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Chromatographic behavior of polar compounds in the transition between reversed-phase and submicellar/micellar liquid chromatography

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ABSTRACT

Micellar liquid chromatography (MLC) is a reversed-phase liquid chromatography (RPLC) mode employing a surfactant, typically sodium dodecyl sulfate (SDS), which is biodegradable, in an aqueous or aqueous-organic mobile phase above its critical micellar concentration (CMC). Under these conditions, substantial alterations in retention, selectivity and peak profile occur in comparison to RPLC using conventional hydro-organic mobile phases. These changes arise from the presence of micelles in the mobile phase and the adsorption of surfactant on the stationary phase. In this work, the chromatographic performance of polar compounds of diverse nature is investigated using mobile phases containing SDS below and above its CMC in the presence of acetonitrile. Three nucleosides, four diuretics, and four sulfonamides, all of biomedical and pharmaceutical significance and possessing distinct acid-base characteristics, were chosen as probe compounds. Modifications in retention and peak shape were investigated using experimental conditions that enable the transition from submicellar to micellar conditions. A comparison was also made with conventional RPLC. Analysing the polar compounds with RPLC under low submicellar or micellar conditions significantly decreases the consumption of organic solvent in comparison to hydrophilic liquid chromatography (HILIC).

1. Introduction

In reversed-phase liquid chromatography (RPLC) using silica-based alkyl-bonded stationary phases, the incorporation of reagents into hydro-organic mobile phases is a common practice that leads to significant modifications in the chromatographic behavior [1]. These additives function as modifiers by establishing secondary equilibria, which mitigate or eliminate the undesired effects of residual silanols on the chromatographic performance of basic drugs [2], thereby improving peak profiles. Amines, surfactants, and ionic liquids are typical examples of ionic mobile phase additives. All these reagents can interact with the alkyl-bonded stationary phase and with ionic solutes via ion-pair interactions in the mobile phase, resulting in alterations in retention and peak shape.

Of particular interest is the addition of ionic surfactants, such as the biodegradable anionic sodium dodecyl sulfate (SDS) [3], to the mobile phase for the analysis of cationic compounds. When surfactants are employed as additives, the monomers exhibit strong adsorption on the surface of the alkyl-bonded stationary phase, forming an asymmetrical bilayer that functions as ion-exchanger for ionic analytes with opposite charges, thereby enhancing their retention. Nevertheless, various chromatographic system environments can be observed depending on the surfactant concentration in the mobile phase:

(i) When the surfactant is introduced below its critical micellar concentration (CMC), the concentration of unbound surfactant molecules in the mobile phase remains low. As long as the adsorbed quantity of surfactant does not reach the maximum capacity of the column, the surfactant coating on the stationary phase increases with the monomer concentration in the mobile phase [4]. Under such circumstances, the system operates under submicellar conditions as micelle formation is not possible.
(ii) Above the CMC, the stationary phase reaches saturation, but the more significant changes occur in the mobile phase due to micelle formation [5–7]. Under micellar conditions (in the so-called micellar liquid chromatography, MLC), solute partitioning occurs across three phases: the modified stationary phase, the hydro-organic mobile phase, and the micelles. In MLC, micelles alter solute solubility and the transfer of solutes between the mobile and stationary phases, leading to changes in the behavior

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in terms of selectivity, analysis time and efficiency [8]. Nevertheless, with conventional reversed-phase columns, the elution strength of micellar mobile phases is insufficient, requiring the addition of a small amount of organic solvent to the mobile phase to enhance the elution strength and efficiency.

In addition to modifying the mobile phase, the organic solvent causes partial desorption of the surfactant coating, consequently impacting the solute distribution between the bulk solvent and micellar aggregates [9]. The presence of a thinner coating when an organic solvent is present (referred to as hybrid micellar mobile phases) is also the primary reason for the improvements in efficiency observed in MLC [5,10]. Among the organic solvents commonly used as co-surfactants in MLC, 1-propanol is widely employed, followed by 1-butanol and 1-pentanol [9,10]. More recently, acetonitrile (a common modifier in RPLC) has been shown to be a beneficial modifier [11].

(iii) In MLC, it is crucial to restrict the concentration of organic solvent to prevent micelle disruption [9]. Once a certain threshold of organic solvent content is exceeded, surfactant monomers are liberated. This leads to the predominance of ion-pair interactions between the solutes and surfactant monomers in both mobile and stationary phases, resulting in the emergence of another chromatographic mode known as High submicellar liquid chromatography (HSLC) [12]. In HSLC, the surfactant concentration is above its CMC, but the substantial amount of organic solvent prevents micelle formation, offering advantages in terms of analysis time, selectivity, and peak shape [13–17].

The chromatographic behavior of basic compounds during the transition between micellar and high submicellar conditions has been recently investigated [11,15–17], revealing significant improvements in efficiency and peak symmetry [18]. However, there is still a need to explore the changes in the chromatographic behavior of compounds with diverse characteristics as the surfactant concentration increases during the transition from low submicellar to micellar conditions. In this work, we aim to provide valuable insights into the underlying processes within the chromatographic system during both transitions: from low submicellar to micellar mode, and from micellar to high submicellar mode. The investigation utilizes the anionic surfactant SDS across a wide range of concentrations to study these transitions for compounds characterized by high polarity and of biomedical and pharmaceutical interest. Three distinct groups of compounds have been selected: nucleosides (basic compounds), diuretics (acidic compounds), and sulfonamides (amphoteric compounds). Their behavior was compared to that observed in conventional RPLC.

