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Effects of pH and the presence of micelles on the resolution of diuretics by reversed-phase liquid chromatography

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Abstract

A comparative study on the performance of two RPLC modes on the separation of 18 diuretics with diverse acid–base behaviour (acetazolamide, althiazide, amiloride, bendroflumethiazide, benzthiazide, bumetanide, canrenoic acid, chlorothiazide, chlorthalidone, ethacrynic acid, furosemide, hydrochlorothiazide, piretanide, probenecid, spironolactone, triamterene, trichloromethiazide and xipamide) was carried out. A conventional octadecylsilane column and acidic acetonitrile–water mobile phases, in the absence and presence of micelles of the anionic surfactant sodium dodecyl sulphate (SDS), were used. The effects of pH and the modifiers acetonitrile and SDS on peak asymmetry, efficiency, selectivity, resolution and analysis time, were examined. The comparison of both RPLC modes (aqueous- and micellar-organics) was done using the same processing tools, applying several polynomial and mechanistic equations to describe the retention. The best separations were obtained by maximising the product of peak purities, considering a wide range of experimental conditions. The study illustrates that, despite the theoretical and practical complexity of the problem, the predicted optimal chromatograms can be reproduced experimentally with great accuracy. None of the examined RPLC modes was able to yield baseline separation of the 18 diuretics. However, their selectivity was complementary, being appropriate for different combinations of a smaller number of the assayed diuretics.

Keywords: Mobile phase composition; pH effects; Micelles; Resolution; Selectivity; Diuretics

1. Introduction

Trial-and-error strategies are frequent in the optimisation of chromatographic separations, despite the numerous reports that prove the superiority of interpretive optimisations. Diverse software have been marketed to facilitate their implementation, especially for reversed-phase liquid chromatographic separations [1–3]. In isocratic elution, only one factor (i.e. the concentration of organic solvent) is usually optimised. However, methodologies for the optimisation of two or three factors affecting the separation, such as the concentrations of organic solvent and amine [4], organic solvent and surfactant [5], the concentration of organic solvent and temperature [6], or pH [7–12], and the concentrations of organic solvent, surfactant and pH [13], have been reported.

Reliable predictions require mainly models describing the retention as accurate as possible, peak profile and other factors being less relevant. However, non-ideal chromatographic peaks are quite common in practice, which frequently obliges to consider asymmetrical peaks and eventual variations in peak profile with mobile phase composition [14]. The optimisation strategy developed in our laboratory [15,16] can be applied to any chromatographic mode, provided the particular retention model for each system is known.

In addition to resolution purposes, predictions obtained by interpretive methods are very useful to compare the particularities and performance of different chromatographic systems. In previous work, we presented several studies comparing aqueous- and micellar-organic RPLC separations for several groups of drugs: β-blockers [17], sulphonamides, steroids [18], and tricyclic antidepressants [19]. In aqueous-organic RPLC, the concentration of organic solvent was optimised, whereas in micellar-organic RPLC, besides the organic solvent, the effect of surfactant concentration and the interrelations between both factors were studied. Usually, micellar-organic mobile phases yielded shorter analysis times, being solvent cost appreciably

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smaller and the resolutions comparable or even better. In these examples, the pH of the mobile phase was kept constant.

In this work, a comparative study of both chromatographic modes was carried out to assess their performance on the separation of compounds showing diverse acid–base behaviour. The simultaneous effect of pH and concentration of both modifiers (organic solvent and surfactant) is considered. Retention of ionisable compounds depends on the ratio of both acid–base species, which makes acidity a worthy factor to be taken into account in RPLC separations. Changes in retention and selectivity with pH may be extremely large for some acidic compounds, being difficult to model accurately from a reduced number of experiments. The difficulties are increased since changes in organic solvent concentration affect solute acid–base constants. Not surprisingly, fixing the pH at a convenient value is a widely extended practice, which means that a worthy experimental factor is not profited. On the other hand, the inherent practical difficulties of getting accurate pH values in aqueous-organic solutions are not often emphasised sufficiently in most reports where the pH is optimised.

The compounds studied are 18 drugs with diuretic activity. Numerous analytical procedures have been reported for their determination [20–24], mainly using octadecylsilane columns with acetonitrile as mobile phase modifier. These conditions were also chosen for our study. Chromatographic elution was performed in the absence or presence of sodium dodecyl sulphate (SDS), which is the most widely used surfactant in this type of separations. The addition of SDS to the mobile phase decreased substantially the amount of acetonitrile needed to elute the solutes in adequate retention times.

Our study illustrates that, despite the theoretical and practical complexity of the problem, the predicted optimal chromatograms can be reproduced experimentally with great accuracy. For the development of this study, the works of Barbosa and coworkers [11] and Barbosa et al. [25–28] on the acid–base equilibria of diuretics in mixed acetonitrile–water solvents, and on the rapid optimisation of the resolution of mixtures of diuretics in aqueous-organic RPLC considering the concentration of organic-solvent and pH, were particularly useful. We applied here an optimisation methodology that improves the accuracy in the prediction of retention and considers the variations in peak shape with eluent composition. This allowed an appropriate comparison of the performance of the aqueous- and micellar-organic systems for the analysis of diuretics.

2. Experimental

2.1. Reagents

Both aqueous- and micellar-organic mobile phases contained acetonitrile (HPLC grade, Scharlab, Barcelona, Spain), whose concentration is given as percentage in volume fraction. Sodium dodecyl sulphate was also used in the micellar mode (99% purity, Merck, Darmstadt, Germany). The pH was buffered with 0.1 M citric acid (Prolabo, Paris, France) and NaOH (Probus, Badalona, Spain).

Stock solutions containing 100 μg/ml of the following diuretics were prepared: althiazide, benzthiazide, bumetanide, canrenoic acid, chlorothiazide, furosemide, hydrochlorothiazide, probenecid, triamterene, trichloromethiazide (Sigma, St. Louis, MO, USA), amiloride (ICI-Farma, Madrid, Spain), acetazolamide (Lederle, Madrid), bendroflumethiazide (Davur, Madrid), chlorthalidone (Ciba-Geigy, Barcelona), ethacrynic acid (Merck, Sharp & Dohme, Madrid), piretanide (Cusi, Barcelona), spironolactone (Searle, Madrid), and xipamide (Lacer, Barcelona). The diuretics, except those of Sigma, were kindly donated by the indicated pharmaceutical laboratories. The drugs were dissolved in a few milliliters of ethanol (analytical grade, Prolabo), with the aid of an ultrasonic bath, and diluted to 20 μg/ml with water for the aqueous-organic mode and with 0.1 M SDS for the micellar mode. The solutions were stored in darkness at 4 °C. Althiazide, furosemide and trichloromethiazide solutions were protected from light with aluminium foil. Nanopure water (Barnstead, Sybron, Boston, MA, USA) was used throughout.

2.2. Apparatus

The HPLC system (Model HP 1050, Palo Alto, CA, USA) was equipped with an isocratic pump, an autosampler with 2 ml vials (Series 1100, Model G1313A), and a UV-Vis detector. The signal was monitored at 274 nm, close to an absorption maxima of the diuretics and where the absorbance of the buffer system was negligible. All separations were carried out with a Kromasil C_{18} column (125 mm × 4.6 mm i.d. and 5 μm particle size, 100 Å pore size; Análisis Vínicos, Ciudad Real, Spain), which was connected to a similar 30 mm guard column (Scharlab). A new column was used for each chromatographic mode. The chromatographic runs were carried out at room temperature. The flow-rate was 1.0 ml/min and the injection volume, 20 μl. Duplicate injections were made.

Data acquisition was carried out with the Peak-96 software (Hewlett-Packard, Avondale, PA, USA). The algorithms for modelling and optimisation were implemented with MATLAB 6.5 (Natick, MA, USA).

