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Journal of Chromatography A, 1049 (2004) 171–181
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Separation of basic compounds by capillary electrochromatography on an X-Terra RP18® stationary phase

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Received 13 April 2004; received in revised form 20 July 2004; accepted 21 July 2004

Abstract

In this work we demonstrate that the X-Terra RP18® stationary phase, specially designed for the analysis of basic compounds in liquid chromatography, may be successfully used in capillary electrochromatography. Although this packing material does not afford a sufficient electroosmotic flow with classical hydro-organic mobile phases, the addition of a surfactant that adsorbs onto the stationary phase allows to generate a sustainable electroosmosis flow (EOF), the direction of which depends on the charge of the surfactant. The way of manipulating the electroosmotic flow is described (nature and concentration of the added surfactant, proportion of the organic modifier in the mobile phase, pH). It is then demonstrated that high efficiencies can be reached with this packing material (up to 220 000 plates/m with a mean diameter particles of 3.5 μm) when it is operated at high linear velocities. Then the separations of different classes of compounds such as amphenicol antibiotics, macrolide antibiotics or basic test solutes with mobile phases with pH up to 10.8 are described. The influence of the addition of sodium dodcylsulfate (SDS) to the mobile phase on the retention is described and the selectivity of the X-Terra RP18® stationary phase is compared to that of a more traditional phase, i.e. Hypersil C_{18} stationary phase with SDS added to the mobile phase. However, it is shown that a good repeatability of the retention factors can only be obtained when the ionization of the compounds is totally suppressed since electrolysis of the buffered hydro-organic mobile phase occurs in the buffer reservoirs leading to a variation of the mobile phase pH and consequently to a modification of the ionization degree of the solutes having their pK_a close to the mobile phase pH.

Keywords: Basic solutes; Stationary phases; Capillary electrochromatography; Amphenicol; Macrolides

1. Introduction

Capillary electrochromatography is a hybrid technique combining aspects of both high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). As in capillary electrophoresis, the separation is performed in a capillary column under an applied electrical field, with electroosmotic flow (EOF) as the driving force for bulk liquid movement. As in liquid chromatography, the capillary contains a stationary phase and the separation of the solutes therefore results from the partitioning of the solutes between the mobile and the stationary phases. When ionised solutes are analysed, electromigration can be exploited as an additional mechanism to modulate the separation. This technique has gained considerably in popularity over the last decade and has been demonstrated to give extremely high efficiencies [1–3]. Despite the great variety of stationary phases available for liquid chromatography, only very few of them have been used today in CEC, especially the classical bonded silicas such as Hypersil or Spherisorb as they give excellent linear velocities under electrodriven conditions and allow separation of a great variety of compounds in a useful timeframe. However, the analysis of strongly basic analytes, an analytical challenge

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0021-9673/$ – see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.chroma.2004.07.056

for many years, on such traditional bonded stationary phases which exhibit a high density of acidic silanol groups, results in secondary interactions, severe peak tailing and increased retention [4]. The main problem encountered with the analysis of basic compounds is due to the interaction between their basic nitrogen and the residual silanol groups of the packing materials. To date, the methodology usually used to analyse basic compounds by electrochromatography relies on the addition of small competiting bases such as triethylamine [5], triethanolamine [5], hexylamine [6], ethylenediamine [7,8] in the mobile phases although this addition slows down the EOF. An other approach to analyse basic compounds has also been described by several authors using unbonded silica in CEC with simple aqueous-organic mobile phase with or without the need for competiting bases in the mobile phase, with good efficiencies and separation performance [9–14]. To overcome the problem of peak tailing encountered with silica materials several authors proposed to use a strong cation-exchange stationary phase but a rapid, unpredictable and unexplainable loss of efficiency of the stationary phases was observed [15–18].

In liquid chromatography, these problems have been addressed by the introduction of a wide range of stationary phases where the silanol groups have either been deactivated or shielded and by the manufacturing of very pure silica (free of metal impurities), thus reducing the number of reactive acidic silanol groups responsible for peak tailing. As a result though, such stationary phases dedicated to the analysis of basic compounds yield a low or even no EOF in capillary electrochromatography when compared to the EOF generated with the more acidic, older silica [13,18,19]. Moreover, these extensively end-capped, pure silica-based phases are often unusual in CEC since they do not possess a sufficient wettability and keep drying out. As a consequence, capillary electrochromatography is plagued by a number of critical issues, one of the main issue of contention being the absence of CEC stationary phase suitable for the analysis of basic compounds [19].

