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Micellar liquid chromatography: suitable technique for screening analysis ☆

M.J. Ruiz-Angel, R.D. Caballero, E.F. Simó-Alfonso, M.C. García-Alvarez-Coque*

Departamento de Química Analítica, Facultad de Química, Universitat de València, Dr. Moliner 50, 46100 Burjassot, València, Spain

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Abstract

The screening capability of micellar liquid chromatography (MLC) is discussed using the reported chromatographic data of several sets of compounds (amino acids, β-blockers, diuretics, phenethylamines, phenols, polynuclear aromatic hydrocarbons, steroids and sulfonamides) and new results (sulfonamides and steroids). The chromatographic data are treated with an interpretive optimisation resolution procedure to obtain the best separation conditions. Usually, the pH and the concentration of surfactant (sodium dodecyl sulfate, SDS, or cetyltrimethylammonium bromide) for the optimal mobile phase were 2.5–3 and <0.12 *M*, respectively. The nature and concentration of organic solvent depended on the polarity of the eluted compounds: a low volume fraction of propanol (~1%, v/v) was useful to separate the amino acids, with $\log P_{o/w}<-1$ (where $P_{o/w}$ is the octanol–water partition coefficient). A greater concentration of this solvent (~5–7%) was needed for compounds in the range $-1<\log P_{o/w}<2$, as with the studied diuretics and sulfonamides, and a high concentration of propanol (~15%) or a low concentration of butanol (<10%) had to be used for less polar compounds with $1<\log P_{o/w}<3$, such as the β-blockers. Pentanol (<6%) was more suitable for the even less polar compounds with $\log P_{o/w}>3$, such as the steroids. For basic drugs such as the phenethylamines ($0<\log P_{o/w}<1.7$), eluted with a micellar eluent of anionic SDS, propanol was too weak. A study is also shown for mixtures of sulfonamides ($\log P_{o/w}=-1.2$ to 1.7) and steroids ($\log P_{o/w}=3.0$–8.1) eluted from conventional C_{18} columns with SDS mobile phases containing acetonitrile and 1-pentanol, respectively, which are compared with classical acetonitrile–water and methanol–water mixtures. The results complement a previous study on β-blockers ($\log P_{o/w}=-0.03$ to 2.8) and reveal that MLC is a very competitive technique for the screening of compounds against conventional RPLC, due to its peculiar behaviour with regard to the selectivity and elution strength. The concentration of organic solvent needed to obtain sufficiently low retention times (even for highly hydrophobic steroids with $\log P_{o/w}=7$–8) is also appreciably smaller for MLC, which reduces the environmental impact of the mobile phases.

Keywords: Micellar liquid chromatography; Mobile phase composition; Resolution; Efficiency; Sulfonamides; Steroids; Amino acids; Phenols; Beta-blockers; Diuretics; Phenethylamines

1. Introduction

Micellar liquid chromatography (MLC) appears to be a real alternative to classical reversed-phase liquid chromatography (RPLC) with aqueous–organic mobile phases. The versatility of the technique is due to

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* Corresponding author. Tel.: +34-96-398-3003; fax: +34-96-386-4436.

E-mail address: celia.garcia@uv.es (M.C. García-Alvarez-Coque).

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the variety of interactions between solutes, stationary phase, aqueous phase and micelles [1]. The elution strength characteristics make the analysis of solutes in a wide range of polarities possible, using isocratic elution. Retention is highly reproducible and can be modelled accurately to predict retention changes as the mobile phase composition (concentration of surfactant and volume fraction of organic modifier) [2] and pH [3] are varied. This facilitates the optimisation of the separation conditions [4,5].

Pure micellar solutions are generally useless as mobile phases. An organic solvent, frequently an alcohol, should be added to decrease the analysis time to acceptable values and enhance the efficiencies. However, the concentration of organic solvent needed is appreciably smaller than in classical RPLC. Moreover, organic solvents are highly retained in the micellar medium, which decreases the risk of evaporation. Micellar phases are stable for a longer time, are inflammable and their toxicity is reduced.

The anionic surfactant sodium dodecyl sulfate (SDS) and 1-propanol are by far the most common components of micellar mobile phases. Only a few examples can be found which employ other surfactants, such as the cationic cetyltrimethylammonium bromide (CTAB) and the non-ionic Brij-35, or modifiers such as 1-butanol, 1-pentanol and acetonitrile [1]. SDS is commercially available in high purity and is less expensive, it efficiently solubilises proteins in biological matrices (urine, plasma, serum and milk), allowing direct injection of the samples into the chromatograph without any treatment other than filtration, which is not possible with cationic surfactants [6]. Conventional SDS-modified C_{18} columns can accommodate hundreds of injections of biological matrices without any increase in back-pressure or decrease in column performance. Therefore, the cost is reduced with respect to classical RPLC. Brij-35 has been used in clinical analysis [7], but it has the disadvantage of being strongly adsorbed on alkyl-bonded stationary phases.

According to the literature, MLC is a suitable technique for the screening of compounds. In this work, this possibility is discussed critically, and the mobile phase characteristics needed to reach the best separations are outlined. The experimental work reported in the MLC literature for amino acids, β-blockers, diuretics, polycyclic aromatic hydrocarbons (PAHs), phenethylamines, phenols and sulfonamides, is used, along with new results for sulfonamides and steroids, separated with SDS mobile phases containing acetonitrile and 1-pentanol, respectively, which are compared with classical acetonitrile–water and methanol–water mixtures. The efficiencies, optimal mobile phase compositions, chromatograms and resolutions are given.

2. Experimental

2.1. Test compounds

The chromatographic data for the following two groups of compounds were obtained in several micellar–organic and aqueous–organic mobile phases:

(a) 13 sulfonamides: sulfacetamide, sulfachloropyridazine, sulfadiazine, sulfadimethoxine, sulfaguanidine, sulfamerazine, sulfamethazine sulfamethizole, sulfamethoxazole, sulfamonomethoxine, sulfanilamide, sulfaquinoxaline and sulfisoxazole, from Sigma (St Louis, MO, USA), except sulfamethazine which was from Aldrich (Milwaukee, WI, USA).

(b) 12 steroids: clostebol acetate, dehydrotestosterone, metenolone enanthate and methyltestosterone (Sigma), dydrogesterone (Kalifarma, Barcelona, Spain), medroxyprogesterone acetate (Cusí, Barcelona), nandrolone (Fher, Barcelona), nandrolone decanoate (Organón, Barcelona), progesterone (Seid, Barcelona), testosterone, testosterone enanthate and testosterone propionate (Schering, Madrid, Spain).

The stock standard solutions of sulfonamides and steroids contained 100 μg/ml of the drugs and were prepared by dissolving the solids in ethanol and diluting with 0.10 *M* SDS (99% purity, Merck, Darmstadt, Germany) for the micellar mode, or with water for the aqueous–organic mode, to obtain the working solutions. The standard solutions remained stable during at least 2 months at 4 °C.

2.2. Other reagents and columns

The micellar mobile phases used in the separation of sulfonamides and steroids were prepared with SDS and acetonitrile or 1-pentanol (HPLC grade, Scharlab, Barcelona), respectively. Aqueous–organic mobile phases containing acetonitrile or methanol (Scharlab) were also prepared for both groups of compounds. The mobile phases were buffered in all cases at pH 3 with 0.01 *M* citric acid (Sigma, Barcelona, Spain) and NaOH (Panreac, Barcelona, Spain). Nanopure water (Barnstead, Sybron, Boston, MA, USA) was used throughout. Drug solutions and mobile phases were filtered through 0.45-μm membranes of 47 mm diameter (Micron Separations, Westboro, MA, USA).