3. Prediction of retention

3.1. Aqueous-organic RPLC

Lopes Marques and Schoenmakers reported a detailed study about retention modelling in aqueous-organic RPLC [29]. The dependence of the logarithm of the retention factor with the concentration of organic solvent, φ, was demonstrated to be appropriately described by a quadratic

relationship, that can be expressed as:

$$k = k^0 \, e^{(S\varphi + T\varphi^2)} \quad (1)$$

where k^0 is the retention factor in the absence of modifier, and S and T are coefficients related to the elution strength. The additional effect of pH on retention was introduced by substituting Eq. (1) for the basic and acidic species, and a similar dependence for the acid–base dissociation constant, in the model that relates k with the concentration of hydrogen ions, h [29]. We applied the same approach, but with several modifications. First, the treatment was simplified by outlining it in terms of protonation:

$$k = k_A \frac{1}{1 + Kh} + k_{HA} \frac{Kh}{1 + Kh} = \frac{k_A + k_{HA} Kh}{1 + Kh} \quad (2)$$

where k_A and k_{HA} are the retention factors for the basic and acidic species, respectively, and K is the protonation constant. According to this, the retention model is given by:

$$k = \frac{k_A^0 \, e^{(S_A\varphi + T_A\varphi^2)}}{1 + K^0 h \, e^{(Q_1\varphi + Q_2\varphi^2)}} + \frac{k_{HA}^0 K^0 h \, e^{[(S_{HA}+Q_1)\varphi + (T_{HA}+Q_2)\varphi^2]}}{1 + K^0 h \, e^{(Q_1\varphi + Q_2\varphi^2)}} \quad (3)$$

Eq. (3) is hard to fit for some solutes (i.e. the equation is non-linear, initial values close to the solution are required, colinearity between parameters is present, and overflow and divergence may happen during the fittings). All these difficulties are decreased by rewriting the equation as follows:

$$k = \frac{e^{(k_A^{0'} + S_A\varphi + T_A\varphi^2)} + h \, e^{[(K'' + S''\varphi + T''\varphi^2)}}{1 + h \, e^{(K^{0'} + Q_1\varphi + Q_2\varphi^2)}} \quad (4)$$

It should be noted that the aim of our study was to obtain predictions of retention as accurate and reliable as possible. We were not interested in the evaluation of the original parameters in Eq. (3).

More recently, Rosés and Bosch proposed an alternative retention model, which is focused on polar interactions inside the column [30]:

$$\log k = (\log k)_0 + p(P_m^N - P_s^N) \quad (5)$$

Eq. (5) separates the contributions of solute, mobile phase and stationary phase by means of the relative polarity descriptors p, P_m^N, $(\log k)_0$ and P_s^N, where the superindex N indicates that the respective descriptor have been normalised; the subindexes m and s refer to the mobile and stationary phase, respectively. The descriptor p depends mainly on solute polarity, although it also considers the nature of the mobile and stationary phases. P_m^N represents the contribution of the mobile phase polarity, and $(\log k)_0$ and P_s^N are descriptors that depend mainly on the working column. The intercept $(\log k)_0$, represents the retention associated to a hypothetical mobile phase with the same polarity as the stationary phase. For a given acetonitrile–water system, the polarity of the mobile phase is given by:

$$P_m^N = 1 - \frac{2.068\varphi}{1 + 1.341\varphi} \quad (6)$$

An interesting advantage of Eq. (5) is that it only needs knowledge of one parameter by solute plus two additional descriptors for the column. This means that, once the column is characterised, the retention of any solute can be predicted from only one experiment. When individually fitted for each solute, the performance is similar to that of the quadratic equation between $\log k$ and φ [31].

The dependence of the retention with pH and φ was introduced in the polarity approach [32]. By dividing all the terms in Eq. (2) by k_{HA}, making $f = k_A/k_{HA}$ and expressing the relationship in logarithmic form, the following results:

$$\log k = \log k_{HA} + \log \left[f - \frac{Kh}{1 + Kh}(f - 1) \right] \quad (7)$$

Finally, combining Eqs. (5) and (7), and considering a linear relationship between K and φ:

$$\log k = (\log k)_0 + p(P_m^N - P_s^N) + \log \left[f - \frac{e^{(K^{0'} + m\varphi)} h}{1 + e^{(K^{0'} + m\varphi)} h}(f - 1) \right] \quad (8)$$

In both Eqs. (4) and (8) the factors are pH and φ. The former equation contains nine parameters ($k_A^{0'}$, S_A, T_A, K'', S'', T'', Q_1, Q_2 and $K^{0'}$), and the latter, six ($(\log k)_0$, P_s^N, p, f, $K^{0'}$ and m).

3.2. Micellar-organic RPLC

The complexity of the problem grows substantially when a third factor is added to the design, even more if one considers that the three factors interact. We have published several studies on the description of the retention in micellar RPLC [33]. A mechanistic model was developed first at fixed pH [34], which was further combined with Eq. (2) to obtain the simultaneous dependence of k with surfactant and organic solvent concentrations, and pH [13]:

$$k = \frac{(K_{AS}/(1 + K_{AD}\varphi)) + (K_{HAS}/(1 + K_{HAD}\varphi))Kh}{(1 + ((1 + K_{MD}\varphi)/(1 + K_{AD}\varphi))K_{AM}\mu) + (1 + ((1 + K_{HMD}\varphi)/(1 + K_{HAD}\varphi))K_{HAM}\mu)Kh} \quad (9)$$

where μ is the concentration of surfactant forming micelles (total concentration of surfactant minus critical micellar concentration), K_{AS} and K_{AM} are the solute-stationary phase and solute-micelle partition constants in pure micellar eluents, and K_{AD}, K_{MD} and K_{SD} measure the relative variations in solute concentration in bulk water, micelle and stationary phase, respectively, in the presence of organic solvent, taking the pure micellar solution as reference. K_{AS}, K_{AM}, K_{AD} and K_{MD} are constants associated to the basic species,

whereas K_{HAS}, K_{HAM}, K_{HAD} and K_{HMD} are associated to the acidic species. These constants, together with the protonation constant, K, constitute the nine parameters that should be fitted to describe the retention of each solute. It is interesting to note that the number of parameters is similar to that in aqueous-organic RPLC (at least nine experiments are required), although three factors are now available to tune the selectivity in optimisations.

When the protonation happens only partially in the selected experimental domain, the choice of polynomial models taking into account the pH and the concentrations of both modifiers (organic solvent and surfactant) becomes an interesting alternative to model the retention in micellar-organic systems. Several equations were checked where $1/k$ and $\log k$ were related with the mobile phase factors. The following equations yielded appropriate results, depending on the extent of the protonation process sampled by the experimental design:

$$\frac{1}{k} = a_0 + a_1\,\mu + a_2\,\varphi + a_3\,\mathrm{pH} + a_{11}\,\mu^2 \quad (10)$$

$$\frac{1}{k} = a_0 + a_1\,\mu + a_2\,\varphi + a_3\,\mathrm{pH} + a_{12}\,\mu\varphi + a_{13}\,\mu\,\mathrm{pH} + a_{23}\,\varphi\,\mathrm{pH} + a_{123}\,\mu\varphi\,\mathrm{pH} + a_{33}\,\mathrm{pH}^2 \quad (11)$$

$$\frac{1}{k} = a_0 + a_1\,\mu + a_2\,\varphi + a_3\,\mathrm{pH} + a_{12}\,\mu\varphi + a_{13}\,\mu\,\mathrm{pH} + a_{23}\,\varphi\,\mathrm{pH} + a_{33}\,\mathrm{pH}^2 + a_{233}\,\varphi\,\mathrm{pH}^2 + a_{333}\,\mathrm{pH}^3 \quad (12)$$

The selection of the most adequate equation for each solute according to its particular acid–base behaviour was carried out by cross-validation.

4. Results and discussion

4.1. Retention behaviour of diuretics

The structures and protonation constants in aqueous medium of the diuretics are listed in Table 1. Octanol–water partition coefficients ($\log P_{o/w}$) are also given. These coefficients are usually between −1.21 (amiloride) and 2.20 (ethacrynic acid, piretanide and xipamide); canrenoic acid (2.40) and spironolactone (2.71) are out of this range. The moderate polarity of most diuretics makes acetonitrile and propanol appropriate to be used as organic solvents in the mobile phases containing micellised SDS [18]. Acetonitrile is a common solvent in conventional RPLC, but scarcely used in the micellar mode. Since the purpose of our study was to assess the effect of the surfactant on the selectivity achieved in RPLC, we chose the same solvent (acetonitrile) for both elution modes. Note, however, that the acetonitrile content required in micellar-organic RPLC was smaller, because SDS expedites the elution strongly.

According to their acid–base behaviour, the diuretics can be classified in four groups: basic (amiloride and triamterene), neutral (spironolactone), weakly acidic (chlorthalidone, acetazolamide and thiazides), and acidic (xipamide, canrenoic acid, piretanide, furosemide, bumetanide, ethacrynic acid and probenecid; Table 1). In this way, when a mixture of diuretics is chromatographed, each compound is expected to show a particular behaviour with mobile phase acidity. This will favour multiple peak crossings. Thus, the search of the most adequate separation conditions will be difficult.