The potential of the X-Terra RP18® stationary phase in CEC has never been explored to the best of our knowledge although it may present several advantages over other conventional stationary phases in CEC and may extend the scope of CEC through the analysis of basic solutes. Indeed, X-Terra RP18® stationary phase has been specifically designed to “shield highly” polar and basic compounds from silanol activity in liquid chromatography. According to the manufacturer, the X-Terra RP18® material combines an extremely low area of underivatized surface because of the methylsiloxane groups incorporated throughout the hybrid particle and a highly shielded surface owing to the incorporated embedded polar group. Its main advantage lies in its stability towards high pH electrolytes, allowing the separation of basic solutes by controlling their ionization degree at high pH without any problem of stationary phase dissolution.

We have evaluated the X-Terra RP18® stationary phase for its ability to generate a sustainable EOF and for its performance in separating neutral and basic compounds by CEC. The influence of several parameters conditioning the generation of the EOF were studied: influence of the addition of a cationic or an anionic surfactant to the mobile phase, influence of the pH and of the hydroorganic content. Then, the separations of a few classes of compounds are described (amphenicol antibiotics, macrolide antibiotics and basic test compounds) and the results compared to those obtained in liquid chromatography in the same experimental conditions. Electrolysis of the mobile phase is demonstrated to be responsible of the lack of repeatability observed when the solutes are separated in the ionisation-control mode, i.e. when the pH of the mobile phase is close to their pK_a values.

2. Experimental

2.1. Chemicals

Stock solutions of the macrolides (Josamycin from ICN Biomedicals (Orsay, France), erythromycin, lincomycin, and tylosin tartrate from Sigma (Paris, France), tilmicosin from Eli Lilly (Suresnes, France), Virginiamycin from Pfeizer (Paris, France), Neospiramycin and spiramycin from Aventis pharma (Paris, France)) were prepared at a concentration of 1 and 10 g L^{-1} in methanol for HPLC and CEC experiments, respectively. These solutions were stored at −20 °C in the dark. All the basic test compounds (protriptylin, diphenhydramin, clozapin and imipramin) were from Sigma. The stock solutions of these compounds were prepared at a concentration of 1 g L^{-1} in methanol and 10 g L^{-1} in water (excepted for clozapin at 5 g L^{-1} in methanol/water, 80/20) for HPLC and CEC experiments, respectively. These solutions were stored at 4 °C in the dark.

The stock solutions of amphenicol antibiotics (Chloramphenicol, Thiamphenicol and Florfenicol) all from Schlering Plough (Paris, France) were prepared in methanol at a concentration of 1 g L^{-1}.

All the stock solutions were diluted in the mobile phase just prior to analysis.

2.2. Mobile phases

The different reagents (sodium tetraborate, tris(hydroxymethyl)methylamine (TRIS), sodium citrate, CAPS, CAPSO, ammonia, sodium dodcylsulfate (SDS) were all of analytical grade from Sigma (Paris, France). Buffers of tetraborate 10 mM at pH 9.3, TRIS 100 mM at pH 7.5, citrate 100 mM at pH 3.5, CAPS 10 mM at pH 10.5, CAPSO 10 mM at pH 10.5, ammonia 10 or 25 mM at pH 10, were prepared at the desired concentration and adjusted using HCl as required. Their pH values refer to the aqueous solution. These aqueous buffer solutions were systematically filtered through 0.45 μm nylon filters prior to use. Then, the hydro-organic mobile phases were prepared by mixing (v/v/v) the organic solvent (HPLC-grade acetonitrile from SDS, Paris, France) with the desired volumes of buffer and

water (if necessary) in order to reach the desirable total ionic strength and the desirable aqueous-organic proportion (v/v).

SDS was added to the hydro-organic mobile phase by dissolving the appropriate amount of SDS in 100 mL of mobile phase. The SDS content refers to the total mobile phase volume.

2.3. Apparatus

2.3.1. Liquid chromatography

The HPLC system consisted of a LC-10AD pump (Shimadzu, France), a model 7125 injection valve (Rheodyne, Paris, France) equipped with an external injection loop of 5 μL, and a model SPD6 wavelength UV detector (Shimadzu, Paris, France). Chromatograms were recorded using a model CR5-A integrator (Shimadzu, Paris, France).

Two commercial columns were used throughout this study. An X-Terra RP18® column (100 mm × 4.6 mm i.d., d_p = 3.5 μm) and an Hypersil ODS column (100 mm × 4.6 mm i.d., d_p = 5 μm). The flow rate was set at 1 mL min^{-1} and the injection volume was 5 μL for all experiments. All the separations were implemented at room temperature (25 °C).

2.3.2. Nano-liquid chromatography

The capillary columns used in nano-liquid chromatography were the same as those used in capillary electrochromatography. The nano-LC system was home-made as following described.