A Hypersil ODS column (5 μm particle size, 100×4.6 mm I.D., Agilent, Waldbronn, Germany) was used for the sulfonamides and a Spherisorb ODS-2 column (5 μm particle size, 125×4.6 mm I.D., Scharlab) for the steroids. Both columns were placed after a Nucleosil ODS guard column (30×4.0 mm I.D., Scharlab) to saturate the mobile phase with silica.

2.3. Apparatus and software

The liquid chromatograph (Agilent, Model HP 1050, Palo Alto, CA, USA) was equipped with an isocratic pump, an autosampler (Model HP 1100), and a UV–visible detector set at 275 nm for the sulfonamides, and 246 nm for the steroids. The signal was acquired by a computer connected to the chromatograph through an integrator (Model HP 3396A), using the PEAK-96 software (Agilent, Avondale, PA, USA). The flow-rate was 1.0 ml/min. Separations were carried out at room temperature, using duplicate injections.

The chromatographic data (retention factors, efficiencies and asymmetries) were measured with MICHROM [8]. The chromatograms and resolution diagrams were simulated with CHROM [9], which includes tools for experimental design, retention modelling, mobile phase optimisation and chromatogram prediction for both classical RPLC and MLC. Resolution was optimised by measuring the free area fractions, which associate a resolution value to each compound in a mixture [4].

3. Results and discussion

3.1. Screening capability

3.1.1. Chromatographic characteristics of the literature selected examples

The compounds studied were the following (C_{18} column length and detection wavelength in parentheses):

(a) 14 amino acids (120×4.6 mm I.D., post-column derivatisation with *o*-phthalaldehyde and *N*-acetyl-L-cysteine, 335 nm) [10]. The optimal mobile phase contains 0.055 *M* SDS–0.8% (v/v) 1-propanol at pH 3. The amino acids appear distributed in three groups in the chromatograms (e.g. Fig. 1a). The analysis time is 40 min and the global resolution low, $R=0.16$ ($R=1$ for baseline resolution [4]), due to the partial overlap of several amino acids. However, screening is feasible.

(b) 15 phenols (125×4 mm I.D., 254 nm) [11]. They were optimally resolved with 0.10 *M* CTAB–10% 2-propanol at pH 2.5 in 20 min and with a global resolution of $R=0.70$ (Fig. 1b). The separation, although not reaching the baseline, was satisfactory for most phenols.

(c) 14 β-blockers (125×4.6 mm I.D., 225 nm) [12]. They were optimally resolved in 30 min using 0.10 *M* SDS–15% 1-propanol at pH 3, with $R=0.93$ (Fig. 2a). The separation is very satisfactory, with only a small overlap between nadolol and pindolol (peaks 4 and 5).

(d) 12 diuretics (125×4.6 mm I.D., 274 nm) [13]. The optimal mobile phase was 0.05 *M* SDS–6% 1-propanol at pH 3, with $R=0.61$ and analysis time of 23 min (Fig. 2b). There is more extensive overlap than with the β-blockers, but the resolution is adequate for screening purposes.

(e) 10 PAHs (150×3.9 mm I.D., 254 nm) [14]. They were resolved in 25 min using 0.15 *M* SDS–15% 2-propanol at neutral pH. No data were available to calculate the resolution.

(f) 12 sulfonamides (250×4.6 mm I.D. hydrophylic

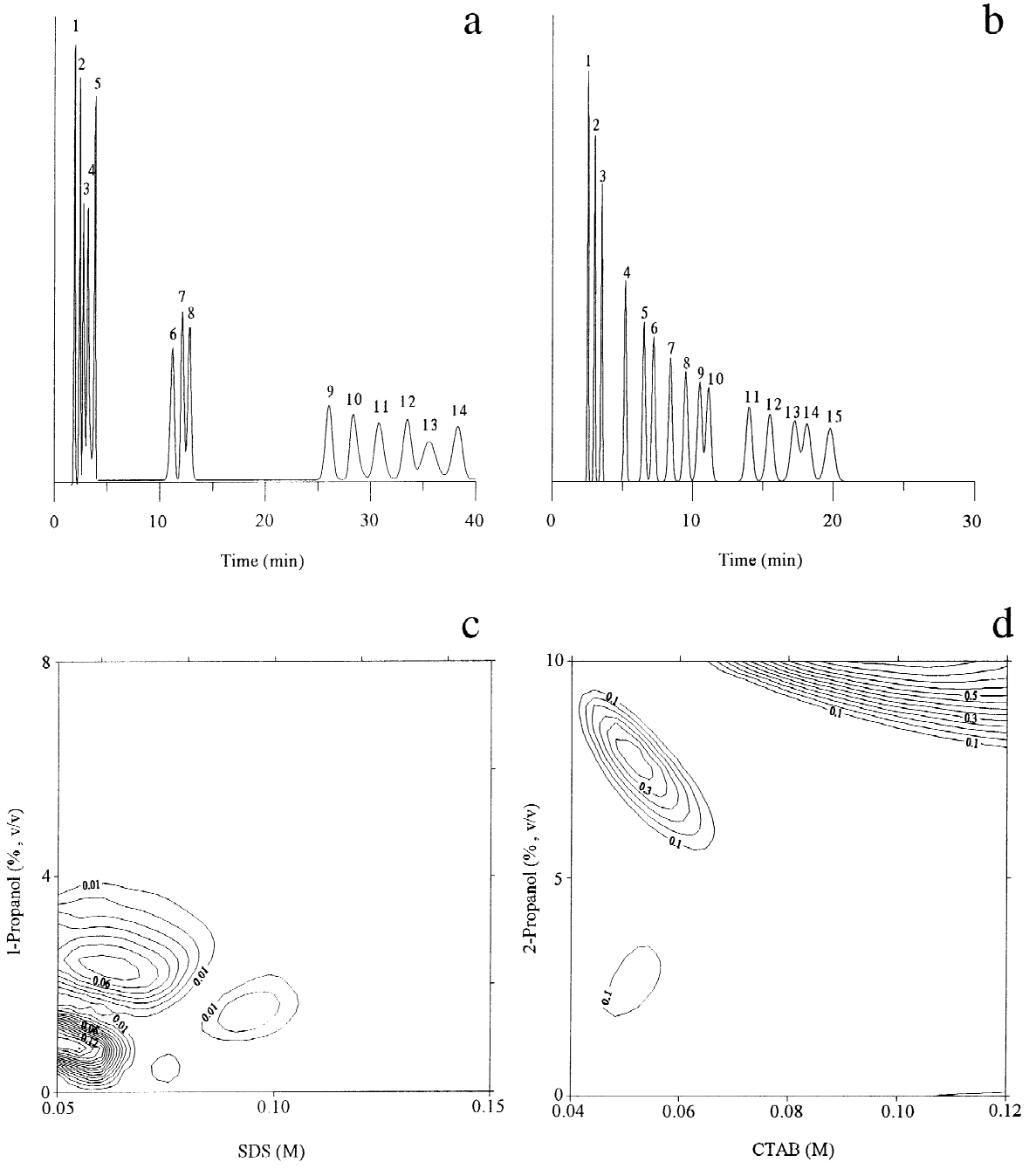

Fig. 1. Chromatograms of mixtures of: (a) 14 amino acids eluted with 0.055 *M* SDS–0.8% (v/v) 1-propanol: (1) aspartic acid, (2) threonine, (3) glutamine, (4) cysteine, (5) alanine, (6) tyrosine, (7) valine, (8) methionine, (9) phenylalanine, (10) leucine, (11) lysine, (12) histidine, (13) tryptophan, and (14) arginine; (b) 15 phenols eluted with 0.10 *M* CTAB–10% (v/v) 2-propanol: (1) 4-benzamidephenol, (2) 4-hydroxybenzyl alcohol, (3) 4-hydroxyphenemethyl alcohol, (4) 4-hydroxybenzylcyanide, (5) 4-hydroxyacetophenone, (6) phenol, (7) 4-hydroxybenzaldehyde, (8) 4-fluorophenol, (9) 4-methylphenol, (10) 4-hydroxypropiophenone, (11) 4-nitrophenol, (12) 4-isopropylphenol, (13) 4-hydroxybenzophenone, (14) 4-hydroxydiphenylmethane and (15) 4-*tert*.-butylphenol. Resolution diagram for the mixture of: (c) amino acids, and (d) phenols.

