 12. Response surface vs. $^{w}_{w}$pH and percentage of acetonitrile for the second example of optimization. Conditions are given in Table 3.

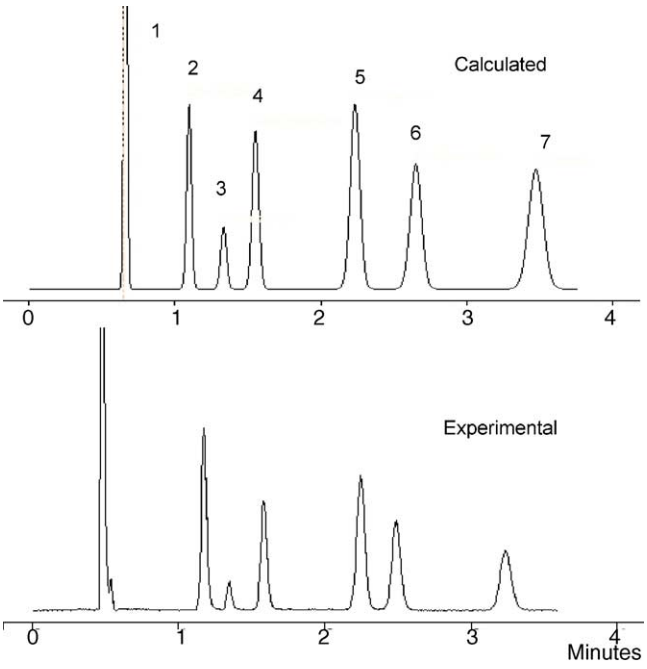

Fig. 13. Experimental and calculated separations for the optimum conditions of Fig. 12. Conditions and solutes are given in Table 3.

5. Conclusion

The determination of the solute pK_a from retention data related to the pH measured in aqueous medium provides a quantity that we have called chromatographic pK_a:p$K_{a,chrom}$. The study of the variation of p$K_{a,chrom}$ with the solvent composition has highlighted the effect of various factors on the ionization of acid and basic compounds. These factors include the type of organic modifier, the type of buffer and the column temperature. It has been shown that for a given compound, p$K_{a,chrom}$ is highly dependent of the particular buffer employed. It has been shown for basic solutes that log(k) versus the solvent composition are neither linear nor quadratic as it is for neutral ones but sigmoidal once the pH of the aqueous medium is lower than the solute pK_a. However, in most cases, the retention data are well fitted with a quadratic model. The retention modeling system consists in describing k first as a function of solvent composition and then as a function of the pH measured in the aqueous medium. This procedure has two main advantages: it allows first to make use of gradient data that are much more appropriate for modeling k as a function of solvent composition and secondly to have the possibility of choosing between two models the more suitable for describing k as a function of pH. It has been shown that the predicted retention times, using this procedure are very close to the experimental ones. We have incorporated this effective procedure into an optimization software and it has proved to provide reliable results for ionizable compounds.

References

[1] M.R. Buchmeiser, J. Chromatogr. A 918 (2001) 233.
[2] J. Nawrocki, J. Chromatogr. A 779 (1997) 29.
[3] M.R. Euerby, P. Patersson, J. Chromatogr. A 914 (2003) 13.
[4] C. Stella, S. Rudaz, J.L. Veuthey, A. Tchapla, Chromatographia 53 (2001) 113.
[5] R.G. Bates, Determination of pH Theory and Practice, second edition, Wiley, New York, 1973.
[6] D. Sykora, E. Tesarova, M. Popl, J. Chromatogr. A 758 (1997) 37.
[7] I. Canals, J.A. Portal, E. Bosch, M. Roses, Anal. Chem. 72 (2000) 1802.
[8] IUPAC, Compendium of Analytical Nomenclature, Definitive rules, 1997.
[9] J. Barbosa, V. Sanz-Nebot, Anal. Chim. Acta 283 (1993) 320.
[10] I. Canals, F. Oumada, M. Roses, E. Bosch, J. Chromatogr. A 911 (2001) 191.
[11] S. Espinosa, E. Bosch, M. Roses, Anal. Chem. 72 (2000) 5193.
[12] I. Canals, K. Valko, E. Bosch, A. Hill, M. Roses, Anal. Chem. 73 (2001) 4937.
[13] P.J. Schoenmakers, S. Van Molle, C.M. Hayes, L.G. Unk, Anal. Chim. Acta 250 (1991) 1.
[14] J.A. Lewis, D.C. Lommen, W.D. Raddatz, J.W. Dolan, L.R. Snyder, I. Molnar, J. Chromatogr. 592 (1992) 183.
[15] P.J. Schoenmakers, R. Tijssen, J. Chromatogr. A 656 (1993) 577.
[16] S. Goga, S. Heinisch, J.L. Rocca, J. Chromatogr. A 868 (2000) 13.
[17] S. Espinosa, E. Bosch, M. Roses, J. Chromatogr. A 947 (2002) 47.
[18] C. Horvath, W. Melander, I. Molnar, Anal. Chem. 49 (1977) 142.
[19] The Merck Index, 11th ed., 1989.
[20] M. Roses, E. Bosch, J. Chromatogr. A 982 (2002) 1.
[21] M. Roses, C. Rafols, E. Bosch, Anal. Chem. 65 (1993) 2294.
[22] E. Espinosa, P. Bou, H. Alleman, M. Roses, Anal. Chem. 68 (1996) 3651.
[23] J. Barbosa, D. Barron, S. Buti, Anal. Chim. Acta 389 (1999) 31.
[24] H.A. Claessens, M.A. Van Straten, J.J. Kikland, J. Chromatogr. A 728 (1996) 259.
[25] L.R. Snyder, J.W. Dolan, J.R. Gant, J. Chromatogr. 165 (1979) 3.
[26] R.J. Tijssen, H.A.H. Billiet, P.J. Schoenmakers, J. Chromatogr. 122 (1976) 185.
[27] K. Valko, L.R. Snyder, J.L. Glajch, J. Chromatogr. A 656 (1993) 501.
[28] J.W. Dolan, L.R. Snyder, M.A. Quarry, Chromatographia 24 (1987) 261.
[29] P.J. Schoenmakers, H.A.H. Billiet, L. De Galan, J. Chromatogr. 205 (1981) 13.
[30] S. Heinisch, J.L. Rocca, M. Feinberg, J. Chemometrics 3 (1988) 127.
[31] M.A. Quarry, R.L. Grob, L.R. Snyder, Anal. Chem. 58 (1986) 907.