2.3.2.1. The pumping system. A LC-10AD pump (Shimadzu, France) was used in the microliter per minute flow rate range. The nano-LC flow velocity was determined measuring the retention time of thiourea, an unretained compound. It corresponded to nanoliter per minute flow rate. Such low flow rates were obtained by splitting the pump flow. Part of the flow goes in the nano-LC column, the other part goes in a restrictor whose length and diameter were adjusted to obtain the desired split ratio. Fig. 1 shows the experimental set-up.

2.3.2.2. Injection procedure. A six-port valve (Rheodyne 7125) from (Interchim, Montluçon, France) was used to perform sample injections. In the "load" position, the valve connects the pump flow directly to the restrictor through ports 2 and 3 (Fig. 1A). The sample is introduced through ports 5 and 4 using a classical syringe. The sample solution flows through ports 4, 1 and 6, sweeping the inlet frit of the LC column without entering it (Fig. 1A, inset). Then the sample solution is let standing in the system for a measured time (typically 30 s). During this contact time, solute molecules can diffuse through the column inlet frit (Fig. 1A, inset). After the contact time was elapsed, the sample solution was rapidly flushed with about 1 mL of mobile phase pushed through port 5 using a second syringe. The valve is immediately switched to its "inject" position resuming the mobile phase flow through the column (Fig. 1B). This procedure allowed for reproducible injections of very small amounts of sample minimizing external dispersion.

2.3.2.3. Detection. The separation column was inserted in the detection cell of a Hewlett-Packard HP^{3D} CE UV–vis detector. The detection window of the capillary allowed to perform in situ detection.

2.3.3. Capillary electrochromatography

CEC experiments were performed on a HP^{3D}CE (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector operated at the desired wavelength. All experiments were carried out at 25 °C. A pressure of 10 bars provided by pressurized nitrogen was applied at both ends of the capillary to prevent bubble formation. Samples were injected electrokinetically by application of a voltage of 5 kV during 2 s. The columns used throughout this study (l = 8.5 cm, L = 32 cm or l = 23.5 cm, L = 32 cm) were home-made as described in the procedure section. In the short-end injection mode, the separation was realized with a 8.5 cm effective separation length (8.5 cm packed portion) and the voltage polarity was inverted so that the solutes migrated towards the detection window located immediately after the retaining frit. Thiourea was used as electroosmotic flow marker.

2.3.4. Capillary electrophoresis (CZE)

CZE experiments were performed on a HP^{3D}CE (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector operated at the desired wavelength. Fused silica capillaries (375 μm o.d.× 50 μm i.d. total length 32 cm, effective length 23.5 cm) were purchased from Thermo Separation Products (Fremont, CA, USA). Samples were injected hydrodynamically by applying a pressure of 25 mbar during 2 s.

2.4. Column preparation

Fused silica capillary columns columns (Thermo Separation Products, Fremont, CA, USA) of approximately 33 cm in length with 375 μm out diameter and 75 μm inner diameter were slurry packed. The length of the packed bed was 23.5 cm (classical configuration) or 8.5 cm (short-end injection mode). The stationary phase (X-Terra RP18® d_p = 3.5 μm from waters or Hypersil ODS d_p = 5 μm from Thermohypersil) was immobilised with two frits, one frit of Nucleosil C_{18} (7 μm 1000 Å) (Macherey-Nagel, Düren, Germany), one frit of the packing material (X-Terra RP18® or Hypersil ODS) because problems of bubble formation and stationary phase drying occurred during analyses when the two frits were made of the packing material.

Prior to the packing procedure with the X-Terra RP18® (or Hypersil ODS) stationary phase, the outlet frit has to be created from the (Nucleosil C_{18}) stationary phase and located at a distance of ca 10 cm of the capillary extremity. In this purpose, the capillary was packed with 1000-7 C_{18} and then the packing was sintered under water circulation by using