As indicated above, chromatographic retention of ionisable compounds depends on the pH of the mobile phase, being a weighted mean of the elution behaviour of the acidic and basic species. Since the intrinsic retentions of both species are different, a sudden change in retention times will happen at pH values close to the logarithm of the acid–base apparent constant in the mobile phase medium. The full protonation process covers several pH units and is shifted according to the apparent constant. However, the change in retention was only properly observed for the acidic diuretics, since the working pH range of a conventional octadecylsilane stationary phase—such as the Kromasil C_{18} column used in this work—is rather reduced (pH 3–7). For this reason, the retention plots of the diuretics versus pH (Fig. 1) showed different patterns and the fittings were in some instances rather difficult.

For most diuretics chromatographed in both mobile phase systems, a decreased retention was observed with pH, at least close to neutral medium. For some weakly acidic diuretics and the neutral compound spironolactone, the retention did not change in the column pH range, whereas for the basic diuretics amiloride and triamterene eluted with acetonitrile–water without the surfactant the retention increased with pH. This behaviour can be explained by considering the ionic nature of the eluted compounds, which is affected by the presence of the modifiers. Acidic diuretics are neutral at low pH and become negatively charged upon dissociation. In mobile phases without surfactant, the neutral species establish hydrophobic interactions with the stationary phase, being retained depending on their polarity. When the negative species dominate, the affinity of the compounds towards the stationary phase is reduced. Consequently, the retention decreases at increasing pH (Fig. 1a and b). When SDS is added, strong hydrophobic interactions are also established between the acidic species and the stationary phase, which is now covered by surfactant molecules and exhibits negative charge. The formation of the anionic basic species, which is repelled by this negative charge, produces again a decreased retention (Fig. 1d and e), although the curve is shifted with respect to aqueous-organic RPLC due to the presence of micelles, that stabilises the protonated neutral species increasing the protonation constant.

For the basic diuretics amiloride and triamterene, the acidic species are positively charged and the basic species,

Table 1
Structures, protonation constants and octanol–water partition coefficients of the studied diuretics

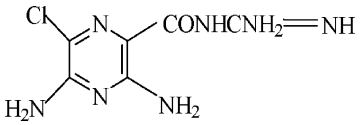
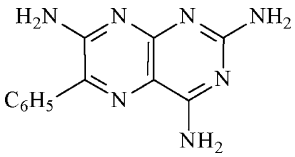
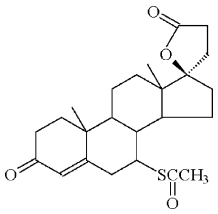
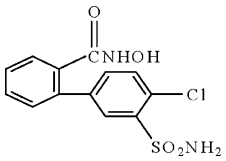
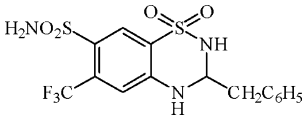
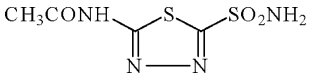
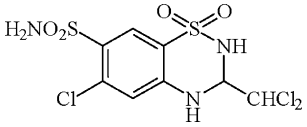
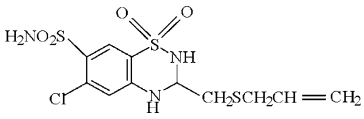
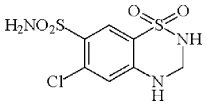
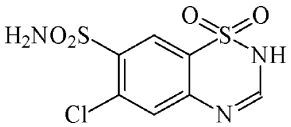
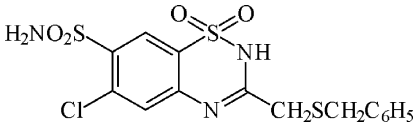
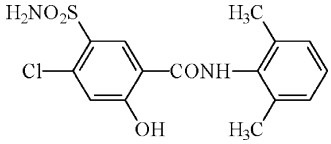
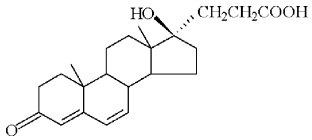
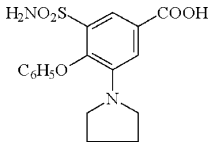
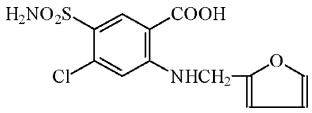
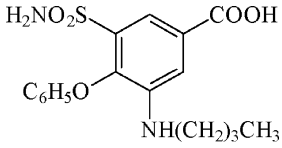
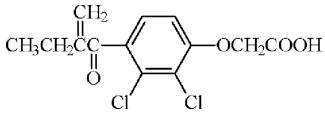
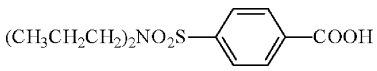
Compound		Structure	log K[a]	log $P_{o/w}$[b]
Amiloride	AMI		8.7	−1.21
Triamterene	TAT		6.2	1.22
Spironolactone	SPI		–	2.71
Chlorthalidone	CHLOR		9.3	0.24
Bendroflumethiazide	BEN		9.0	1.95
Acetazolamide	ACE		7.4	−0.30
Trichloromethiazide	TRIZ		10.6, 8.6, 7.3	1.00
Althiazide	ALTZ		–	1.01
Hydrochlorothiazide	HYDZ		7.0	−0.11
Chlorothiazide	CHLZ		6.7	−0.35
Benzthiazide	BENZ		6.0	1.73

Table 1 (*Continued*)

Compound		Structure	log K[a]	log $P_{o/w}$[b]
Xipamide	XIP		10.0, 4.8	2.19
Canrenoic acid	CAN		4.6	2.40
Piretanide	PIR		4.1	2.20
Furosemide	FUR		7.5, 3.8	1.81
Bumetanide	BUM		7.7, 3.6	2.09
Ethacrynic acid	ETHAC		3.5	2.20
Probenecid	PROB		3.4	1.40

[a] [35,36].
[b] [37].

neutral. In aqueous-organic RPLC, the interaction is stronger for the neutral species, producing an increased retention with pH (Fig. 1c). In micellar-organic RPLC, both acid–base species interact again hydrophobically with the column, but the interaction is stronger for the positively charged species, due to the additional electrostatic attraction. Therefore, the elution profile is similar to that found for the acidic diuretics in the presence of SDS, and opposed to the behaviour of basic diuretics in the aqueous-organic system (Fig. 1f). Moreover, the additional electrostatic interaction of the cationic protonated species towards the anionic SDS-modified stationary phase explains the strong retention. This is a general behaviour observed for basic drugs in SDS mobile phases [18].

Fig. 1 depicts also the influence of acetonitrile concentration on retention. For both RPLC modes, the organic solvent shifts the curves due to the modification of the protonation constants, in addition to the decreased retention. These constants have been determined for some diuretics experimentally. For instance, log K for furosemide is 3.8 in aqueous medium and increases to 4.8, 5.0 and 5.4 for 30, 40 and 50% acetonitrile, respectively [26]. This trend is also observed for the same acetonitrile concentrations in other diuretics, such as trichloromethiazide (log K = 7.3, 7.9, 8.3 and 8.8), and amiloride (log K = 8.7, 8.9, 9.0 and 9.3) [25]. Although the protonation process is only partially covered by the column working pH range, curves in Fig. 1a–c show shifts to larger pH values.