2. Experimental

2.1. Reagents

The probe compounds consisted of three nucleosides (adenosine, cytidine, and xantosine), four diuretics (althiazide, chlorothiazide, hydrochlorothiazide, and trichloromethiazide), and four sulfonamides (sulfachlorpyridazine, sulfadiazine, sulfamerazine, and sulfisoxazole), all from Sigma (St. Louis, MA). The structures, pK_a and log $P_{o/w}$ values of these compounds are provided in the Supplementary Material (Tables S1 to S3). Stock solutions of approximately 100 μg/ml for each drug were prepared by dissolving the solids in 40:60 (*v/v*) acetonitrile–water mixture (Scharlab, Barcelona, Spain). These solutions remained stable for at least two months when stored at 4 °C. Working solutions were prepared by diluting the stock solutions with water to a final concentration of 40 μg/ml, and subsequently filtered through 0.45 μm Nylon syringe filters (Labbox, Barcelona), prior to injection into the chromatograph. The dead time was determined from the initial baseline perturbation observed in the chromatograms.

All mobile phases consisted of 10%, 20%, and 30% (*v/v*) acetonitrile, with or without the addition of SDS (99% purity, Merck, Darmstad, Germany), at concentrations of 1, 4, 8, 10, 20, 30, 60, 80, and 150 mM (the mobile phases were run in ascending order of concentration). The selection of the minimum and maximum concentrations of SDS and acetonitrile in the mobile phase aimed to achieve sufficient retention for the most polar compounds, while avoiding excessive retention for the least polar compounds. In all cases, the mobile phases were buffered at pH 3.0 using 0.01 M sodium dihydrogenphosphate (Sigma) and hydrochloric acid, with the pH adjusted prior to organic solvent addition.

The mobile phases were filtered through 0.45 μm Nylon membranes from Micron Separations (Westboro, MA), and degassed using an Elmasonic S15 H ultrasonic bath from Elma (Singen, Germany). Nanopure water obtained with an Adrona system (Riga, Latvia) was used throughout.

2.2. Apparatus and column

An Agilent chromatograph (Waldbronn, Germany) was used, consisting of an isocratic pump (Series 1260), a solvent selector valve (Series 1290), an automatic injector (Series 1260), a column compartment (Series 1260) set at 25 °C, and a UV–visible wavelength detector (Series 1100). The system was controlled and data acquisition performed using the OpenLAB CDS LC Chemstation software (Agilent C.01.08). Chromatographic peaks were analysed using the MICHROM software to determine peak parameters, such as retention times and peak half-widths [19]. The nucleosides were monitored at 260 nm, the diuretics at 273 nm, and the sulfonamides at 254 nm. The retention data were acquired using a flow rate of 1 ml/min, and duplicate injections of 20 μl were made.

The chromatographic column used in this study was an Agilent Zorbax Eclipse C18 with the following specifications: 150 mm × 4.6 mm internal diameter, 5 μm particle size, 80 Å mean pore diameter, 180 m^2/g surface area, and 10 wt% total carbon. To protect the analytical column, a similar 30 mm guard column was installed.

To minimize reagent consumption and waste generation, the mobile phases were recycled between runs and during analysis. Periodic rinsing of the chromatographic system was performed using 10 volumes of a methanol–water solution (5:95, *v/v*), followed by 10 volumes of pure methanol (approximately 30 ml) (Panreac, Castellar del Vallès, Barcelona) to remove any residual surfactant from the stationary phase. During the weekends, the column was stored in methanol to maintain its condition.

3. Results and discussion

3.1. Comparison of the retention capability of low submicellar, micellar and high submicellar systems containing SDS

3.1.1. Column environment

The addition of SDS to a reversed-phase liquid chromatography (RPLC) mobile phase results in the coverage of the apolar C18 surface, creating a negatively charged layer on the stationary phase. The retention of solutes is influenced by the coating effect of this column, which depends on the concentration of the surfactant, whether it is above or below the CMC, and on the concentration of organic solvent in the mobile phase. It is important to note that the presence of high concentrations of organic solvent in SDS solutions reduces the aggregation number of micelles and can eventually lead to micelle breakdown. This also results in the partial desorption of surfactant monomers associated with the stationary phase.

Acetonitrile has been investigated as a co-surfactant for SDS in low submicellar, micellar, and high submicellar liquid chromatography. The CMC of SDS, which is 8.2×10^{-3} M in water, increases to approximately 0.01 M and 0.03 M at acetonitrile concentrations of 10% and 20%, respectively [20]. However, when acetonitrile concentrations exceed approximately 30%, SDS micelles disaggregate. In the presence of SDS