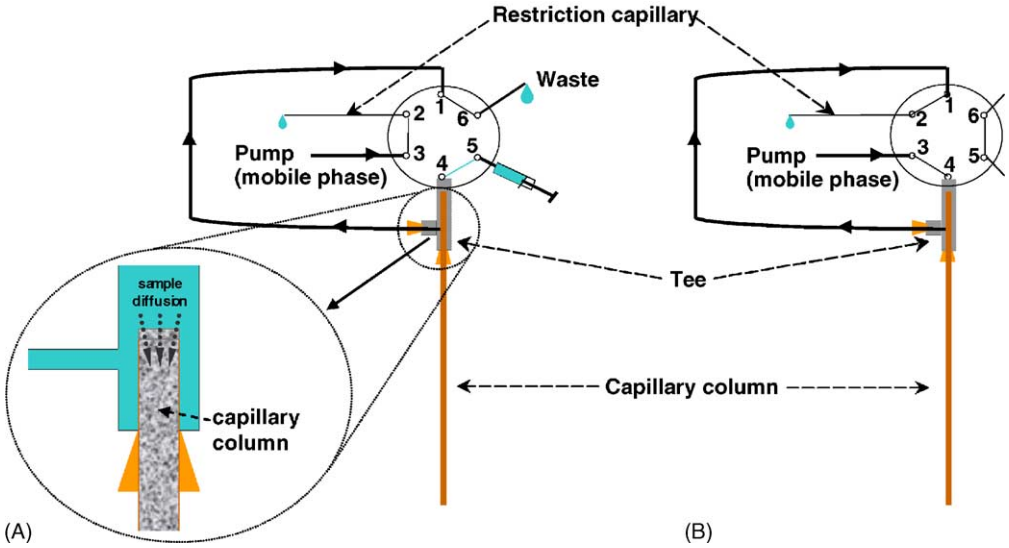

Fig. 1. Experimental set-up for nano-LC experiments.

the frit fusing device and then the excess the Nucleosil C_{18} stationary phase was removed from both sides of the frit as previously described [20]. The X-Terra RP18® (or Hypersil ODS) stationary phase was slurried in acetonitrile at a concentration of 20–30 mg $mL^{-1}$and the suspension placed in an ultrasonic bath for 5 min. The slurry was then poured into the glass lined tube connected to the capillary and to the LC pump. During the packing procedure, the slurry reservoir was placed in the ultrasonic bath to prevent settling out of the stationary phase. The initial flow rate was set at 60 μL min^{-1} until the pressure reached 400 bars. The packing pressure was further maintained at 400 bars until the desired length of capillary had been reached, the system was then allowed to depressurise. Then, the packing material (X-Terra RP18® or Hypersil ODS) was sintered under water circulation. No sodium was added in the fritting solution in order to create the inlet frit.

A detection window was made at 2 mm from the outlet frit (in the open section) by burning the polyimide coating using the same hot filament device. The column was then flushed with the mobile phase with the LC pump.

3. Results and discussion

3.1. Electroosmotic flow on X-Terra RP18® stationary phase

Since the ability to support electroosmosis flow (EOF) is one of the most important properties of a column packing material for CEC, it is of utmost importance to investigate the conditions of generation of EOF and the ways for its controlling and manipulating. In fact, owing to its particular surface structure the X-Terra RP18® material may not possess a high density of residual silanol groups to generate a sustainable EOF allowing to work in the right range of linear velocity. First, experiments were carried out in classical experimental conditions, i.e. with buffered or unbuffered hydro-organic mobile phases and the results compared with those obtained with an Hypersil ODS stationary phase operated in the same experimental conditions. Results are summarized in Table 1.Whatever the mobile phase composition many problems of drying out of the column were encountered with the X-Terra RP18® stationary phase and the column had to be regularly flushed at a high pressure (200 bars) with an LC pump. The electroosmotic mobility determined on the X-Terra RP18® stationary phase in presence of 85% acetonitrile at pH 7.6 (1.3×10^{-4} cm^2 s^{-1} V^{-1}) was in the same order of magnitude of the electroosmotic mobility calculated by Channer et al. [14] on the X-Terra unbonded phase at the same pH ($\mu_{eo} = 10^{-4}$ cm^2 s^{-1} V^{-1} with a 8/2 (v/v) ACN/TRIS 50 mM pH 7.8/water). Whereas the Hypersil ODS material allowed generating a high EOF in a wide range of pH and acetonitrile composition, the EOF generated by the X-Terra RP18® material was at least the half of the EOF generated by the Hypersil ODS packing material. Moreover, the EOF on the X-Terra RP18® material was much more dependant on pH and dropped off considerably in a weak acidic medium indicating the residual silanols remaining at the surface of this material are weak acids. The same phenomenon has already been reported by Smith [13] who studied the behaviour of two other packing materials specially designed for the analysis of basic compounds (Symmetry Shield RP 8 and Develosil ODS UG-5), although the EOF generated by these two materials in similar experimental conditions were significantly higher ($\mu_{eo} \cong 2 \times 10^{-4}$ cm^2 s^{-1} V^{-1}), even in buffered aqueous organic mobile phase. On the X-Terra RP18® material, the highest electroosmotic mobility ($\mu_{eo} = 1.5 \times 10^{-4}$ cm^2 s^{-1} V^{-1}, i.e. $v_{eo} = 0.14$ cm s^{-1} at $V =$ 30 kV for $L = 32$ cm), was obtained with unbuffered mobile phases, i.e. with the lower ionic strength (higher double layer thickness) and with the higher organic solvent content, i.e. the

Table 1
Electroosmotic mobilities measured in various mobile phase conditions for two different packing materials