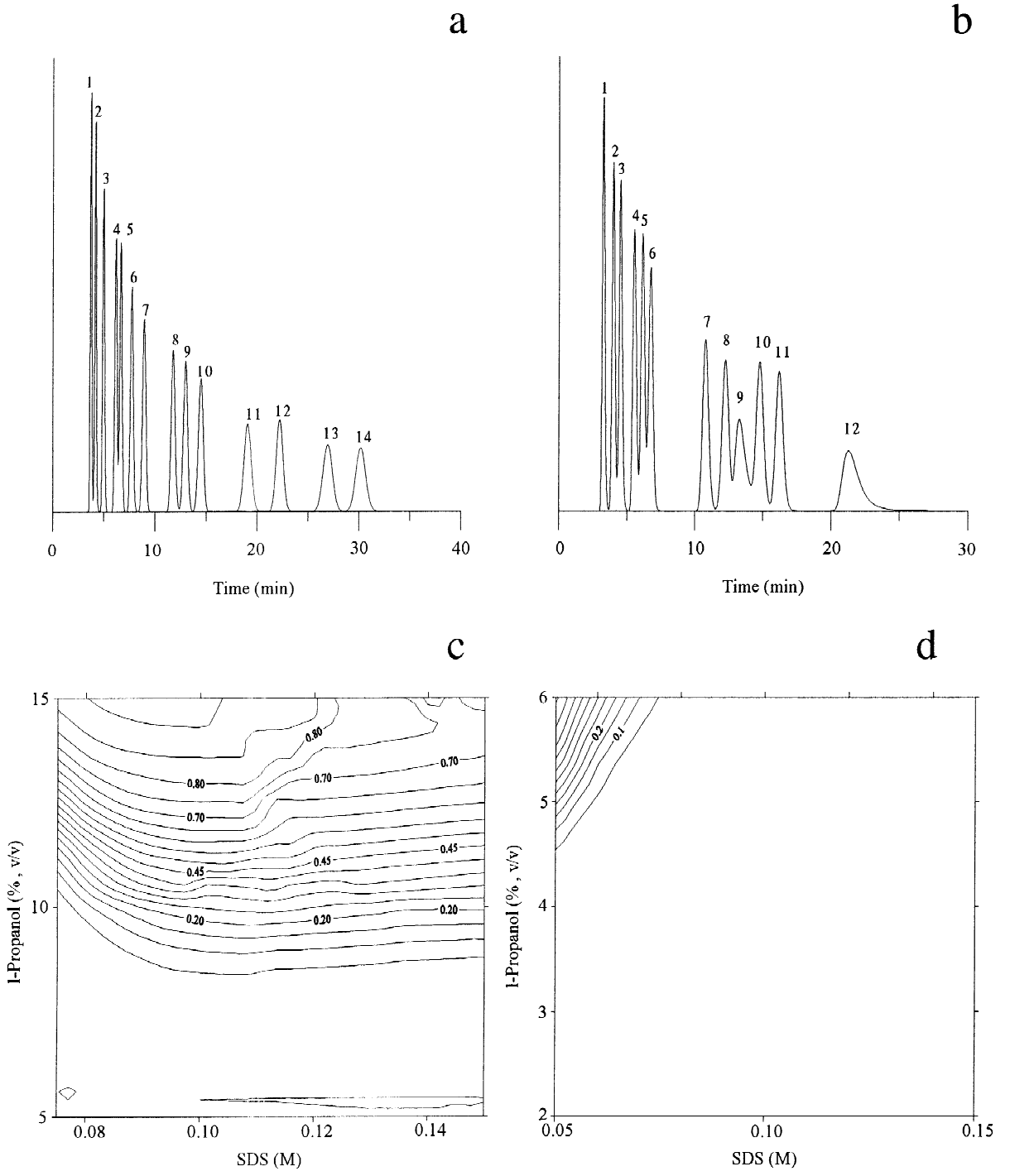

Fig. 2. Chromatograms of mixtures of: (a) 14 β-blockers eluted with 0.10 *M* SDS–15% (v/v) 1-propanol: (1) atenolol, (2) sotalol, (3) carteolol, (4) nadolol, (5) pindolol, (6) acebutolol, (7) celiprolol, (8) esmolol, (9) timolol, (10) bisoprolol, (11) labetalol, (12) oxprenolol, (13) propranolol, and (14) alprenolol; (b) 12 diuretics eluted with 0.05 *M* SDS–6% (v/v) 1-propanol: (1) tricloromethiazide, (2) chlorthalidone, (3) althiazide, (4) benzthiazide, (5) furosemide, (6) bendroflumethiazide, (7) piretanide, (8) bumetanide, (9) amiloride, (10) xipamide, (11) ethacrynic acid, and (12) triamterene. Resolution diagrams for the mixtures of: (c) β-blockers, and (d) diuretics.

endcapped column, 254 nm) [15]. The mobile phase giving optimal resolution is 0.07 *M* SDS–6% 1-propanol at pH 3 with $R=0.95$ in 15 min.

(g) Nine phenethylamines (120 mm×4.6 mm I.D., 274 nm for arterenol, methoxyphenamine and tyramine, and 256 nm for the other compounds) [16]. The optimal mobile phases were 0.065 *M* SDS–6% 1-butanol (Fig. 3a) and 0.12 *M* SDS–3% 1-pentanol (Fig. 3b), both at pH 3. The resolutions were $R=0.65$ and $R=0.44$, respectively.

3.1.2. Composition of the mobile phase

In most examples, 1-propanol or 2-propanol were added to the micellar mobile phases. However, their elution strength is rather small and non-polar solutes will elute at long retention times. More hydrophobic alcohols such as 1-butanol and 1-pentanol should be added to decrease the retention of strongly retained solutes. For solutes of intermediate polarity, acetonitrile can also provide satisfactory results, as shown below for sulfonamides.

Good correlation has been demonstrated between solute polarity (measured as log $P_{o/w}$; $P_{o/w}$ is the octanol–water partition coefficient) and retention in MLC [17]. Therefore, the selection of a particular modifier depends mostly on the polarities of the solutes. It is interesting to observe that the log $P_{o/w}$ ranges for the sets of diuretics and sulfonamides are similar: −1.2 to 1.9 [18] and −1.0 to 1.7 [19], respectively, and the same can be said for the mobile phase compositions used to reach the maximal resolution: 0.05 *M* SDS–6% 1-propanol [13] and 0.07 *M* SDS–6% 1-propanol [15], respectively.