The effect of acetonitrile on the acid–base equilibria was smaller in micellar media, at least in the observed acetonitrile concentration range (10–20% for micellar-organic in contrast to 30–50% for the aqueous-organic system) (Fig. 1d–f). On the other hand, a slight shift towards higher pH was observed at increasing concentration of SDS in the mobile phase. The protonation constants of acidic diuretics were measured in a propanol–SDS medium in a previous work and showed decreases and increases by increasing the concentrations of propanol and SDS, respectively [38]. The shifts in acid–base equilibria produced by micelles makes the second

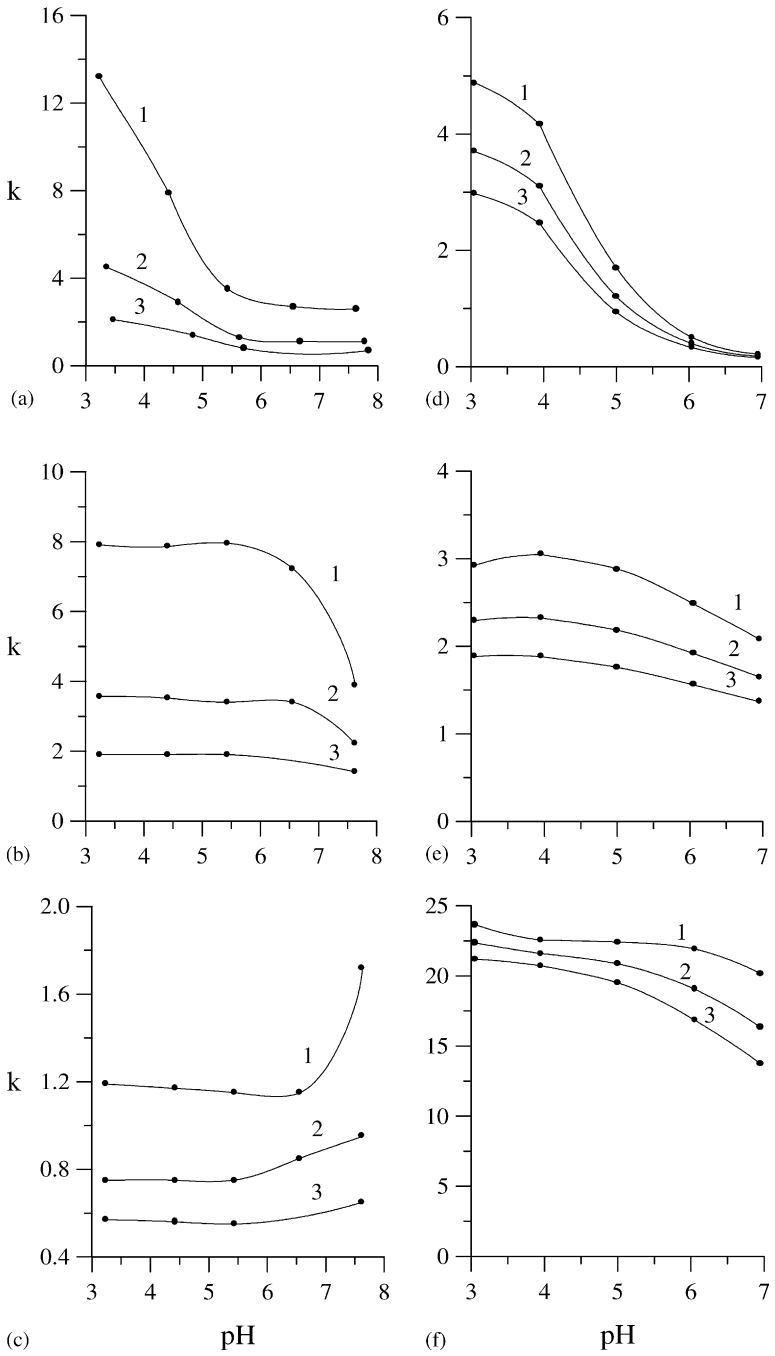


Fig. 1. Effect of pH on the retention of three diuretics in the presence of increasing acetonitrile concentration. (a–c) Aqueous-organic RPLC: (1) 30, (2) 40, and (3) 50% (v/v) acetonitrile. (d–f) Micellar-organic RPLC (0.10 M SDS): (1) 10, (2) 15, and (3) 20% (v/v) acetonitrile. Compounds: (a, d) furosemide, (b, e) trichloromethiazide, and (c, f) triamterene.

(and eventual third equilibria) for polyprotic drug systems less apparent.

The elution strength was measured in both chromatographic modes as the slope of the classical retention models that relate linearly $\log k$ to the concentration of modifier. At pH 3 without surfactant, the elution strength of acetonitrile varied in the range from −1.6 to −6.4. In the micellar-organic system, it ranged between −0.5 and −3.5, whereas the elution strength for SDS varied between −0.9 and −6.7. The mean values for acetonitrile were −4.0 and −1.6, for the aqueous- and micellar-organic systems, respectively, and −4.3 for SDS. Therefore, the surfactant showed greater elution strength than acetonitrile in the micellar-organic mode, but similar to the organic solvent in the aqueous-organic mode. The mean elution strength for acetonitrile was similar at other pH values, whereas for SDS it decreased at pH close to 7.

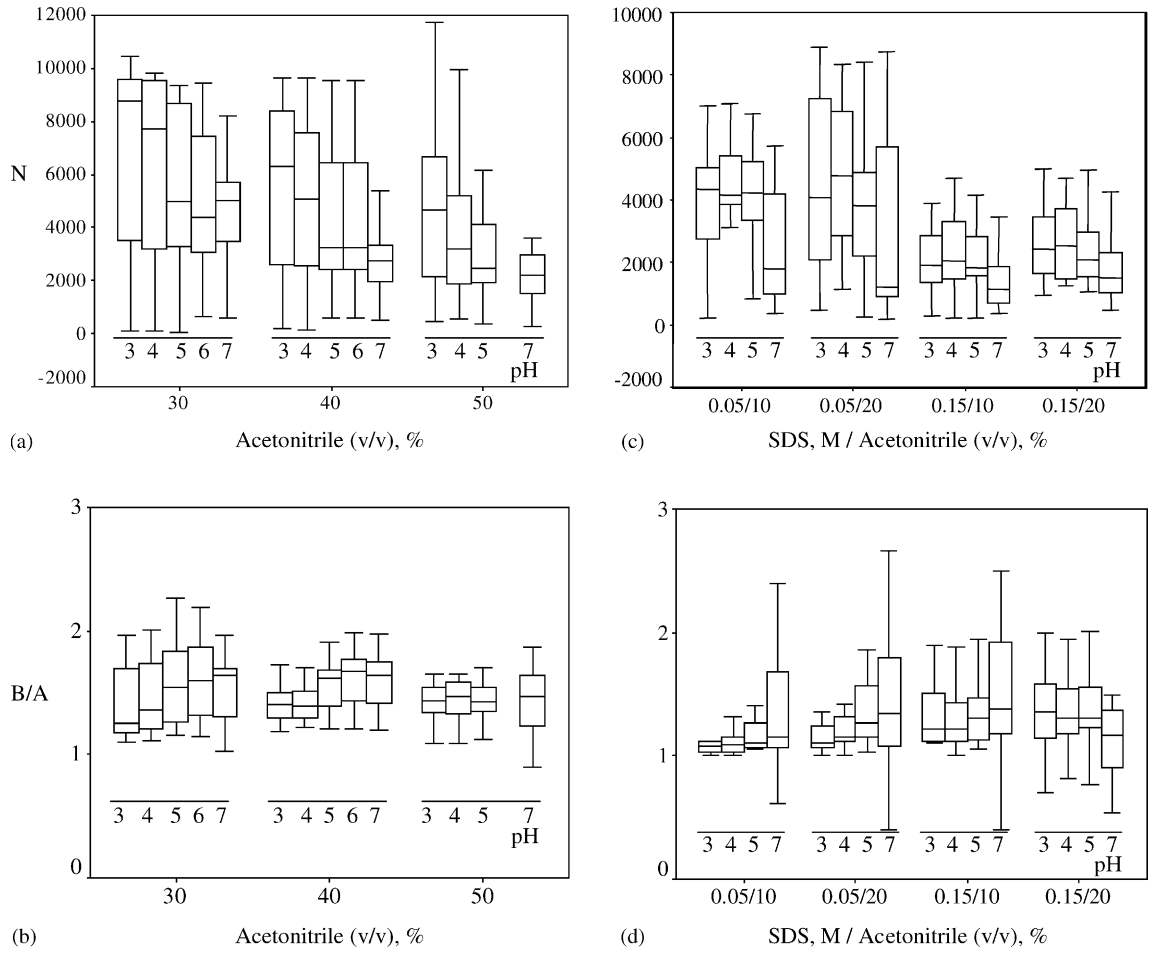


Fig. 2. Efficiencies (*N*) and asymmetry factors (*B*/*A*) for the aqueous-organic (a, b) and micellar-organic (c, d) mobile phases, at diverse mobile phase compositions and pH.

4.2. Efficiency and asymmetry

The efficiency, expressed as plate counts (*N*), was estimated at 10% peak height according to the equation of Foley and Dorsey [39]. The asymmetry factor (*B*/*A*) was calculated as the tailing-to-fronting halfwidths ratio, measured also at 10% peak height. Both *N* and *B*/*A* values are plotted in Fig. 2. As observed for the aqueous-organic mode (Fig. 2a), the efficiencies decreased at increasing concentrations of acetonitrile in the 30–50% range. Also, at each acetonitrile level the individual values were reduced at increasing pH. In the micellar mode, the efficiencies were usually smaller (Fig. 2c). They increased with the amount of acetonitrile and decreased with SDS, which is the usual behaviour in these systems. For a given level of acetonitrile and SDS, the efficiencies varied scarcely with pH, except close to pH 7, where a sharp reduction was observed. Owing to the increase in elution strength and, in a smaller extent, the deterioration of the efficiencies at larger SDS concentrations, the optimal separations in micellar RPLC are usually obtained at low or intermediate surfactant concentrations.