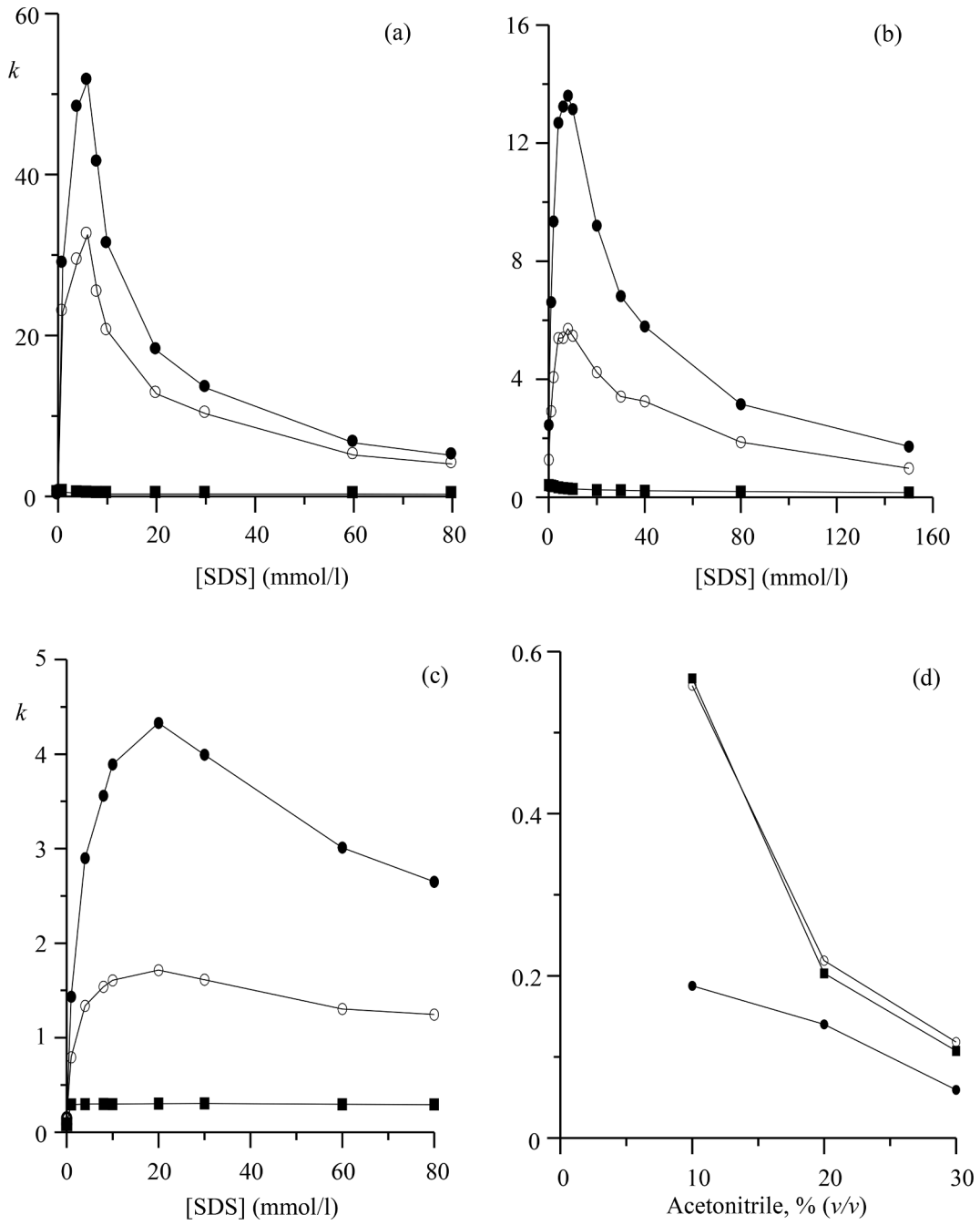

Fig. 1. Change in retention for cytidine (●), adenosine (○) and xantosine (■), at increasing concentration of SDS, in the presence of: (a) 10% acetonitrile, (b) 20% acetonitrile, (c) 30% acetonitrile, and (d) without SDS at increasing concentration of acetonitrile.

concentrations above its CMC, the submicellar mode is obtained at high surfactant concentrations [21].

Therefore, when 20% acetonitrile is present along with SDS concentrations below 0.03 M (1, 4, 8 and 10 mM), the chromatographic mode is considered submicellar. In this mode, the stationary phase is partially coated with SDS monomers, wherein the polar head groups are oriented away from the surface. This arrangement forms a negatively charged bilayer, while the mobile phase consists of surfactant monomers at low concentrations. As the concentration of SDS increases from 1 to 10 mM, the extent of surfactant coating on the column increases, but column saturation is not achieved. The maximum capacity of the column is attained when the SDS concentration exceeds its CMC (0.03 M at 20% acetonitrile), resulting in the micellar chromatographic mode. Under these circumstances, the stationary phase still maintains a negative net charge, but secondary equilibria are established with the formed micelles in the mobile phase due to electrostatic and hydrophobic interactions. Once the column becomes saturated with surfactant, further addition of surfactant up to 80 mM or more increases the amount of micelles in the mobile phase. Additionally, in this work, mobile phases containing SDS and 10% acetonitrile were also prepared. Increasing the concentration of acetonitrile, from 10% to 20% leads to a thinner coverage of the stationary phase.