The log $P_{o/w}$ values for the β-blockers are between −0.03 and 2.8 [20]. The ratio of the extreme $P_{o/w}$ values is similar to that for sulfonamides, but the polarity of the most retained β-blockers is significantly lower. For this reason, the separation of these compounds required a high volume fraction of 1-propanol (15%). However, the retention time for the most retained β-blocker (alprenolol) was 30 min. Therefore, 1-butanol instead of 1-propanol could be a better choice to increase further the resolution and decrease the analysis time.

The separation of mixtures of steroids and PAHs illustrates the chromatographic behaviour of non-polar compounds. The log $P_{o/w}$ values for the steroids and PAHs range between 3.0 and 8.1 [21], and between 3.3 and 7.2 [22], respectively. For steroids, adequate retention was obtained using a high concentration of 1-pentanol in the SDS mobile phase. The PAHs were separated using high concentrations of 2-propanol and SDS (0.15 *M* SDS–15% 2-propanol) in 25 min.

Finally, strong solvents, such as 1-butanol or 1-pentanol, are needed in MLC to separate polar compounds that show a high affinity for the surfactant adsorbed on the stationary phase, such as phenethylamines (Fig. 3). The log $P_{o/w}$ values for the studied phenethylamines range between −0.09 for pseudoephedrine and 1.7 for methoxyphenamine [19]. They elute quickly with conventional acetonitrile–water and methanol–water mobile phases, using a C_{18} column, but are highly retained in the SDS micellar system. The retention times of phenethylamines were still excessive when 1-propanol was added to the SDS micellar mobile phase. The optimal separation required a relatively high concentration of 1-butanol (Fig. 3a) or 1-pentanol (Fig. 3b) [16].

The pH of the mobile phase in the examined examples was 2.5–3, except for the PAHs, which were chromatographed at neutral pH. For weak acids, such as amino acids, diuretics, phenols and sulfonamides, a wider separation space is obtained in acidic conditions where the protonated species dominates. This favours the resolution. The retention of β-blockers and phenethylamines does not change in the working pH range of the C_{18} columns [16,23], but a low pH is selected to enhance the efficiencies of these basic drugs through the protonation of free silanol groups on the column.

The best separation conditions are found for surfactant concentrations ≤ 0.12 *M*, with the exception of the PAHs, which needed more surfactant to decrease their retention. In MLC, the volume fraction of the organic solvent in the mobile phase should be limited to ensure the integrity of the micelles, which is approximately 15% for 1-propanol, 10% for 1-butanol, 6% for 1-pentanol and 20% for acetonitrile [24]. In the examples, the volume fraction of 1-propanol (the most common and weaker modifier) was above 6%, except for the amino acids, which are highly polar ($\log P_{o/w} \leq -1$, with most amino acids having $\log P_{o/w} \leq -2$) [25,26].

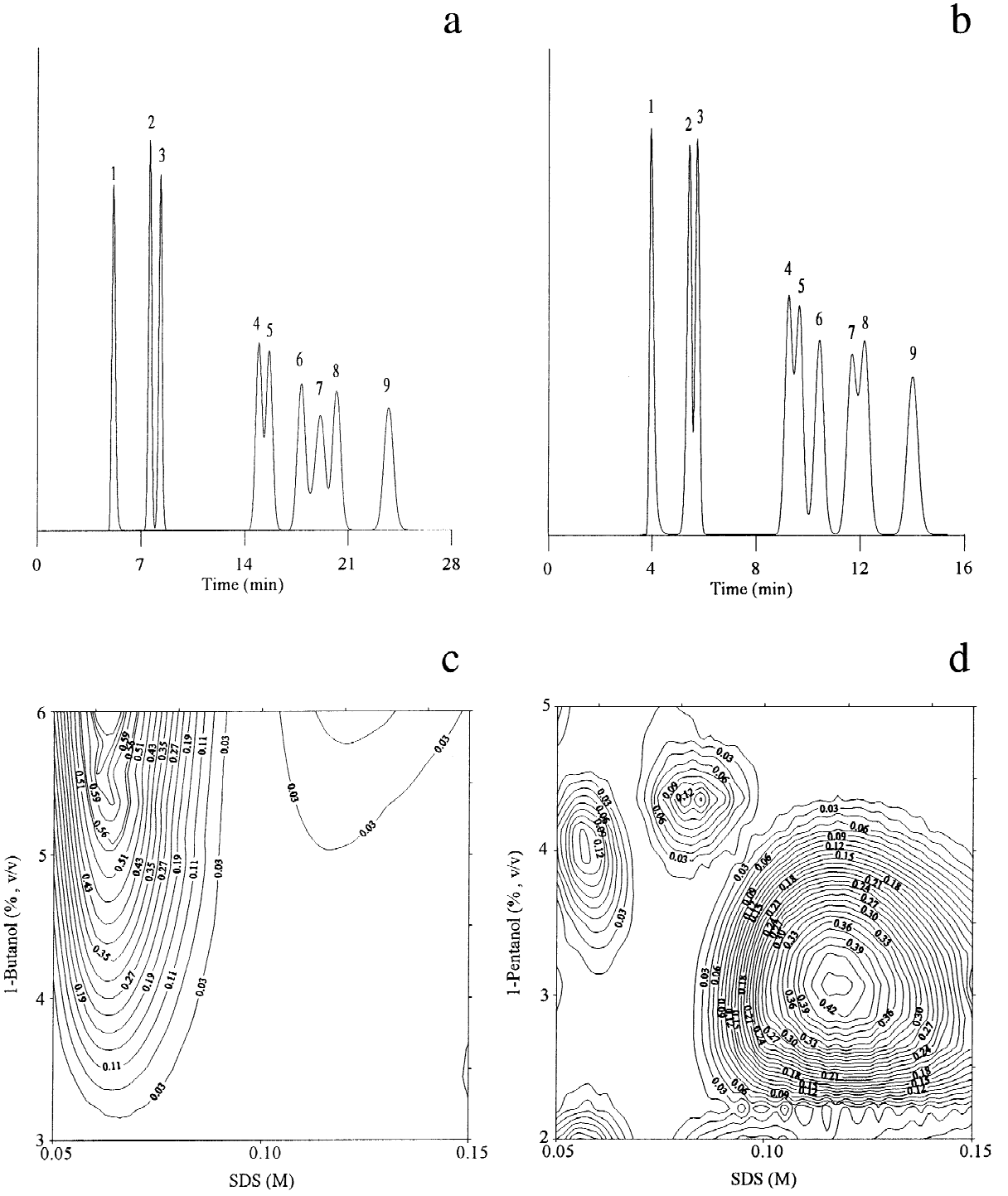

Fig. 3. Chromatograms of mixtures of nine phenethylamines eluted with: (a) 0.065 *M* SDS–6% (v/v) 1-butanol, and (b) 0.12 *M* SDS–3% (v/v) 1-pentanol. Compounds: (1) artenerol, (2) tyramine, (3) phenylephrine, (4) pseudoephedrine, (5) ephedrine, (6) methoxyphenamine, (7) mephentermine, (8) phenylpropanolamine, and (9) amphetamine. Resolution diagrams for the phenethylamines eluted with: (c) SDS–butanol, and (d) SDS–pentanol. From Ref. [16].

3.1.3. Resolution diagrams

The chromatographic behaviour of the micellar systems is best viewed through the resolution diagrams of the mixtures, in a wide region of concentrations of surfactant and organic solvent. The lowest surfactant concentrations were 0.05 *M* for SDS and 0.04 *M* for CTAB, well above the critical micellar concentrations. Surfactant concentrations above 0.20 *M* were not considered due to the solubility of the surfactant, the viscosity of the mobile phase and the degradation of the efficiency. The maximal volume fraction of organic solvent was limited by the stability of the micelles.