In the aqueous-organic mode, the asymmetry factors (Fig. 2b) varied slightly with the concentration of acetonitrile, and at a given concentration of organic solvent, peak shape was deteriorated with pH. In the micellar mode (Fig. 2d), the asymmetry factors were similar at varying acetonitrile and SDS concentrations, but the peaks deteriorated at pH 7. The mean asymmetry factors were 1.52 and 1.37 in the pH range of 3–5, in the absence and presence of surfactant, respectively. Therefore, the peaks, although wider, were slightly more symmetrical in micellar RPLC. Enhanced peak shape, in a larger extent, was previously reported for basic drugs, such as β-blockers and phenethylamines, eluted with hybrid SDS mobile phases [17,40].

4.3. Prediction of retention

The studied diuretics exhibit diverse acid–base behaviour. The retention of some compounds does not change with pH. However, since a slight decrease in retention was observed at increasing pH due to changes in ionic strength—changes that can be correlated with pH—a similar mathematical treatment was assayed to describe the retention for all diuretics. The complexity of the problem adviced to develop an exhaustive experimental design to assure a good

description, including more experiments than those required attending exclusively to the number of parameters in the fitted equations (e.g. nine for Eq. (4) and 10 for Eq. (12)). The experimental design in aqueous-organic RPLC contained 15 mobile phases at 30, 40 and 50% acetonitrile, in each case at pH close to 3, 4, 5, 6 and 7. In micellar RPLC, data from 20 mobile phases (acetonitrile/SDS) were taken: 10%/0.05 M, 20%/0.05 M, 15%/0.10 M, 10%/0.15 M, and 20%/0.15 M, at pH values close to 3, 4, 5 and 7.

In both systems, pH was buffered with citric acid/citrate. Measurements of pH were first carried out in the buffered aqueous solution, before adding the organic solvent to the mobile phase, and afterwards, in the aqueous-organic mixture. In this medium, acid–base equilibria of the buffer are inhibited and the pH values are larger. The standardisation of the pH-meter was always carried out using aqueous buffers [41].

The difference in pH between aqueous and aqueous-organic solutions was greater at increasing acetonitrile concentration and larger pH, ranging between 0.55 for 30% acetonitrile at pH 3 and 1.13 for 50% acetonitrile at pH 7, with a mean value of 0.88. The difference in pH between pure micellar (i.e. before mixing the organic solvent) and micellar-organic media also increased, although in a lesser extent, ranging between 0.08 for 10% acetonitrile/0.05 M SDS at pH 4 and 0.59 for 15% acetonitrile/0.10 M SDS at pH 7. The mean value was 0.40. No clear trend was observed in the pH values at varying SDS concentration.

In RPLC, the most extended practice consists of measuring the pH in aqueous medium before the addition of the organic solvent. This has the advantage of reducing the number of measurements, since the pH value will be the same for all mobile phases prepared with the same buffered solution. However, from these measurements, the fitted retention models will not provide correct values of acid–base constants. The determination of constants with physicochemical meaning should be made using pH values measured in the aqueous-organic medium. Recently, aqueous buffers have demonstrated to be useful in the calibration step when pH is measured in aqueous-organic solutions, although a particular correction should be made for each organic solvent [41]. All the results shown in this work were obtained with pH values measured in the aqueous-organic medium, using aqueous buffers without making any correction in the pH.

For each diuretic, the mean relative fitting error for the prediction of retention was obtained according to:

$$\mathrm{RE} = \frac{\sum_{i=1}^{n} |k_{i,\mathrm{exp}} - k_{i,\mathrm{calc}}|}{\sum_{i=1}^{n} k_{i,\mathrm{exp}}} \quad (13)$$

where $k_{i,\mathrm{exp}}$ and $k_{i,\mathrm{calc}}$ are the experimental and calculated retention factors for the n mobile phases included in the experimental design. This calculation reduces the impact of differences in magnitude among k values for mobile phases of extreme elution strength.

Table 2 lists the correlation coefficients of the fittings for the assayed models, together with the mean relative errors. The errors were below 4% for most compounds and retention models. Chlorothiazide in the micellar mode gave rise however to errors abnormally large with Eq. (11) (11%). Note that this diuretic is the least retained in micellar media, with

Table 2
Prediction errors with different retention models

Compound	Aqueous-organic RPLC				Micellar-organic RPLC			
	Eq. (4)		Eq. (8)		Eq. (9)		Eqs. (10)–(12)	
	r	RE (%)	r	RE (%)	r	RE (%)	r	RE (%)
Amiloride[a]	0.9991	0.7	0.9820	2.9	0.9994	1.7	0.9999	0.9
Triamterene[b]	0.9984	1.4	0.9954	2.8	0.9984	2.3	0.9980	3.0
Spironolactone[c]	0.9998	1.2	0.9992	2.9	0.9994	1.2	0.9997	0.9
Chlorthalidone[c]	0.9999	0.4	0.9979	2.5	0.9915	4.3	0.9998	0.8
Bendroflumethiazide[c]	0.9955	3.3	0.9965	4.0	0.9987	2.3	0.9994	1.2
Acetazolamide[a]	0.9991	0.8	0.9988	0.9	0.9970	1.5	0.9937	2.4
Trichloromethiazide[a]	0.9998	0.8	0.9997	1.1	0.9984	2.0	0.9933	3.8
Althiazide[c]	0.9957	3.1	0.9970	3.3	0.9983	2.2	0.9997	0.9
Hydrochlorothiazide[c]	0.9955	1.6	0.9985	1.1	0.9757	4.0	0.9766	3.5
Chlorothiazide[a]	0.9987	1.0	0.9960	2.2	0.9986	1.6	0.9283	11.0
Benzthiazide[b]	0.9996	1.9	0.9994	2.4	0.9979	2.4	0.9982	2.5
Xipamide[b]	0.9998	1.9	0.9996	3.1	0.9980	4.2	0.9987	3.2
Canrenoic acid 1[b]	0.9993	2.5	0.9987	4.6	0.9976	4.1	0.9998	1.2
Canrenoic acid 2[c]	–	–	–	–	0.9997	0.9	0.9999	0.6
Piretanide[b]	0.9999	1.6	0.9996	3.1	0.9976	5.2	0.9995	2.2
Furosemide[b]	0.9999	0.9	0.9990	3.7	0.9972	6.0	0.9996	1.9
Bumetanide[b]	0.9999	1.1	0.9998	2.3	0.9992	2.9	0.9996	2.0
Ethacrynic acid[b]	1.0000	0.9	0.9996	3.3	0.9980	5.5	0.9994	2.9
Probenecid[b]	1.0000	0.9	0.9998	2.4	0.9974	5.2	0.9995	2.4

[a] Eq. (11).
[b] Eq. (12).
[c] Eq. (10).

retention times close to the void volume. The larger error for this compound is thus quite logical. In the aqueous-organic mode, the mean relative error considering the 18 diuretics was smaller for Eq. (4) (1.4%) than for Eq. (8) (2.7%). However, the latter equation includes a smaller number of parameters (i.e. it requires fewer experiments) and yields results accurate enough. Consequently, it may be preferable.

Eq. (9) has been used in previous work for the micellar mode. Nevertheless, it has the drawback of requiring estimations of the retention factors for both the acidic and basic species, which are often not available simultaneously within the measured pH range (see Fig. 1). Knowledge of these values requires, therefore, extrapolations, which are translated in predictions that lack frequently the desired accuracy. We observed, in effect, that for some compounds exhibiting incomplete displacement of the acid–base equilibrium (e.g. furosemide and probenecid), fitting of Eq. (9) yielded out-of-range parameters, due to the insufficient information available. Predictions were consequently sometimes inappropriate. The mean relative error for the 18 diuretics was larger for this model (3.2%) than for the polynomial ones (Eqs. (10)–(12)) (2.0%). The latter models are linear and thus easier to fit. Finally, convergence of the fittings in both systems was always rapid, in the range of seconds to a few minutes.