3.1.2. *Retention behavior of nucleosides*

The retention times of nucleosides in submicellar and micellar chromatographic systems are influenced by the characteristics of the eluted solutes. Nucleosides possess a positive charge at pH 3, and in low submicellar RPLC, they can establish hydrophobic interactions with the

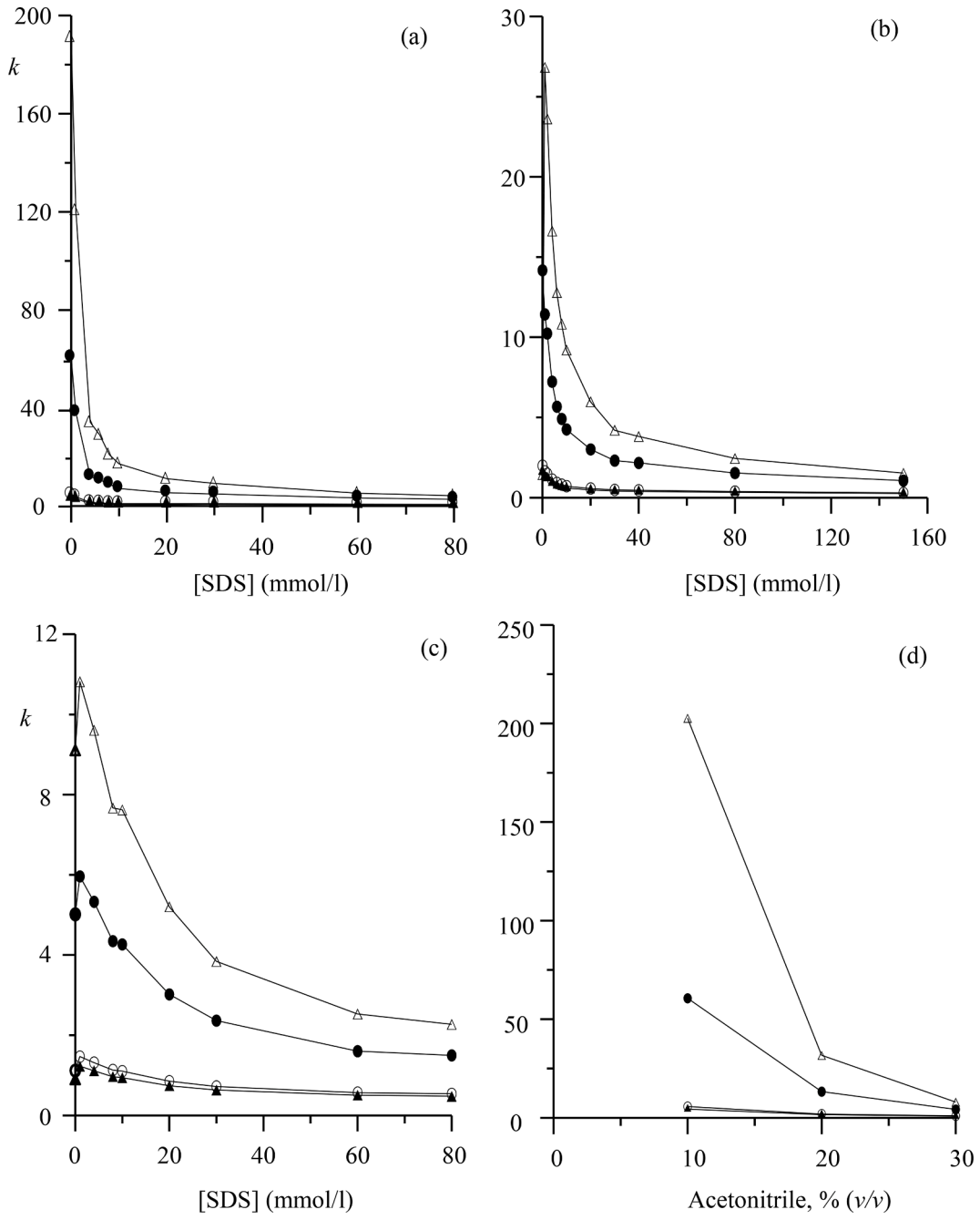

Fig. 2. Change in retention for althiazide (Δ), chlorothiazide (▲), hydrochlorothiazide (○), and trichloromethiazide (●), at increasing concentration of SDS, in the presence of: (a) 10% acetonitrile, (b) 20% acetonitrile, (c) 30% acetonitrile, and (d) without SDS at increasing concentration of acetonitrile.

silica-based stationary phase and electrostatic interactions with the adsorbed SDS monomers. These interactions lead to an increase in nucleoside retention as the concentration of SDS rises. Fig. 1 illustrates this behavior below the SDS CMC, in the presence of 10% (Fig. 1a) and 20% acetonitrile (Fig. 1b). The figures also show that the introduction of surfactant at concentrations above the CMC significantly reduces retention. This decrease can be attributed to the presence of micelles in the mobile phase, which interact with the solutes and result in reduced retention times.

Fig. 1c illustrates the changes in retention of nucleosides in the high submicellar mode, when 30% acetonitrile is present, where micelles are not formed. In these conditions, the retention mechanism is dependent on the amount of remaining surfactant adsorbed onto the stationary phase. The retention factors observed are significantly shorter compared to those shown in Fig. 1a and b. This decrease can be attributed to desorption of surfactant from the stationary phase caused by the high concentration of organic solvent, leading to a decrease in the polarity of the mobile phase as well. However, the retention factors of nucleosides in the high submicellar mode (at 30% acetonitrile) are much higher than those observed in hydro-organic RPLC without SDS (Fig. 1d). This confirms that a certain amount of SDS remains adsorbed on the column in the high submicellar mode, allowing for ionic exchange with the cationic nucleosides to occur.