The resolution diagrams for the mixtures of amino acids (Fig. 1c), phenols (Fig. 1d), β-blockers (Fig. 2c), diuretics (Fig. 2d), and phenethylamines (Fig. 3c,d) were drawn with data obtained in our laboratory or reported by other authors. The shape of the diagrams is complex, with several local optima, except for the β-blockers and diuretics. The location of the optimal mobile phases given above (Section 3.1.1) can be observed on the diagrams, together with other secondary maxima. The best maximum appears inside the studied experimental domain for the amino acids and phenethylamines eluted with 1-pentanol. In other cases (β-blockers, diuretics, phenols, and phenethylamines eluted with 1-butanol), the maximal resolution is located in an extreme of the experimental domain, and probably outside.

The best resolution for the mixture of 14 amino acids corresponded to a very low concentration of 1-propanol (0.055 *M* SDS–0.8% 1-propanol). The concentration of organic solvent could not be increased to diminish the analysis time since the less retained amino acids (aspartic acid, threonine, glutamine, cysteine and alanine) were pushed to the dead time. Meanwhile, the most hydrophobic amino acids (lysine, histidine, tryptophan and arginine) eluted at times above 30 min (Fig. 1a). The separation of the amino acids in three groups suggests the possibility of reducing the analysis time without decreasing the resolution, using a gradient of organic solvent.

The resolution diagrams for the phenols (Fig. 1d) and diuretics (Fig. 2d) indicate that the selected experimental domain should be enlarged to higher volume fractions of organic solvent for better resolution, especially for the diuretics. Although the optimal composition for β-blockers is in an extreme of the factor domain, the use of an organic solvent of higher elution strength may deteriorate the resolution.

3.2. Comparison of MLC and classical RPLC

In previous work, MLC was shown as a very competitive technique for the screening of β-blockers [12]. The performance of MLC (with SDS and 1-propanol) and classical RPLC (with acetonitrile or methanol) to resolve a set of 14 β-blockers (log $P_{o/w} = -0.03$ to 2.8) was compared using a conventional C_{18} column. MLC was better in terms of efficiency and elution strength. The resolution attained for the β-blockers was remarkably improved with respect to that found with aqueous–organic mobile phases, even when special columns designed for basic compounds were used. A comparison of MLC and classical RPLC in the analysis of other groups of compounds showing a different range of polarities, 13 sulfonamides (log $P_{o/w} = -1.2$ to 1.7) and 10 steroids (log $P_{o/w} = 3.0$ to 8.1) is considered next (see test compounds in Section 2.1).

3.2.1. Screening of sulfonamides

The retention behaviour of the sulfonamides with the micellar SDS–acetonitrile mobile phases was studied using an experimental design consisting of six runs arranged as follows (concentration of SDS–volume fraction of acetonitrile): 0.02 *M*, 0.12 *M*, 0.02 *M*–3%, 0.02 *M*–6%, 0.12 *M*–6% and 0.075 *M*–3%. For classical RPLC, the experimental design consisted of five mobile phases with the following volume fraction of organic solvent (acetonitrile or methanol): 10, 15, 20, 25 and 30%. The resolution was optimised at pH 3 since the separation space at larger pH was smaller.

Fig. 4a,c shows the efficiencies and asymmetry factors for several sulfonamides eluted with mobile phases containing the same concentration of SDS (0.02 *M*) and varying volume fraction of acetonitrile. For all sulfonamides, the efficiencies improved almost linearly when acetonitrile was added. Symmetry enhancements were also achieved. In contrast, an increase in SDS diminished the efficiencies and deteriorated the symmetries (not shown). For acetoni-

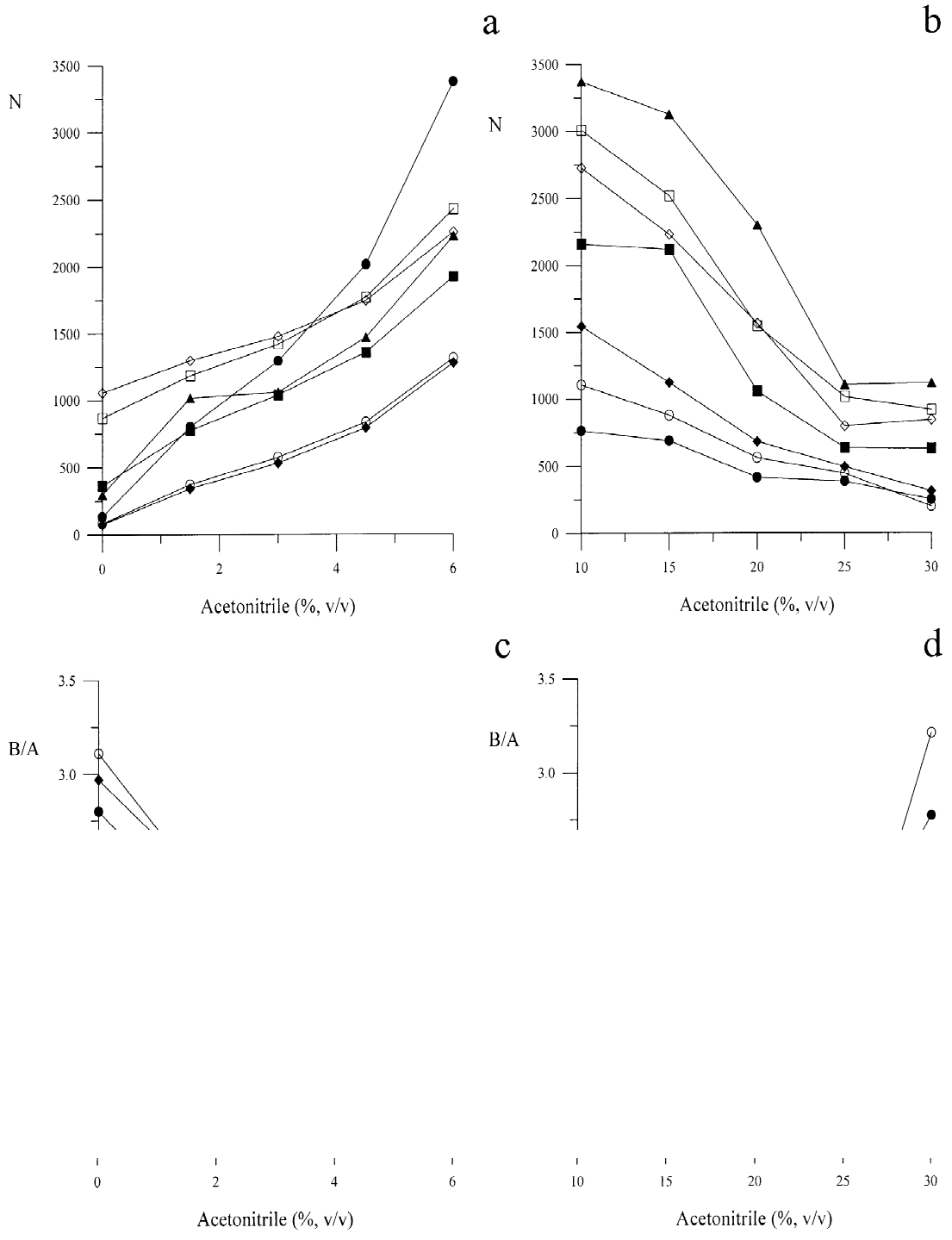

Fig. 4. Efficiencies (N) and asymmetry factors (B/A) at varying volume fractions of acetonitrile and pH 3: (a, c) micellar mobile phases containing 0.02 M SDS; and (b, d) aqueous–organic mobile phases. Compounds: sulfadiazine (●), sulfamerazine (○), sulfamethazine (⧫), sulfamethoxazole (◇), sulfamonomethoxine (■), sulfisoxazole (□), and sulfadimethoxine (▲).

trile–water, the behaviour was opposite to that obtained for MLC: the efficiencies decreased at increasing percentage of the modifier (Fig. 4b), except for sulfacetamide, sulfanilamide, sulfaguanidine and sulfaquinoxaline, for which no trend was observed. The asymmetry factors with acetonitrile–water remained approximately constant or increased with the addition of more modifier (Fig. 4d). For methanol–water, no trend was observed in the efficiencies and asymmetries at varying methanol concentrations.