Canrenoic acid showed a double peak in the micellar mode except for the weaker eluents. In addition, the peak at lower retention time could not be detected for the strongest eluents. The retention of this species was modelled considering only mobile phases at which its peak could be adequately measured. These are the results shown in Table 2.

4.4. Screening of diuretics

The performance of both chromatographic systems on the separation of complex mixtures of diuretics (15–18 compounds) was examined using the retention models described above. The resolution was evaluated as the product of peak purities, R, where $R = 1$ for peak arrangements where baseline resolution is achieved for all compounds, and $R = 0$ when at least two peaks are entirely overlapped [15]. R decreases strongly with the number of solutes in situations of partial overlapping. Peak shape, which is needed for the calculation of peak purities was predicted according to a polynomially-modified Gaussian [14]. The optimal mobile phases that resolved the mixtures of diuretics were achieved through the simulation of chromatograms for a regular distribution of the experimental factors: 101 levels by factor in the aqueous-organic mode and 31 levels by factor in the micellar-organic mode.

Due to the complexity of the separation, the regions of acceptable resolution were in all cases critical. This fact stands out clearly in the small size of the regions of high resolution in the contour maps shown in Fig. 3, which describe the separation of two mixtures of 15 diuretics (mixtures A and B, see figure caption for compositions). The

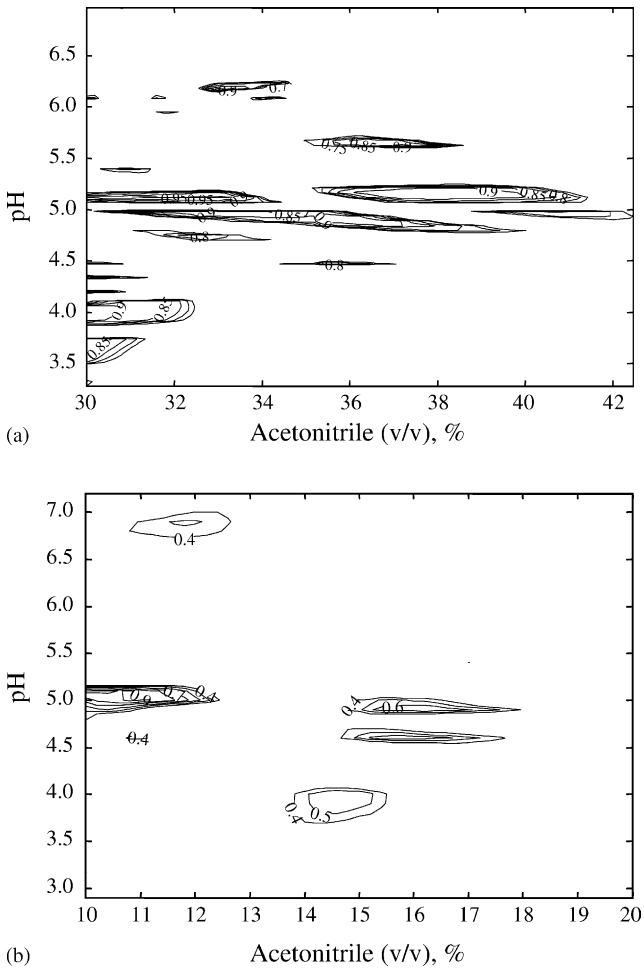


Fig. 3. Contour maps for: (a) aqueous-organic and (b) micellar-organic separations of two mixtures (A and B, respectively) of 15 diuretics. For micellar-organic RPLC, the 0.075 M SDS level, which is the optimal, is shown. Only contour levels larger than 0.7 were drawn. Mixture A: althiazide, amiloride, bendroflumethiazide, bumetanide, canrenoic acid, chlorothiazide, chlorthalidone, ethacrynic acid, furosemide, hydrochlorothiazide, probenecid, spironolactone, triamterene, trichloromethiazide and xipamide. Mixture B has similar composition, but it contains acetazolamide, benzthiazide and piretanide instead of canrenoic acid, ethacrynic acid and hydrochlorothiazide.

map corresponding to the aqueous-organic RPLC separation (Fig. 3a) shows several regions where the resolution is $R > 0.9$. However, although these regions are wide enough in the direction of acetonitrile—which means that this factor is not critical—they are extremely narrow (i.e. unrugged) in the direction of pH. Therefore, achievement of the predicted optima would require the pH to be fixed with an uncertainty smaller than 0.1 units. The situation is harder in the micellar mode (Fig. 3b), since there is only one region of acceptable resolution, and both acetonitrile and pH are critical. In this case, only the optimal surfactant level (0.075 M) is represented to draw a contour map comparable to Fig. 3a. This SDS concentration coincides with the optimal separation of mixture B. The resolution level at other SDS concentrations was lower. The strong diminution of the resolution far from

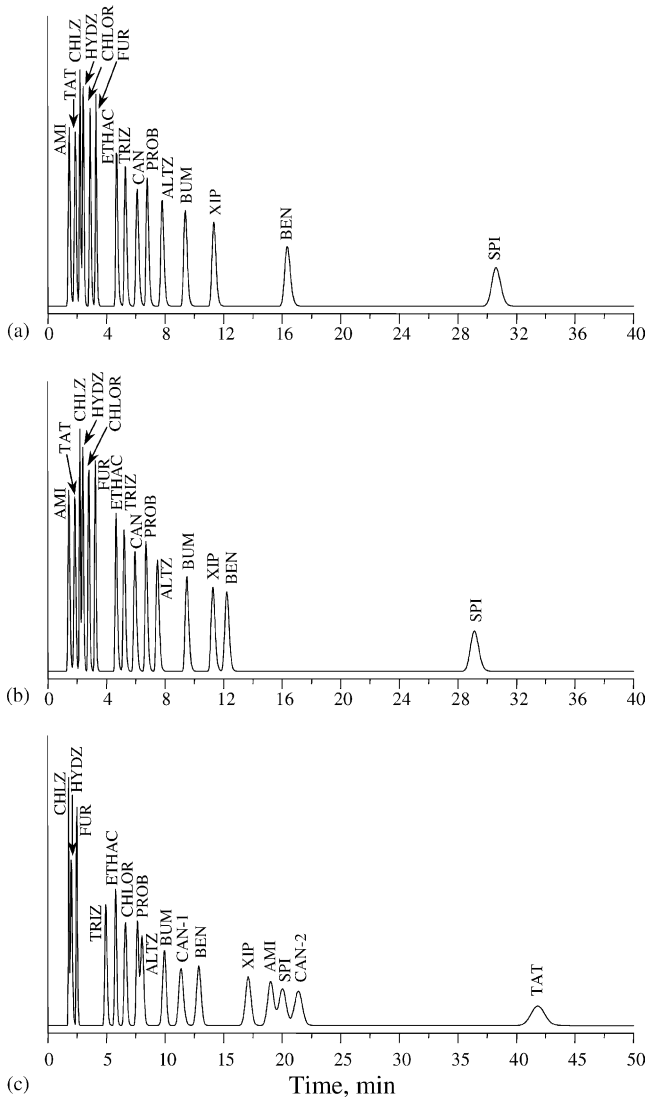


Fig. 4. (a) and (b) Predicted chromatograms for mixture A at 37.5% acetonitrile and pH 5.19 (optimal composition according to Eq. (8)). Peak positions were obtained with: (a) Eq. (8), and (b) Eq. (4). (c) Predicted optimal chromatogram according to Eqs. (10)–(12) for the same mixture in the micellar-organic mode (11.2% acetonitrile/0.07 M SDS at pH 5.45). See Table 1 for peak identity.