In the high submicellar mode (Fig. 1c), the retention behavior of nucleosides follows a similar pattern to the transition observed between the low submicellar and micellar modes in the presence of 10% and 20% acetonitrile (Fig. 1a and 1b): Initially, the retention increases at low concentrations of SDS and then decreases after reaching a maximum.

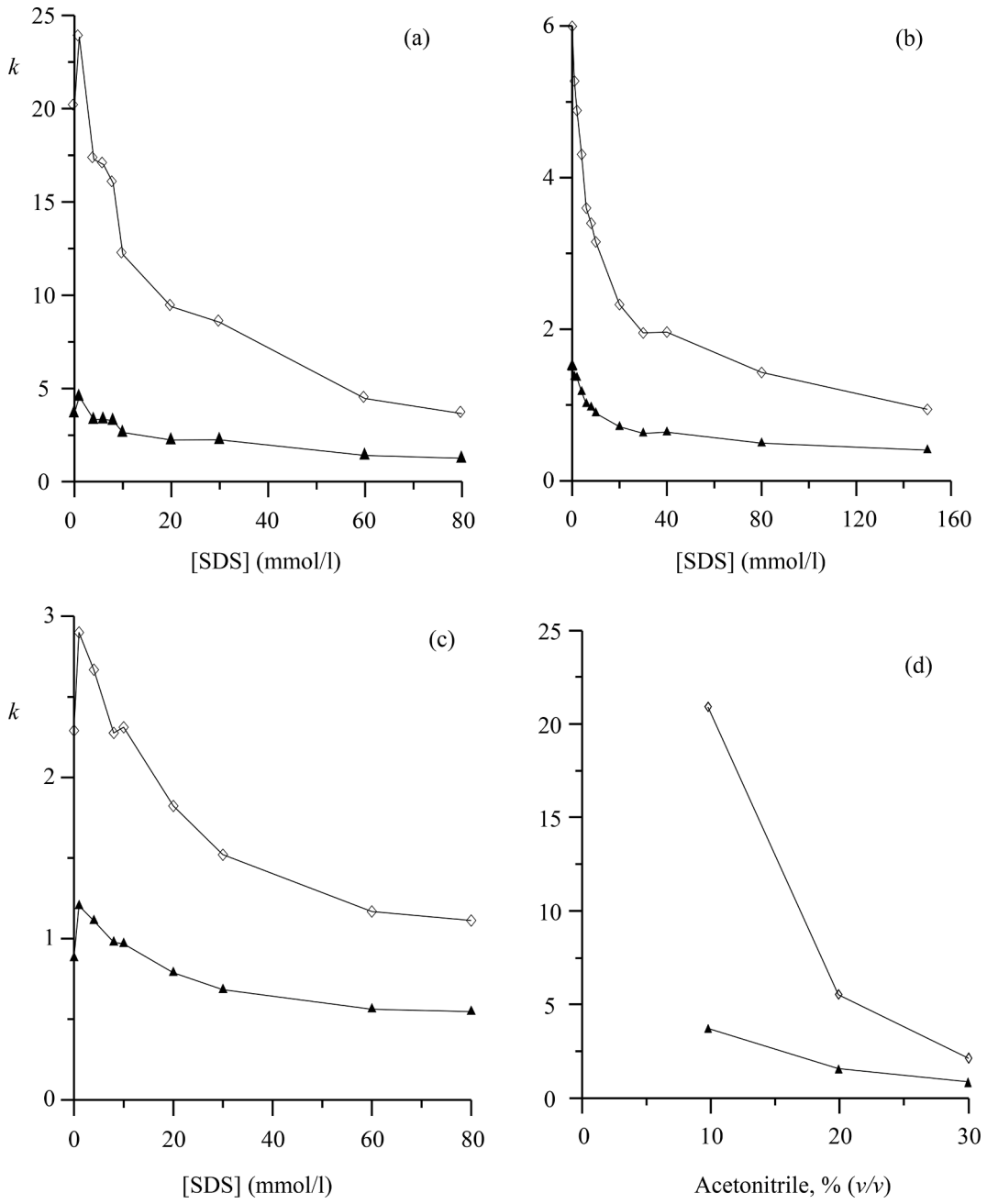

Fig. 3. Change in retention for sulfachloropyridazine (◇), and sulfadiazine (▲), at increasing concentration of SDS, in the presence of: (a) 10% acetonitrile, (b) 20% acetonitrile, (c) 30% acetonitrile, and (d) without SDS at increasing concentration of acetonitrile.

However, in a mobile phase containing surfactant monomers but no micelles, the slope of the ascending and descending regions of the retention curve is smaller. It is important to note that the increased retention observed at lower SDS concentrations in Fig. 1c (obtained at 30% acetonitrile) is produced by the presence of a significant concentration of adsorbed monomers on the stationary phase, which attract the cationic solutes. Under these conditions, micelles are not present, but there are SDS monomers in the mobile phase that associate with the cationic solutes. This association leads to a decrease in retention at higher SDS concentrations. Due to the weaker association between the cationic solutes and SDS monomers compared to micelles, the retention is shorter, and the slopes of the retention curves are smaller under these conditions.