μ_{eo} ($\times 10^4$ cm^2 s^{-1} V^{-1})	Percentage acetonitrile	Citrate buffer pH 3.5	TRIS buffer pH 7.5	Unbuffered mobile phase
X-Terra RP18®	85	$<0.3 \times 10^{-4}$	$1.30 \pm 0.11 \times 10^{-4}$	$1.55 \pm 0.06 \times 10^{-4}$
	30	$<0.3 \times 10^{-4}$	$0.90 \pm 0.05 \times 10^{-4}$	$1.10 \pm 0.05 \times 10^{-4}$
Hypersil ODS	85	$1.60 \pm 0.05 \times 10^{-4}$*	$2.80 \pm 0.05 \times 10^{-4}$	$3.30 \pm 0.28 \times 10^{-4}$
	30	$1.50 \pm 0.10 \times 10^{-4}$*	$2.35 \pm 0.05 \times 10^{-4}$	$2.7 \pm 0.50 \times 10^{-4}$**

$n = 2$ excepted for (**) $n = 4$. The electroosmotic mobility value is the mean of the n values obtained for the n capillaries tested, each value being the mean of at least three EOF measurements on the same capillary. The total ionic strength was kept constant for all experiments (5 mM) excepted for (*) total buffer concentration: 2 mM. Capillary columns: total length, 32 cm; packed length, 23.5 cm.

lower viscosity. However, since unbuffered mobile phases do not allow the control of the ionisation degree of basic solutes and consequently their retention in chromatography, their use is usually prohibited.

3.1.1. *Influence of the addition of an ionic surfactant*

In order to increase the EOF on the X-Terra RP18® stationary phase and to be able to reach the optimal mobile phase linear velocity (about 0.14 cm/s with 3.5 μm silica particles), the addition of an anionic surfactant to the mobile phase was studied. Such an addition of an ionic surfactant to the mobile phase has already been reported in the literature with two different purposes [21–24]. First, the addition of SDS allows stabilizing the current and the electroosmotic flow by improving the wettability of the stationary phases (by decreasing the surface tension at the solid–liquid interface) especially for non-porous particles [21]. Secondly, such an addition, at a concentration below the critical micelle concentration, may allow to increase the EOF by increasing the charge density of the packing material, the variation of the EOF being closely related to the organic content of the mobile phase [22,24] and to the nature of the stationary phase (porous or non-porous).

First and as expected, the addition of SDS allowed stabilizing the separation current and the EOF and the current failures consecutive to the drying out of the packing material were no longer observed. Furthermore, increasing the sodium dodecylsulfate concentration in the mobile phase from 0 to 10 mM led to a progressive increase of the cathodic EOF (+33%, +89% and +144% at 2 mM SDS, 5 mM SDS and 10 mM SDS, respectively) consecutive to the adsorption of the anionic surfactant onto the surface of the packing material and allowed to reach an electroosmotic mobility of 2.2×10^{-4} cm^2 s^{-1} V^{-1}, i.e. to reach a linear velocity of 0.17 cm/s at 25 kV. The same values of EOF were reached when SDS was replaced by dodecyltrimethyl ammonium bromide (DTAB), a cationic surfactant with the same alkyl chain length, but the polarity had to be reversed owing to the positive charge of the modified surface (EOF directed towards the anode).

As illustrated in Fig. 2, an increase of the acetonitrile proportion led to an increase of the EOF when no SDS was added to the mobile phase whereas it led to its decrease when SDS was present. In absence of SDS, the increase of the EOF is due to the predominant effect of the increase of the (ε/η) ratio, i.e dielectric constant/viscosity ratio, whereas in presence of SDS, the decrease of the zeta potential consecutive to the decrease of the adsorption of the surfactant with the increased acetonitrile ratio is responsible for the EOF diminution. Increasing the surfactant concentration or its alkyl chain length counterbalanced this decrease of the EOF with the acetonitrile ratio: with 70% acetonitrile in the mobile phase, an electroosmotic mobility of 1.7×10^{-4} cm^2 s^{-1} V^{-1} was reached by increasing simultaneously the alkyl chain length of the surfactant from C_{12} to C_{16} and its concentration from 10 to 15 mM.

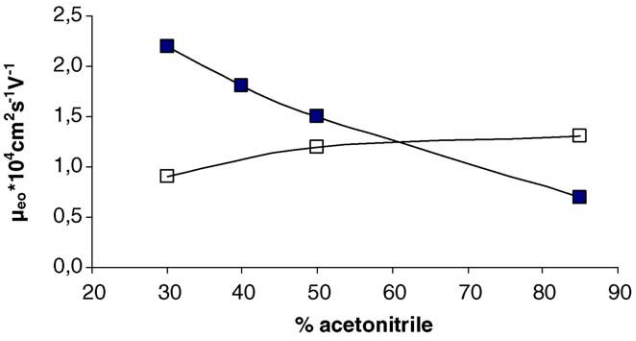

Fig. 2. Effect of acetonitrile proportion on the electroosmotic mobility. Column: L = 32 cm, l = 8.5 cm and 75 μm in i.d. (short-end injection mode), stationary phase: X-Terra RP18® (d_p = 3.5 μm), mobile phase: (acetonitrile/water, x/100-x, v/v) injection 2.5 kV, 2 s. (■): with SDS 10 mM; (□): without SDS.