The elution strength or solute sensitivity to changes in the modifier concentration of the mobile phases was also examined. This parameter was measured as the slope (c_1 coefficient) of the classical elution model [27], applied to micellar–organic and aqueous–organic mobile phases:

$$\log k = c_0 + c_1\varphi \quad (1)$$

$$\log k = c_0 + c_1 [M] \quad (2)$$

where φ is the volume fraction of organic solvent and $[M]$ the concentration of surfactant. The c_1 coefficients are listed in Table 1. Although the quality of the fittings is better for acetonitrile than for SDS, it is evident that the elution strength of the mobile phases in MLC is determined mainly by the surfactant, and increases as the polarity of the compounds decreases. On the other hand, in aqueous–organic RPLC, the elution strength of methanol is much larger than for acetonitrile, but for the assayed mobile phases the retention factors with methanol were longer than those with acetonitrile at the same volume fraction.

Chromatograms corresponding to the optimal separation are shown in Fig. 5 for the 13 sulfonamides eluted using the MLC and classical RPLC modes. In MLC, the compounds are evenly distributed according to their retention times, while in RPLC with acetonitrile–water, although the total run time is shorter than in MLC, most sulfonamides are partially or completely overlapped at the beginning of the chromatogram. This behaviour can also be observed with methanol–water, with the additional drawback that the two most retained sulfonamides appear at long times (>60 min). In MLC, the resolution value was $R=0.64$, much better than in classical RPLC ($R=0.21$ for acetonitrile and $R=0.016$ for methanol). The elution order obtained with the micellar–

Table 1
Elution strength of alcohol and surfactant in micellar and aqueous–organic mobile phases for a set of sulfonamides[a]

	Compound	MLC				Classical RPLC			
		Acetonitrile[b]		SDS[c]		Acetonitrile		Methanol	
		c_1[d]	r	c_1[e]	r	c_1[d]	r	c_1[d]	r
(1)	Sulfacetamide	−1.60	0.998	−3.88	0.982	−2.44	0.997	−6.15	0.974
(2)	Sulfanilamide	0.15	0.810	−4.68	0.987	−1.38	0.998	−3.52	0.968
(3)	Sulfadiazine	−3.43	0.996	−4.93	0.983	−2.91	0.996	−7.24	0.979
(4)	Sulfamerazine	−3.33	0.998	−6.34	0.981	−3.46	0.996	−8.53	0.982
(5)	Sulfamethazine	−2.77	0.999	−7.56	0.949	−3.98	0.993	−9.83	0.980
(6)	Sulfachloropyridazine	−3.36	0.999	−7.11	0.981	−4.33	0.996	−10.69	0.982
(7)	Sulfamethizole	−2.39	0.999	−7.36	0.980	−4.42	0.994	−11.01	0.977
(8)	Sulfamethoxazole	−3.34	0.998	−7.10	0.982	−4.54	0.996	−11.31	0.978
(9)	Sulfamonomethoxine	−3.29	0.998	−7.44	0.979	−4.78	0.995	−11.87	0.979
(10)	Sulfisoxazole	−3.43	0.998	−7.70	0.963	−4.92	0.996	−12.20	0.980
(11)	Sulfaguanidine	−3.53	0.998	−7.58	0.950	−1.77	0.996	−4.53	0.964
(12)	Sulfadimethoxine	−3.39	0.997	−8.26	0.967	−6.03	0.996	−14.90	0.980
(13)	Sulfaquinoxaline	−1.19	0.999	−8.89	0.966	−6.42	0.996	−15.87	0.980

[a] Sulfonamides are listed according to their elution order with 0.024 *M* SDS–6% (v/v) acetonitrile.
[b] At 0.02 *M* SDS.
[c] At 6% (v/v) acetonitrile.
[d] Slope of Eq. (1).
[e] Slope of Eq. (2).