3.1.3. Retention behavior of diuretics and sulfonamides

The retention behavior of diuretics and sulfonamides is depicted in Figs. 2 and 3, at varying concentrations of SDS and acetonitrile. These compounds possess weakly acidic and amphoteric characteristics, respectively. At pH 3, neutral species dominate in the mobile phase. Consequently, elution of these compounds under low submicellar, micellar, and high submicellar conditions consistently leads to a gradual decrease in retention times as the concentration of SDS increases (in comparison to the retention behavior of nucleosides shown in Fig. 1). It is worth noting that hydrochlorothiazide and chlorothiazide exhibit very similar retention behavior. The retention behavior of the pairs sulfadiazine/sulfamerazine, and sulfachlorpyridazine/sulfisoxazole is also similar.

Diuretics and sulfonamides are effectively separated in the presence of SDS, primarily due to hydrophobic interactions with the modified

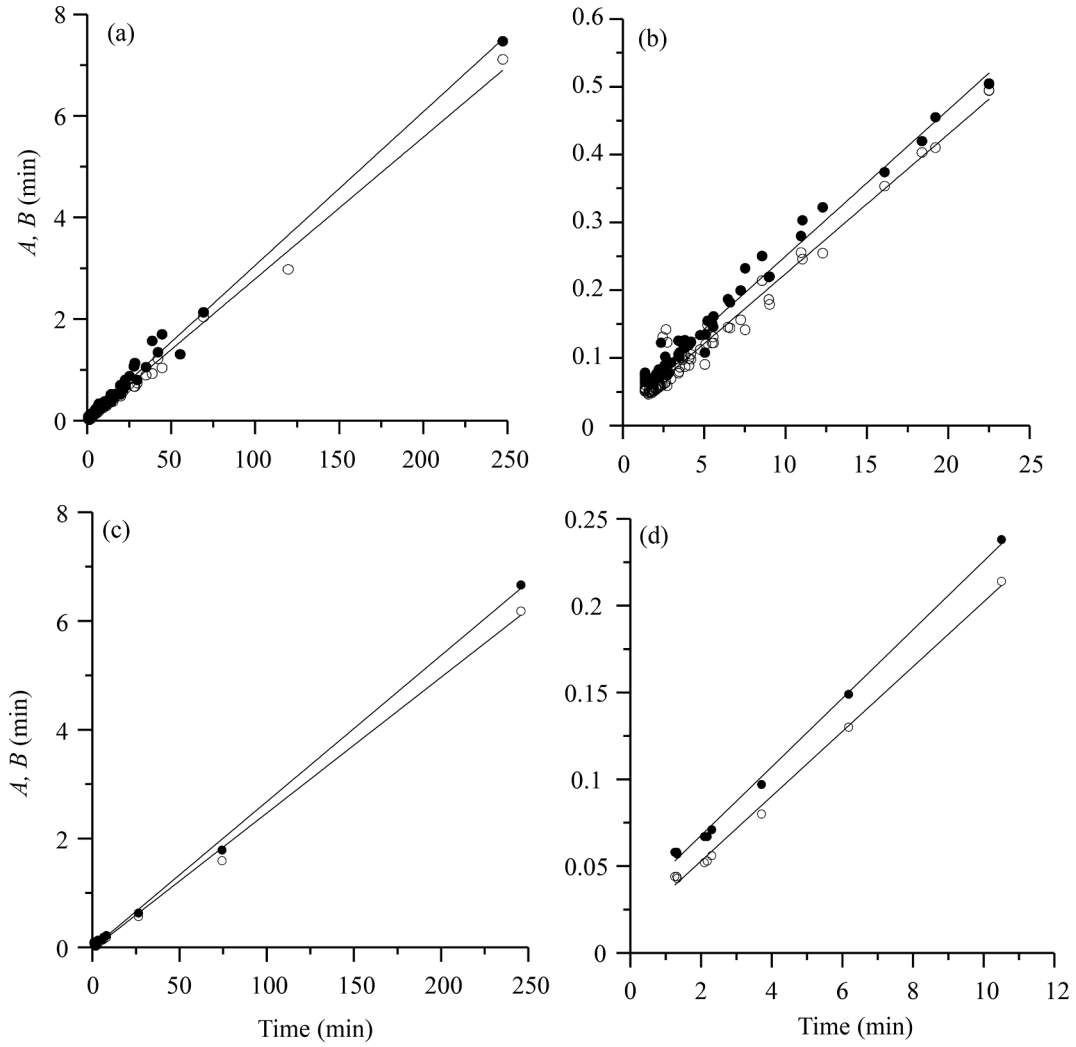

Fig. 4. Half-width plots (*A* (○) and *B* (●)) for the whole set of probe compounds, eluted with mobile phases in the presence of increasing concentrations of SDS at: (a) 10% acetonitrile, and (b) 30% acetonitrile; and with a mobile phase without SDS at: (c) 10% acetonitrile, and (d) 30% acetonitrile.

stationary phase and the solubilization capacity of SDS monomers and micelles in the mobile phase. It should be noted that the changes in retention, as well as the absolute retention, are smaller in the high submicellar mode (Fig. 2c and 3c). Meanwhile, Fig. 2d and 3d illustrate the retention of diuretics and sulfonamides in the hydro-organic mode, which is higher in comparison to the modes involving the anionic surfactant at the same acetonitrile concentration.