Moreover, and as can be seen in Table 2, the EOF became quite independent of the pH of the mobile phase when a surfactant was added to the mobile phase, indicating that the residual silanol groups that supported the EOF in absence of SDS, no longer participate to the EOF in such conditions and that the EOF is only supported by the surfactant adsorbed onto the packing material. It was also verified (by conductivity measurements) that no micelle formation occurred in

Table 2
Electroosmotic mobilities measured at different pH values

	μ_{eo} ($\times 10^4$ cm^2 s^{-1} V^{-1})
Citrate buffer pH 3.5 (n = 2)	$2.00 \pm 0.13 \times 10^{-4}$
TRIS buffer pH 7.5 (n = 2)	$2.20 \pm 0.04 \times 10^{-4}$
Unbuffered mobile phase (n = 2)	$2.20 \pm 0.14 \times 10^{-4}$

Capillary column: total length, 32 cm; packed length, 23.5 cm. Stationary phase: X-Terra RP18®, d_p = 3.5 μm. Each electroosmotic mobility value is the mean of the n values obtained for the n capillaries tested, each value being the mean of at least three EOF measurements on the same capillary. Mobile phase: acetonitrile/water/buffer (100 mM, 30/68/2, v/v/v), SDS = 10 mM.

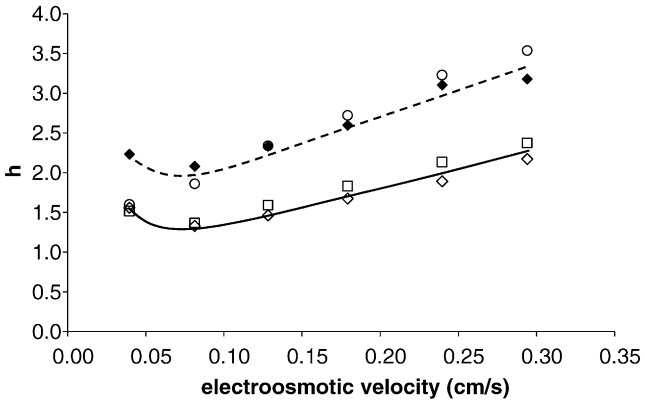

Fig. 3. Effect of the mobile phase linear velocity on the reduced plate height. Column: L = 32 cm, l = 8.5 cm and 75 μm in i.d. (short-end injection mode), stationary phase: X-Terra RP18® (d_p = 3.5 μm), mobile phase: acetonitrile/water (30/70, v/v), SDS = 10 mM, injection 2.5 kV, 2 s. (♦): thiourea (k = 0); (◯): TAP (k = 0.4); (□): FLF (k = 1.4); (◇): CAP (k = 2).

the experimental conditions used throughout this study, i.e. with surfactant concentration lower than 15 mM and with acetonitrile percentage ranging from 30% to 70%. In conclusion, a satisfactory EOF can be obtained on the X-Terra RP18® stationary phase, in the whole range of organic modifier composition and pH, by adjusting the alkyl chain length of the surfactant and its concentration. The direction of the EOF depends on the charge, cationic or anionic one, of the surfactant. Moreover, one may notice that there was problem associated with EOF reproducibility (Table 2) as long as a sufficient equilibration time allowed to reach the surfactant adsorption/desorption equilibrium when the organic modifier content of the mobile phase was modified.

3.1.2. Evaluation of the performances of X-Terra RP18® home-made columns

In order to evaluate the performances of the home-made columns packed with the X-Terra RP18® packing material, their efficiencies were determined for thiourea (unretained solute) and for three neutral retained solutes (Thiamphenicol (TAP), Chloramphenicol (CAP) and Florfenicol (FLF)) in presence of an hydro-organic mobile phase (acetonitrile/water, 30/70), SDS 10 mM at different mobile phase velocities. The applied voltage was varied from 5 to 30 kV on columns of 8.5 cm effective length and 32 cm total length (in the short-end injection mode) and on columns of 23.5 cm effective length and 32 cm total length. The effect of mobile phase velocity on the separation efficiency was studied by evaluation of the h–u curve and the corresponding results represented in Fig. 3 for the short column (8.5 effective length). Theoretical reduced plate heights ($h = H/d_p$) values as low as 1.3 (i.e. around 220 000 plates m^{-1}) can be reached for retained solutes ($k > 1$) at a mobile phase velocity of ca. 0.08 cm s^{-1}. The higher reduced plate heights determined for the EOF marker and the less retained solute were due to extra-broadenings effect (presence of two frits on a short column), the influence of which are more important for species having low retention factors and for short length columns: for the longer columns (packed length 23.5 cm) the efficiencies were the same (around 220 000 plates m^{-1}) for the unretained and retained solutes. As can be seen Fig. 3, reduced plate heights remained quite low (less than 2.2) when higher linear velocities (up to 0.3 cm s^{-1}) were used. The principal advantage of this is the possibility of realizing fast separations without a great loss in efficiency.