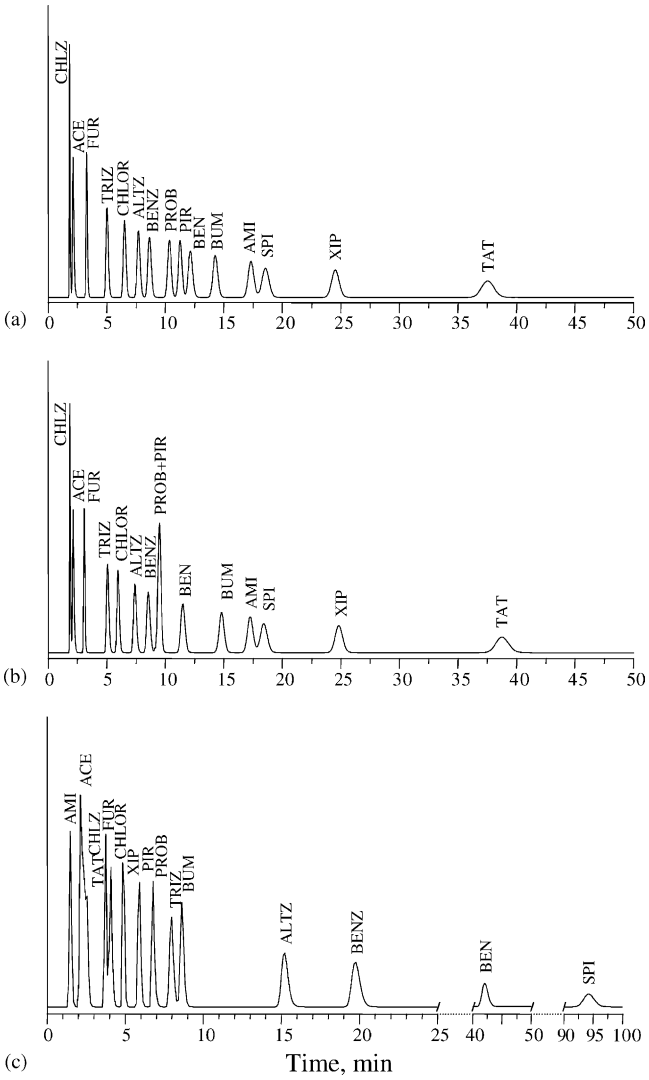


Fig. 5. (a) and (b) Predicted chromatograms for mixture B at 11% acetonitrile/0.075 M SDS and pH 5.00 (optimal composition according to Eqs. (10)–(12)). Peak positions were obtained with: (a) Eqs. (10)–(12), and (b) Eq. (9). (c) Predicted optimal chromatogram according to Eq. (8) for the same mixture in the aqueous-organic mode (30% acetonitrile at pH 6.74). See Table 1 for peak identity.

the optima, especially in the direction of pH, is due to multiple peak reversals.

Figs. 4 and 5 depict several predicted chromatograms for the mixtures of 15 diuretics. Fig. 4a and b show the separation of mixture A with acetonitrile–water. The former corresponds to the optimal mobile phase according to Eq. (8), and the latter, to the expected peak arrangement at that composition calculating in this case peak positions according to Eq. (4). The distribution of peaks is similar in both cases (except for bendroflumethiazide), and the analysis times, quite similar (30–32 min, although most diuretics were eluted below 17 min). Note that the selected mobile phase does not belong to the region of maximal resolution in the contour map (Fig. 3a), but to a secondary maximum, less critical than the main one in the direction of pH. Ruggedness in mobile phase preparation was thus greater.

Fig. 5a and b correspond to the separation of mixture B with SDS–acetonitrile–water. In this case, the optimal mobile phase was obtained according to Eqs. (10)–(12) (see Table 2), and the chromatograms at that composition were simulated with these equations (Fig. 5a) or with Eq. (9) (Fig. 5b). The peaks in the chromatograms predicted with the polynomial models appeared better resolved.

The analysis times in both chromatographic modes were similar, although in micellar RPLC the peaks appeared more regularly distributed and yielded different selectivity. The extreme behaviour of the basic diuretics

Table 3
Elementary resolutions (r) at the optimal mobile phase, limiting resolutions (r_{lim}) and associated mobile phases for mixtures of 18 diuretics

Compound	Aqueous-organic RPLC			Micellar-organic RPLC		
	r[a]	r_{lim}	Acetonitrile (%)/pH[b]	r[c]	r_{lim}	SDS (M)/acetonitrile (%)/pH[b]
Amiloride	1.000	1.000	30/6.0	0.871	1.000	0.05/20/4.8
Triamterene	0.975	0.998	32.4/7.1	1.000	1.000	0.05/10/3.5
Spironolactone	1.000	1.000	30/3.3	0.935	1.000	0.05/14.3/5.6
Chlorthalidone	0.972	1.000	30/4.9	1.000	1.000	0.05/10/5.9
Bendroflumethiazide	1.000	1.000	30/4.8	1.000	1.000	0.05/10/6.2
Acetazolamide	0.971	0.996	30/6.4	0.906	0.968	0.05/17/6.9
Trichloromethiazide	0.999	1.000	30/3.2	0.985	1.000	0.05/10/3.0
Althiazide	1.000	1.000	30/3.9	0.989	1.000	0.05/13.3/4.4
Hydrochlorothiazide	0.999	0.999	32.2/6.5	0.914	0.981	0.05/20/6.5
Chlorothiazide	0.947	0.998	30/4.9	0.805	0.995	0.07/15/5.6
Benzothiazide	1.000	1.000	30/3.2	0.989	1.000	0.05/15/3.9
Xipamide	0.999	1.000	30/4.6	0.985	1.000	0.05/10/3.8
Canrenoic acid 1	0.977	1.000	30/6.3	0.999	1.000	0.05/10/6.5
Canrenoic acid 2	–	–	–	0.934	1.000	0.05/0.2/5.6
Piretanide	1.000	1.000	30/5.7	0.981	1.000	0.05/20/3.9
Furosemide	0.994	1.000	30/3.2	0.843	1.000	0.08/10/4.7
Bumetanide	0.862	1.000	30/4.1	0.913	1.000	0.05/15/4.7
Ethacrynic acid	0.860	1.000	30/4.3	0.856	1.000	0.05/10/3.2
Probenecid	0.999	1.000	30/3.2	0.942	1.000	0.08/10/2.9
R	0.628	0.992		0.293	0.945	

[a] 30% acetonitrile/pH 6.67.
[b] Mobile phase compositions to reach the limiting resolutions.
[c] 0.05 M SDS/12% acetonitrile/pH 6.80.

amiloride and triamterene deserves a specific mention. In the aqueous-organic mobile phases these compounds eluted at short retention times, close to the head of the chromatograms, whereas in the micellar mode they were among the most retained diuretics. The strong electrostatic interaction of the protonated solutes with the negatively charged surfactant-modified stationary phase, in the working pH range, is the reason of such a strong retention. For the remaining diuretics, the discrepant behaviour between both chromatographic modes is less remarkable, but some minor changes in elution order are indeed observed. This is the case of bendroflumethiazide, that eluted at the end of the chromatogram with acetonitrile–water before spironolactone, whereas it showed an intermediate retention in the micellar mode.

The differences in selectivity between both chromatographic modes can also be checked in Figs. 4 and 5. Fig. 4c shows the optimal separation when mixture A is eluted with micellar mobile phases, whereas Fig. 5c corresponds to the optimal separation of mixture B eluted with acetonitrile–water. The resolution in these chromatograms is poorer than those at the top of the figures.

Table 3 lists the resolutions obtained for each compound (r) at the optimal mobile phases for a mixture containing the 18 diuretics (30% acetonitrile at pH 6.67 and 12% acetonitrile/0.05 M SDS at pH 6.80). Maximal global resolutions in aqueous- and micellar-organic RPLC were 0.628 and 0.293, respectively. In the former case, high individual resolutions were obtained, except for bumetanide and ethacrynic acid, that overlapped partially. Micellar-organic RPLC, with a poorer global resolution, gave $r > 0.9$ for all compounds, except for amiloride, chlorothiazide, furosemide and ethacrynic acid.

Limiting resolution (r_{lim}) for each compound and RPLC mode is also given. This parameter can be considered formally as an extension of the peak purity concept, and quantifies the maximal expectancies of resolution for each specific diuretic when all the others are considered interferents. Limiting resolutions denote thus what the maximal attainable peak purity for each compound could be, and point out the particular composition that would allow reaching it. Furthermore, similarly to the global resolution, the combined limiting peak purities indicate the maximal value that can be achieved using complementary resolution approaches [42].

Limiting resolutions in Table 3 suggest that there is at least one mobile phase composition that allows complete resolution for each diuretic in each chromatographic mode. In aqueous-organic RPLC, these compositions were always found in a narrow window (30–32% acetonitrile), where retention times are larger. Accordingly, the optimal resolution for the full mixture was found inside this range (30%). In contrast, pH values required for complete resolution of each compound were diverse, which was translated in a difficult separation. In micellar-organic RPLC, the concentration of SDS needed to reach the limiting values was 0.05 M, except for chlorothiazide, furosemide and probenecid, that required a larger concentration (0.07–0.08 M). In this case, acetonitrile and pH varied in the 10–20% and 3.0–6.9 ranges, respectively, for the limiting conditions.