3.2. Peak profile in the submicellar and micellar systems

3.2.1. Chromatograms of the probe compounds

The primary objective in chromatographic separation is to achieve chromatograms with high resolution, characterised by symmetric and narrow peaks. Chromatograms corresponding to a representative compound from each of the three studied sets (cytidine, chlorothiazide, and sulfachloropyridazine) are presented in Figs. S1 to S3 of the Supplementary Material. The chromatograms show the peaks obtained in the low submicellar mode (Figs. a), micellar mode (Figs. b), and high submicellar mode (Figs. c), all utilising mobile phases containing SDS and acetonitrile. Chromatograms of mixtures of the studied nucleosides and diuretics are also illustrated in Figs. S4 and S5, respectively.

Similar chromatographic peaks were observed for the entire set of compounds investigated, including nucleosides with a basic character. The chromatographic peaks exhibited high symmetry, particularly under micellar and high submicellar conditions. However, when the concentration of SDS was below its CMC, the peaks displayed greater asymmetry with slight tailing, as seen in Fig. S1a to S3a. This effect was particularly pronounced for the basic nucleoside cytidine. This observation suggests that under low submicellar conditions, the column did not reach saturation. Consequently, the basic solutes were able to establish slow interaction kinetics with the silanols present on the stationary phase, leading to the formation of tailed peaks.

3.2.2. Half-width plots

Half-width plots are constructed using the left (*A*) and right (*B*) half-widths, measured at 10% peak height, plotted against the retention time. They serve as a valuable tool for evaluating changes in peak width and asymmetry within a chromatographic column. The choice of measuring at 10% peak height allows for the assessment of peak symmetry without being influenced by baseline noise in chromatograms. Recently, these plots have been proven to be useful for comparing the chromatographic behavior of different compounds using various chromatographic columns and/or mobile phases with different characteristics [18,22–25]. The construction of these plots is straightforward, with each line represented by the following equations:

$$A = A_0 + m_A t_R \quad (1)$$

$$B = B_0 + m_B t_R \quad (2)$$

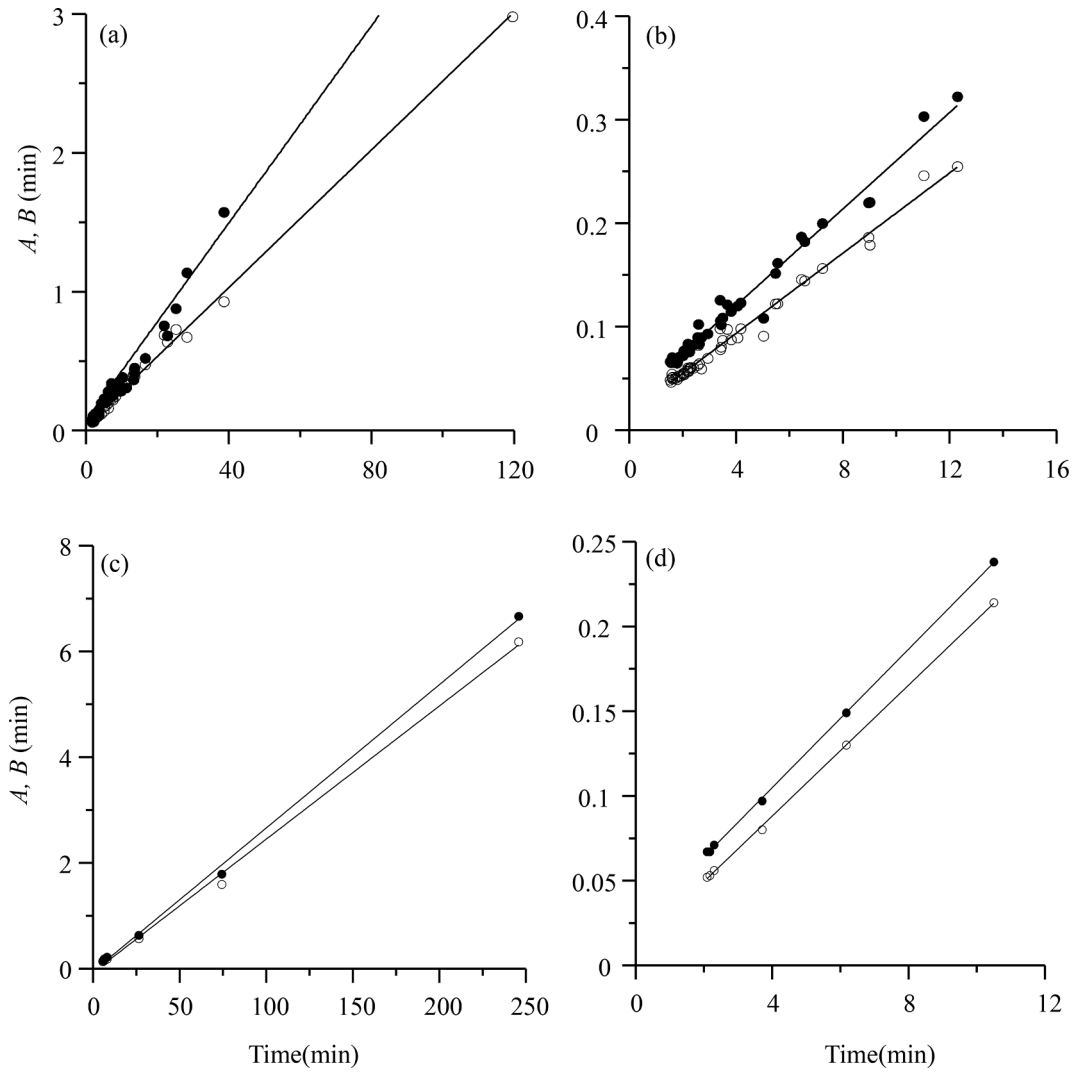

Fig. 5. Half-width plots (*A* (○) and *B* (●)) for the studied diuretics and sulfonamides, eluted with mobile phases in the presence of increasing concentrations of SDS at: (a) 10% acetonitrile, and (d) 30% acetonitrile; and with a mobile phase without SDS at: (c) 10% acetonitrile, and (b) 30% acetonitrile.