Table 3
Retention factors calculated for the amphenicol compounds in CEC and nano-LC on the same X-Terra RP18® capillary column

	k (TAP)	k (FLF)	k (CAP)
CEC	0.46 ± 0.02 (n = 3)	1.41 ± 0.04 (n = 3)	1.85 ± 0.06 (n = 3)
Nano-LC	0.46 ± 0.01 (n = 3)	1.47 ± 0.02 (n = 3)	1.97 ± 0.02 (n = 3)

Capillary column: total length, 32 cm; packed length, 23.5 cm. Mobile phase: acetonitrile/water (70/30, v/v), SDS 10 mM.

3.2. Applications

3.2.1. Separation of amphenicol antibiotics

The first class of compounds studied in CEC with the X-Terra RP18® stationary phase was the amphenicol antibiotics (FLF, CAP, TAP) that are neutral compounds whatever the pH of the mobile phase and that do not present any basic moiety (Fig. 4). As illustrated in Fig. 5a, they were separated in less than 2 min (v_{eo} = 0.237 cm s^{-1}) in presence of an hydro-organic mobile phase containing a 10 mM SDS concentration, in the short end injection mode (l = 8.5 cm). As reported in Table 3, the retention factors calculated in CEC were quite similar to those measured in nano-LC on the same capillary column and with the same mobile phase composition, indicating that the partitioning of these neutral compounds on the reversed-phase is not significantly affected by the elution mode (pressure- or electro-driven mode) as sometimes observed in the literature for other compounds [25–27].

In order to study the influence of SDS on retention, several complementary experiments were conducted. First, the influence of the addition of SDS on retention factors was studied in LC: the obtained results (Table 4) indicate that

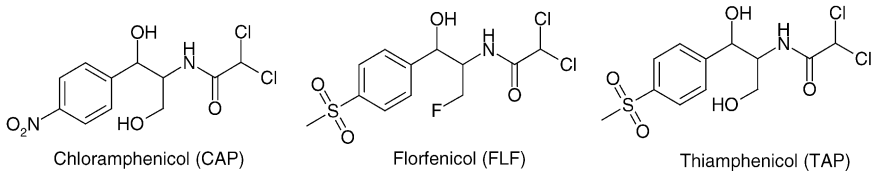

Fig. 4. Structures of the amphenicol antibiotics.

Table 4
Influence of the addition of SDS on the retention factors of amphenicols in LC (experiments without SDS were implemented before experiments with SDS)

	k (TAP)	*k* (FLF)	*k* (CAP)
Without SDS	0.61 ± 0.01 (*n* = 3)	2.01 ± 0.01 (*n* = 3)	2.33 ± 0.01 (*n* = 3)
SDS (10 mM)	0.49 ± 0.01 (*n* = 3)	1.57 ± 0.01 (*n* = 3)	2.13 ± 0.01 (*n* = 3)

Column: X-Terra RP18®, *L* = 10 cm, d_p = 3.5 μm, 4.6 i.d., flow rate 1 mL/min. Mobile phase: acetonitrile/water (70/30, v/v) with or without SDS.

the presence of SDS (at 10 mM) in the mobile phase only slightly reduces the retention factors of the amphenicol antibiotics, the reduction being less pronounced for the more retained compound (−9% for CAP) than for the two other ones (−20% for CAP and TAP). The influence of the mobile phase composition (organic modifier content) on retention was also studied with SDS added to the mobile phase and the logarithms of retention factors of the three neutral amphenicols are plotted against the acetonitrile percentage (Fig. 6, bold lines). As expected in the reversed-phase mode, there is a decrease of the logarithm of the retention factors when the acetonitrile percentage increases. Furthermore, the retention and selectivity on the X-Terra stationary phase were compared to those obtained with a more traditional phase, the Hypersil ODS stationary phase, in presence of SDS and in the same experimental conditions (Fig. 6, dashed lines). The results indicate that higher retention factors are measured with the X-Terra RP18® material (excepted at a high acetonitrile percentage) but that the selectivities are quite similar with both materials). The corresponding chromatograms are represented Fig. 5a and b. At last, it was verified that the CEC separation of amphenicol antibiotics was not due to interactions occurring in solution with the SDS monomers or with SDS aggregates since the amphenicol antibiotics did not possess any apparent electrophoretic mobility in CZE in presence of the charged surfactant added to the mobile phase.The efficiencies attainable in the CEC mode with the X-Terra stationary phase (about 170 000 plates/m at a linear velocity of 0.19 cm s^{-1}) were significantly higher than those obtained with the same column in the nano-LC mode (60 000 plates/m at 0.17 cm s^{-1}) confirming that CEC allows to reach higher efficiencies than LC.