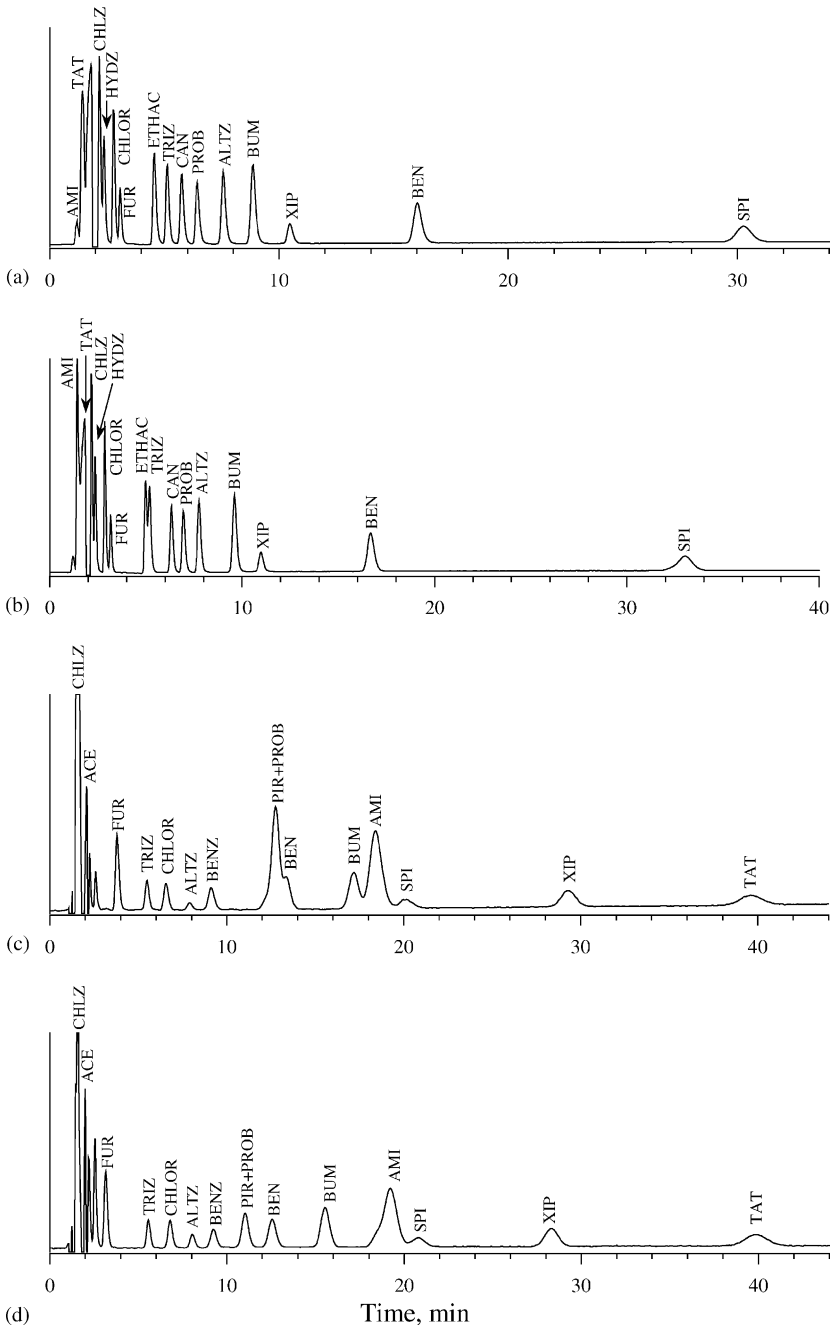


Fig. 6. Experimental chromatograms obtained for mixture A using aqueous-organic mobile phases containing 37.5% acetonitrile at pH: (a) 5.19 and (b) 5.14. Experimental chromatograms for mixture B using micellar-organic mobile phases containing 11% acetonitrile/0.075 M SDS at pH: (c) 5.07, and (d) 5.27. See Table 1 for peak identity.

The reliability of the predictions was checked by obtaining experimental chromatograms at the predicted optimal conditions. The chromatogram of mixture A with 37.5% acetonitrile at pH 5.19 in Fig. 6a shows satisfactory agreement with the prediction for Eq. (8) (Fig. 4a). This evidences the accuracy of the predicting system. Although the polynomial model (Eq. (4)) predicts similar resolution, the agreement in peak position was poorer. The separation of the diuretics in the aqueous-organic mode was otherwise complete, except for the compounds eluting at the head of the chromatogram, for which partial overlapping (amiloride/triamterene and chlorothiazide/hydrochlorothiazide) exists. Fig. 6b shows another chromatogram for mixture A using the same mobile phase composition as above (37.5% acetonitrile), but at a slightly lower pH (5.14). Peak overlap for ethacrynic acid and trichloromethiazide is indicative of the extremely critical resolution in the direction of pH, but also evidences the reliability of the proposed optimisation methodology in a very critical case.

Experimental chromatograms for mixture B eluted with micellar-organic mobile phases at two pH values, containing the same amount of acetonitrile and SDS (11%/0.075 M), are shown in Fig. 6c and d. The agreement between the experimental and predicted chromatograms was poorer (compare Fig. 5a and b with Fig. 6c). This disagreement could be likely due to small uncertainties in the retention model and/or an unaccurate standardisation of the pH-meter. It should be indicated that the chromatograms shown in Fig. 6c and d were run several months after the development of the experimental design used to model the retention. Also, the pH-meter had to be replaced. Whatever the reason, pH was seemingly responsible of the disagreement.

This hypothesis was checked by observing the changes in peak position with pH around the optimal value, through new computer simulations. We found that a simulation obtained at pH 4.82 looked similar to the experimental chromatogram at pH 5.07. Therefore, we decided to add some drops of NaOH to the mobile phase, up to increase its pH to 5.27. The corresponding experimental chromatogram is shown in Fig. 6d. Agreement with the predicted chromatogram at 11% acetonitrile/0.075 M/pH 5.00 according to both polynomial and mechanistic models (Fig. 5a and b) was satisfactory, although for the latter model the overlapping between piretanide and probenecid was described better. However, this nicer agreement should be taken with care, since the incipient protonation for some solutes makes the mechanistic model rather risky.

5. Conclusions

RPLC separation of complex mixtures including compounds with diverse acid–base behaviour, whose dissociation in some instances is poorly sampled by the narrow pH range accessible to conventional octadecylsilane columns, is a big challenge. Achievement of good resolution is really critical and, frequently only possible at very specific pH values. The main problem arises from acidic solutes experiencing strong drops in retention with pH, which produce multiple peak reversals with weakly acidic, neutral and basic solutes whose retentions remain unchanged or vary scarcely with pH. Consequently, a highly accurate description of the elution behaviour is needed, in order to predict the peak positions and eventual overlaps. The most critical factor is therefore pH, which should be buffered with high accuracy. The importance and difficulties in describing retention changes with pH become even more critical when instead of optimising two factors in the mobile phase (e.g. acetonitrile and pH), three factors are considered simultaneously (e.g. acetonitrile, SDS and pH).

The tools used in this work allow, despite these difficulties, satisfactory predictions. Several retention models were used with different success. It is shown that small disagreements between experimental and predicted chromatograms can be corrected by performing simulations at close conditions, which advices the changes in pH that should be made to reach the desired resolution.

The complexity of micellar RPLC optimisations can be reduced by considering only acetonitrile and pH, fixing the concentration of surfactant in the 0.07–0.11 M SDS range. This makes the optimisation similar to the aqueous-organic case. The addition of the anionic surfactant to a conventional acetonitrile–water mixture decreases the amount of organic solvent required to achieve an adequate separation (i.e. from 37.5 to 11.2% for mixture A and 30 to 11% for mixture B). This changes also the selectivity. Although the chromatographic peaks are somewhat wider in the micellar-organic mode, they are almost symmetrical and their distribution in the chromatogram is more even. The analysis times for mixtures of 15–18 diuretics were similar for both modes.

The micellar-organic procedure proposed for diuretics, in this work, presents the additional advantage of allowing the direct injection of physiological samples [43]. It differs from other previously reported procedures in the use of acetonitrile instead of propanol [38,44], which is the traditional organic solvent in this chromatographic mode. When compared with propanol, acetonitrile improves the efficiencies, reduces peak tailing and yields different selectivity. All these factors are translated in a higher resolution.

Neither aqueous-organic nor micellar-organic RPLC could resolve completely the mixture of 18 diuretics. However, since both modes showed different selectivities, they can be considered complementary to achieve full resolution of any mixture of diuretics.

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