Table 1
Half-width plots parameters for the studied compounds eluted with mobile phases containing 10%, 20%, and 30% acetonitrile, in the absence and presence of surfactant.

Compounds	Mobile phase	m_A	m_B	$m_A + m_B$	m_B / m_A
Nucleosides (basic)	10% Acetonitrile	ND	ND	ND	ND
	10% Acetonitrile with SDS	0.0235	0.0264	0.0499	1.12
	20% Acetonitrile	ND	ND	ND	ND
	20% Acetonitrile with SDS	0.0219	0.0193	0.0412	0.88
	30% Acetonitrile	ND	ND	ND	ND
	30% Acetonitrile with SDS	0.0186	0.0190	0.0376	1.02
Diuretics and sulfonamides (neutral)	10% Acetonitrile	0.0252	0.0271	0.0523	1.08
	10% Acetonitrile with SDS	0.0248	0.0361	0.0609	1.46
	20% Acetonitrile	0.0197	0.0210	0.0407	1.07
	20% Acetonitrile with SDS	0.0203	0.0189	0.0379	0.93
	30% Acetonitrile	0.0193	0.0204	0.0397	1.06
	30% Acetonitrile with SDS	0.0194	0.0233	0.0427	1.20

ND: Non-determined. Solutes eluting close to the dead time.

where m_A and m_B represent the slopes of the linear correlations for the left and right half-widths, respectively, while A_0 and B_0 correspond to the intercepts representing the extra-column contribution to peak broadening. Eqs. (1) and (2) allow for the prediction of peak half-widths for compounds eluted at various retention times, and are useful for characterising chromatographic columns. The sum of m_A and m_B reflects the broadening rate of chromatographic peaks within the column, and the ratio (m_B/m_A) indicates the peak asymmetry at high retention times. In this work, half-width plots have been employed to investigate the impact of different elution conditions (specifically in the low sub-micellar, micellar, and high submicellar modes) on peak profiles.

The plots were constructed for sets of mobile phases containing 10%, 20%, and 30% acetonitrile, with increasing concentrations of SDS, considering the entire set of compounds. Fig. 4a and b illustrate the results for 10% and 30%, respectively. For comparison, the half-width plots obtained for mobile phases containing 10% and 30% acetonitrile without SDS are also shown (Fig. 4c and d, respectively). In all cases, the slopes of the half-width plots exhibited a high degree of coincidence, indicating significantly symmetrical peaks. Fig. 5 illustrates the plots for diuretics and sulfonamides for 10% and 30% acetonitrile. Half-width plots for nucleosides in the hydro-organic mode could not be constructed, since the peaks eluted close to the dead time.

Table 1 presents the parameters of the equations that describe the

half-width plots, including the slopes of the left (m_A) and right (m_B) half-widths, as well as the sum and ratio of the slopes for all the plots built for nucleosides, and diuretics and sulfonamides. The m_B/m_A ratio, where the values close to unity indicate symmetrical peaks, was similar for all chromatographic modes. For diuretics and sulfonamides (Fig. 5), there is no significant difference among the peak widths in the hydro-organic and submicellar and micellar modes.

4. Conclusions

In conclusion, this study investigated the changes in the chromatographic behavior of sets of compounds with significant polarity, when a surfactant is added to acetonitrile–water mobile phases, at the typical working pH in RPLC. The results demonstrate that the addition of SDS to a hydro-organic RPLC system leads to notable differences in retention, depending on the solute's nature and the concentration of the surfactant and organic solvent. Thus, in the hydro-organic mode, the retention factors are much smaller for nucleosides, whereas they are significantly higher for diuretics and sulfonamides in comparison to the behavior observed when the anionic surfactant SDS is added to the mobile phase. Changes in peak profile, such as width and symmetry, are less significant.

In the literature, compounds with high polarity are typically analysed using hydrophilic liquid chromatography (HILIC), mode that requires high concentrations of organic solvent (>70%) [26–28]. Among others, HILIC methodologies have been developed for the compounds studied in this work (nucleosides, thiazides and sulfonamides) [29–31]. The use of low submicellar or micellar mobile phases in RPLC for the analysis of such polar compounds significantly reduces organic solvent consumption, making it more environmentally friendly while still providing satisfactory chromatographic peaks. The anionic surfactant SDS has also the advantage of being biodegradable [3].

Furthermore, previous studies have focused on the significant retention of basic compounds (with cationic character) under low submicellar conditions, but there was still limited information on the transition between low submicellar and micellar conditions. In this work, basic compounds with high polarity, specifically three nucleosides, were selected to explore this transition. The observed retention behavior suggests that the anionic surfactant is gradually adsorbed on the stationary phase, at increasing concentration of SDS below the CMC. These findings confirm earlier studies conducted by Berthod et al., based on adsorption isotherms [5,32].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2023.109040.

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