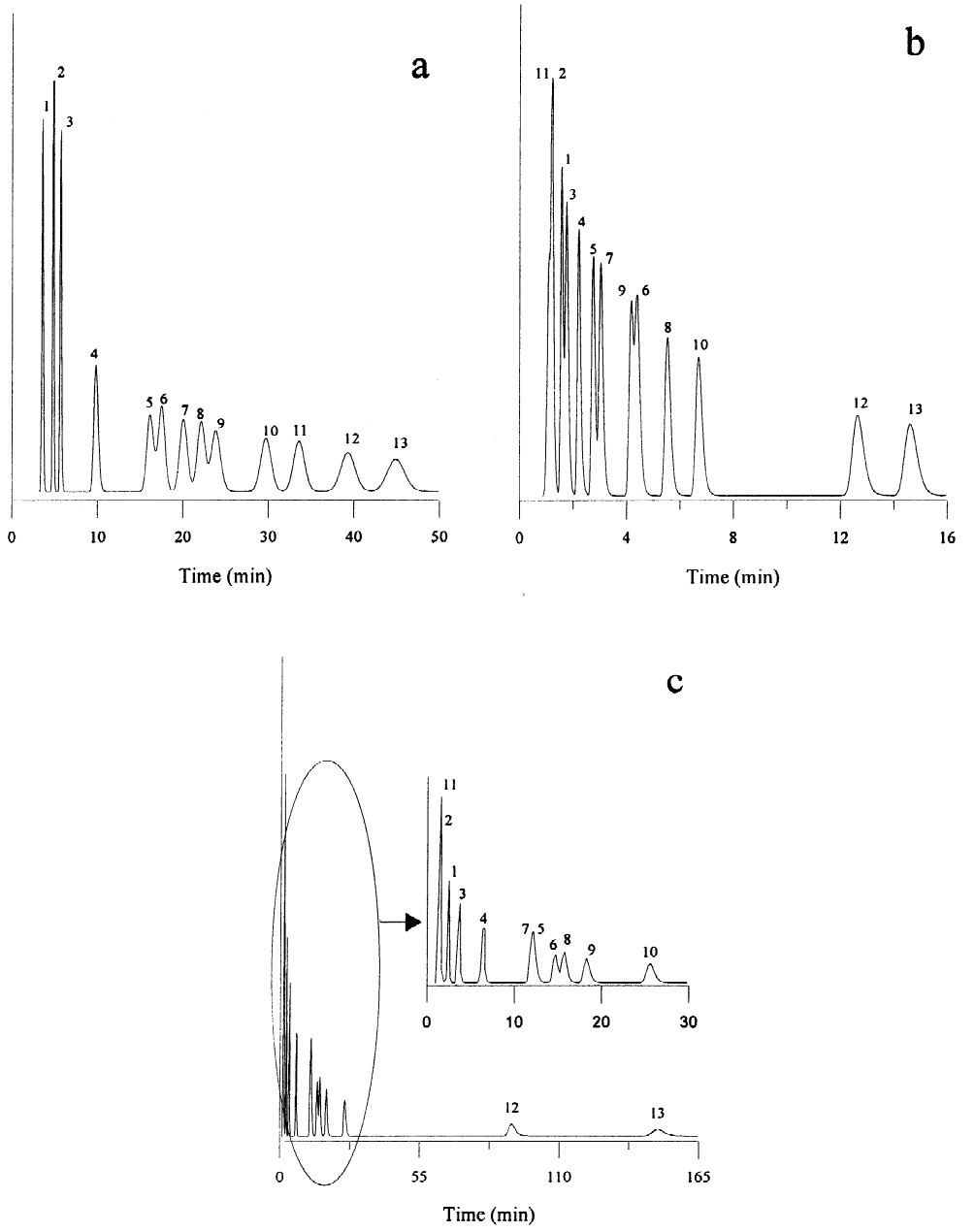

Fig. 5. Chromatograms of a mixture of 13 sulfonamides eluted with: (a) 0.025 *M* SDS–6% (v/v) acetonitrile, (b) 16% (v/v) acetonitrile, and (c) 10% (v/v) methanol. Compounds: (1) sulfacetamide, (2) sulfanilamide, (3) sulfadiazine, (4) sulfamerazine, (5) sulfamethazine, (6) sulfachloropyridazine, (7) sulfamethizole, (8) sulfamethoxazole, (9) sulfamonomethoxine, (10) sulfisoxazole, (11) sulfaguanidine, (12) sulfadimethoxine, and (13) sulfaquinoxaline.

organic mobile phases is different from that of the acetonitrile–water or methanol–water mixtures.

3.2.2. Screening of steroids

Steroids are low polar compounds, strongly associated with C_{18} columns. For this reason, a strong modifier, 1-pentanol, was selected in MLC for their separation. Only 10 steroids of the 12 initially taken were considered for the interpretive optimisation. Metenolone enanthate and nandrolone decanoate, the most hydrophobic steroids (log $P_{o/w}=7.6$ and 8.1, respectively), eluted at too long retention times. The experimental design for MLC consisted of eight mobile phases (concentration of SDS–volume fraction of 1-pentanol): 0.10 *M*–4%, 0.10 *M*–7%, 0.15 *M*–4%, 0.15 *M*–7%, 0.20 *M*–4%, 0.20 *M*–7%, 0.12 *M*–6% and 0.18 *M*–6%. For classical RPLC, the experimental design consisted of five mobile phases, with the following volume fractions of organic solvent: 30, 40, 50, 60 and 80% for acetonitrile, and 50, 55, 60, 70 and 80% for methanol.

In MLC, the efficiencies followed the expected trend: they decreased at increasing concentration of surfactant and increased with the volume fraction of 1-pentanol. In classical RPLC, no clear trend was observed for the efficiencies as in the case of the sulfonamides. However, these were higher than for MLC (compare the chromatograms shown in Fig. 6). The elution strengths for steroids and different modifiers are given in Table 2. In the MLC mode, in contrast to the sulfonamides, the elution strength of the mobile phase was determined mainly by the organic solvent, which presented higher c_1 values than SDS. For the aqueous–organic mode, methanol gave again the greatest elution strength.

For the MLC mode, eight of the 10 steroids could be resolved with almost baseline resolution (Fig. 6a). Clostebol acetate and testosterone enanthate eluted very close to testosterone propionate (peak 7) and nandrolone (peak 8), respectively, producing a strong overlap. For acetonitrile–water (Fig. 6b) and methanol–water (Fig. 6c), nine and eight steroids, respectively, were resolved (although not to the baseline) with shorter analysis times than MLC. In MLC, the efficiencies were poorer than in classical RPLC, but the better distribution of the chromatographic peaks yielded a good resolution ($R=0.93$ for MLC, $R=0.92$ for acetonitrile–water, and $R=0.79$ for methanol–water). Also, 10 steroids were eluted below 22 min using a small volume fraction of 1-pentanol (5.8%).

For acetonitrile–water, testosterone enanthate, and for methanol–water, testosterone enanthate and clostebol acetate, were excessively retained, with retention times >60 min for the optimal conditions (52% acetonitrile and 71% methanol). The retention times were appreciably decreased using stronger mobile phases: testosterone enanthate (40 and 8 min for 60 and 80% acetonitrile, respectively, and 15 min for 80% methanol), and clostebol acetate (4 min for 80% methanol).

Metenolone enanthate and nandrolone decanoate could not be modelled in any system, micellar–organic or aqueous–organic, owing to their high retention. The following retention times were however obtained for these steroids: metenolone enanthate (16 min for 0.07 *M* SDS–7% 1-pentanol, 50 and 11 min for 60 and 80% acetonitrile, respectively, and 20 min for 80% methanol), and nandrolone decanoate (15 min for 0.07 *M* SDS–7% 1-pentanol, 19 min for 80% acetonitrile, and 39 min for 80% methanol).

Therefore, the concentration of organic solvent needed to observe the peaks of highly hydrophobic steroids at sufficiently low times is notably smaller for the micellar system. Although the elution strength could not be measured for some steroids, it is evident from the data in Table 2 that it is much higher for 1-pentanol in the micellar mobile phases ($c_1=6.96$–14.36) than for acetonitrile or methanol in the classical RPLC mode ($c_1=2.39$–3.44 for acetonitrile and 3.04–5.12 for methanol).

Another peculiarity observed in the chromatograms is that while the least retained compound elutes at 1.5–2 min with the aqueous–organic mobile phases (dehydrotestosterone with acetonitrile and nandrolone with methanol), the first compound (dehydrotestosterone) elutes at 5 min in the micellar mode. This result is interesting with regard to the analysis of the steroids in biological fluids, since even after a sample clean-up, a protein band is obtained in the first minutes of the chromatograms. Finally, it should be noted that the elution order changes for the different mobile phases. The most significant change is observed for nandrolone, which appears at very short times in aqueous–organic RPLC, while in MLC it elutes at 18 min.

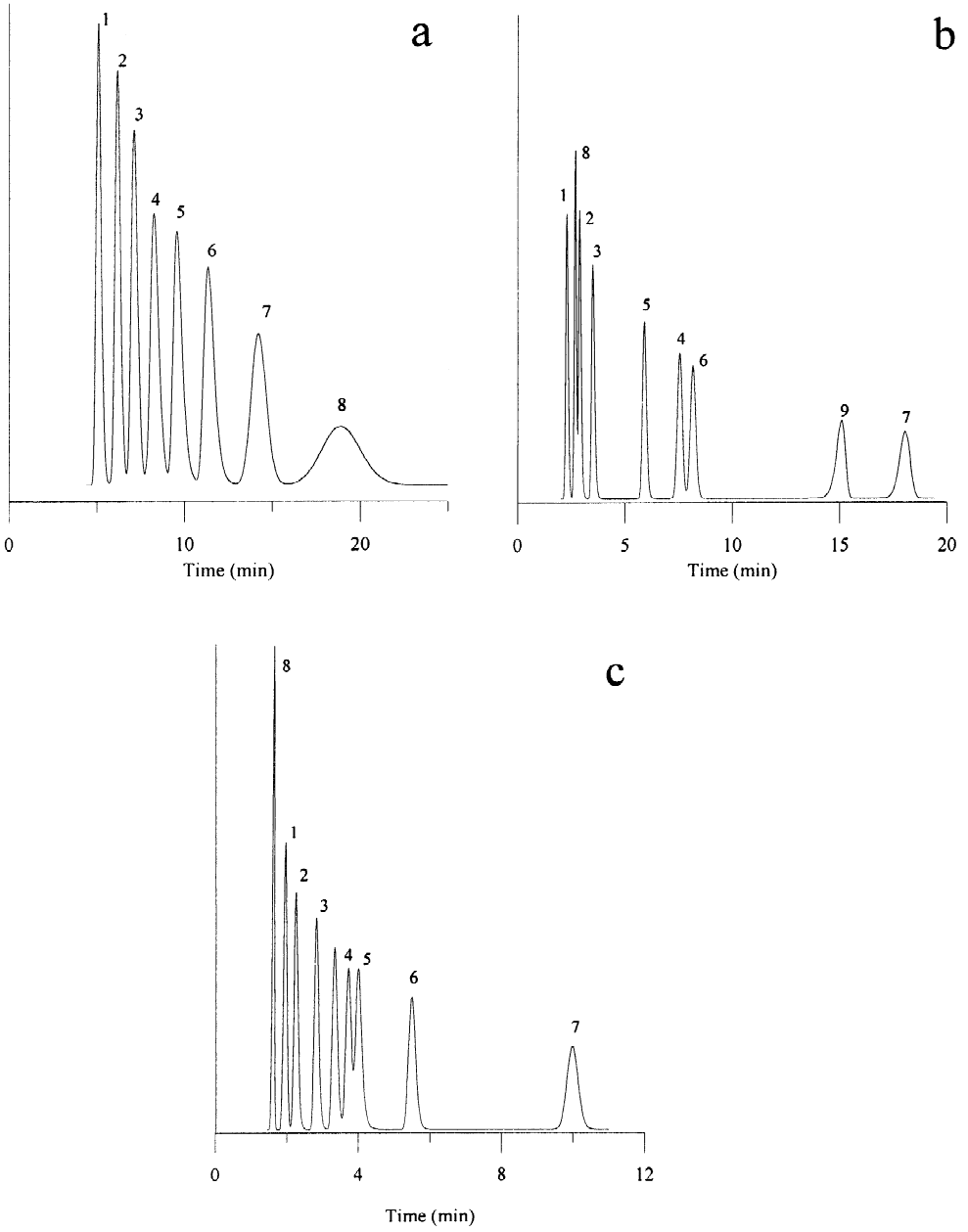

Fig. 6. Chromatograms of mixtures of 8–9 steroids eluted with: (a) 0.12 *M* SDS–5.8% (v/v) 1-pentanol, (b) 52% (v/v) acetonitrile, and (c) 71% (v/v) methanol. Compounds: (1) dehydrotestosterone, (2) testosterone, (3) methyltestosterone, (4) medroxyprogesterone acetate, (5) dydrogesterone, (6) progesterone, (7) testosterone propionate, (8) nandrolone, and (9) clostebol acetate.

4. Conclusions

The resolution optimisation approach used in this work is a useful tool in the development of a screening procedure in chromatography. Its appropriate application depends on the possibility of modelling the retention behaviour with great accuracy, as is the case in MLC and classical RPLC. In MLC, the most suitable organic solvent used as modifier of the mobile phase is mainly determined

Table 2
Elution strength of alcohol and surfactant in micellar and aqueous–organic mobile phases for a set of steroids[a]

	Compound	MLC				Classical RPLC			
		1-Pentanol[b]		SDS[c]		Acetonitrile		Methanol	
		c_1[d]	r	c_1[e]	r	c_1[d]	r	c_1[d]	r
(1)	Dehydrotestosterone	−6.96	0.943	−2.17	0.982	−2.39	0.950	−3.68	0.960
(2)	Testosterone	−7.15	0.954	−2.00	0.985	−2.64	0.953	−4.05	0.964
(3)	Methyltestosterone	−7.53	0.958	−1.77	0.994	−2.79	0.956	−4.40	0.973
(4)	Medroxyprogesterone acetate	−10.94	0.973	−1.37	0.969	−3.23	0.987	−5.12	0.974
(5)	Dydrogesterone	−10.45	0.974	−1.34	0.969	−2.73	0.985	−4.99	0.976
(6)	Progesterone	−10.59	0.979	−1.17	0.988	−2.85	0.986	−4.33	0.990
(7)	Testosterone propionate	−10.26	0.992	−1.20	0.995	−3.23	0.987	−4.01	0.999
(8)	Nandrolone	−14.36	0.989	−1.08	0.996	−3.04	0.978	−3.04	0.966
(9)	Clostebol acetate	−7.86	0.993	−1.63	0.999	−3.44	0.992	–	–

[a] Steroids are listed according to their elution order with 0.12 *M* SDS–5.8% (v/v) 1-pentanol.
[b] At 0.15 *M* SDS.
[c] At 7% (v/v) acetonitrile.
[d] Slope of Eq. (1).
[e] Slope of Eq. (2).

by the polarity of the eluted compounds and their association to the surfactant. Log $P_{o/w}$ values can be used in most cases to select the organic solvent. The results shown in this work can guide for this decision: a low volume fraction of propanol (~1%) is useful to separate compounds with log $P_{o/w} < -1$, such as amino acids. A greater concentration of this solvent (~5–7%) is needed for compounds showing $-1 < \log P_{o/w} < 2$, such as diuretics and sulfonamides. A high concentration of propanol (~15%) or a low concentration of butanol (<10%) can be useful for less polar compounds with $1 < \log P_{o/w} < 3$, such as β-blockers, and pentanol (<6%) is more suitable for non-polar compounds with log $P_{o/w} > 3$, such as steroids. However, for the phenethylamines with $0 < \log P_{o/w} < 1.7$, propanol was too weak due probably to the strong electrostatic association of the charged solutes and the anionic surfactant adsorbed on the stationary phase. A stronger solvent was thus needed. In all examples, compounds having a wide range of polarities were resolved isocratically in relatively short analysis times using a low volume fraction of organic solvent.

Basic compounds, such as phenethylamines and β-blockers yield tailing peaks in classical RPLC because the cationic protonated species interact with the dissociated silanol groups on the silica surface. Special columns are needed to eliminate the poor efficiencies [28]. In MLC, efficiencies are higher and the use of these columns is less necessary. The surfactant layer adsorbed on the column prevents the interaction with the silanol groups and the association kinetics between the charged solutes and surfactant seems to be more facile than the ion-exchange processes involving the silanol groups on the silica surface. For acidic compounds, such as sulfonamides, the efficiencies achieved with both MLC and classical RPLC were comparable using the same column, but for non-polar compounds such as steroids, the efficiencies were smaller. However, in the MLC mode, non-polar compounds are eluted at sufficiently short retention times using a small volume fraction of a strong organic solvent, whereas in classical RPLC, high concentrations of organic solvent are needed.

In the three examples examined in our laboratory, where a comparison was made of the elution behaviour in micellar–organic and aqueous–organic mobile phases using conventional C_{18} columns, the resolution was comparable or better for the MLC mode. The results of this work show that the peculiar behaviour of MLC with regard to the selectivity and elution strength should be taken into account for screening purposes. MLC is an interesting technique

due to the easy preparation of the samples and low consumption of organic solvent. In many cases, direct injection and resolution of complex samples is possible using a conventional alkyl-bonded stationary phase. This gives rise to fast and economical procedures.

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