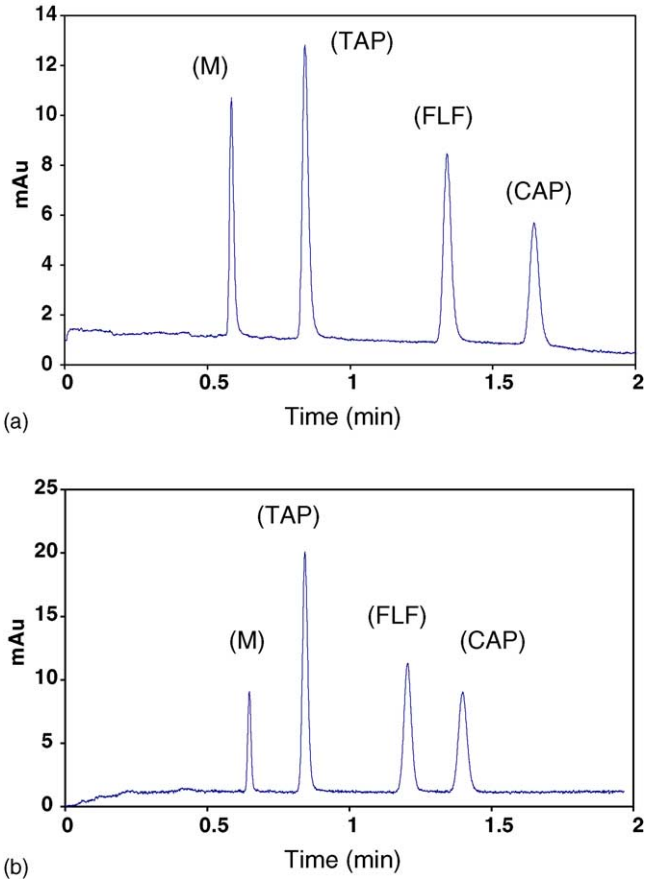

Fig. 5. Separation of amphenicol antibiotics. Mobile phase: acetonitrile/water (30/70), SDS 10 mM. Column: *L* = 32 cm, *l* = 8.5 cm and 75 μm in i.d. (short-end injection mode), *V* = 25 kV, injection 2.5 kV, 2 s. M: EOF marker; TAP: Thiamphenicol; FLF: Florfenicol; CAP: Chloramphenicol. (a) X-Terra RP18® (d_p = 3.5 μm) and (b) Hypersil ODS (d_p = 3 μm).

3.2.2. Separation of macrolide antibiotics

In order to explore the ability of such a packing material to separate basic compounds under their neutral form (by suppressing their ionization in a basic medium) a few macrolide antibiotics (Fig. 7) were chosen for further work. For such compounds, the ionization of which may be controlled by the mobile phase pH, it was necessary to select a buffer that possesses an appropriate buffering capacity at the desired pH, that does not absorb in the desired wavelength range (210–300 nm) and that does not possess a high conductivity in order to avoid Joule effect. The phosphate buffer at a high

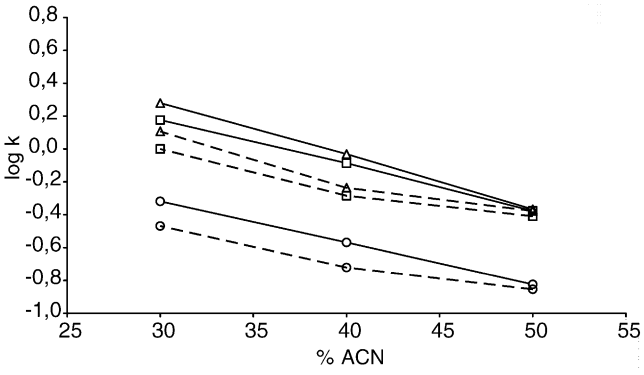

Fig. 6. Influence of the mobile phase composition on the retention. Column: *L* = 32 cm, *l* = 8.5 cm and 75 μm in i.d. (short-end injection mode). Mobile phase: acetonitrile/water (x/100-x, v/v), SDS = 10 mM, injection 2.5 kV, 2 s. (◯): TAP; (□): FLF; (△): CAP. Bold lines (—): stationary phase: X-Terra RP18® (d_p = 3.5 μm); dashed lines (- - - -): stationary phase: Hypersil ODS (d_p = 5 μm).

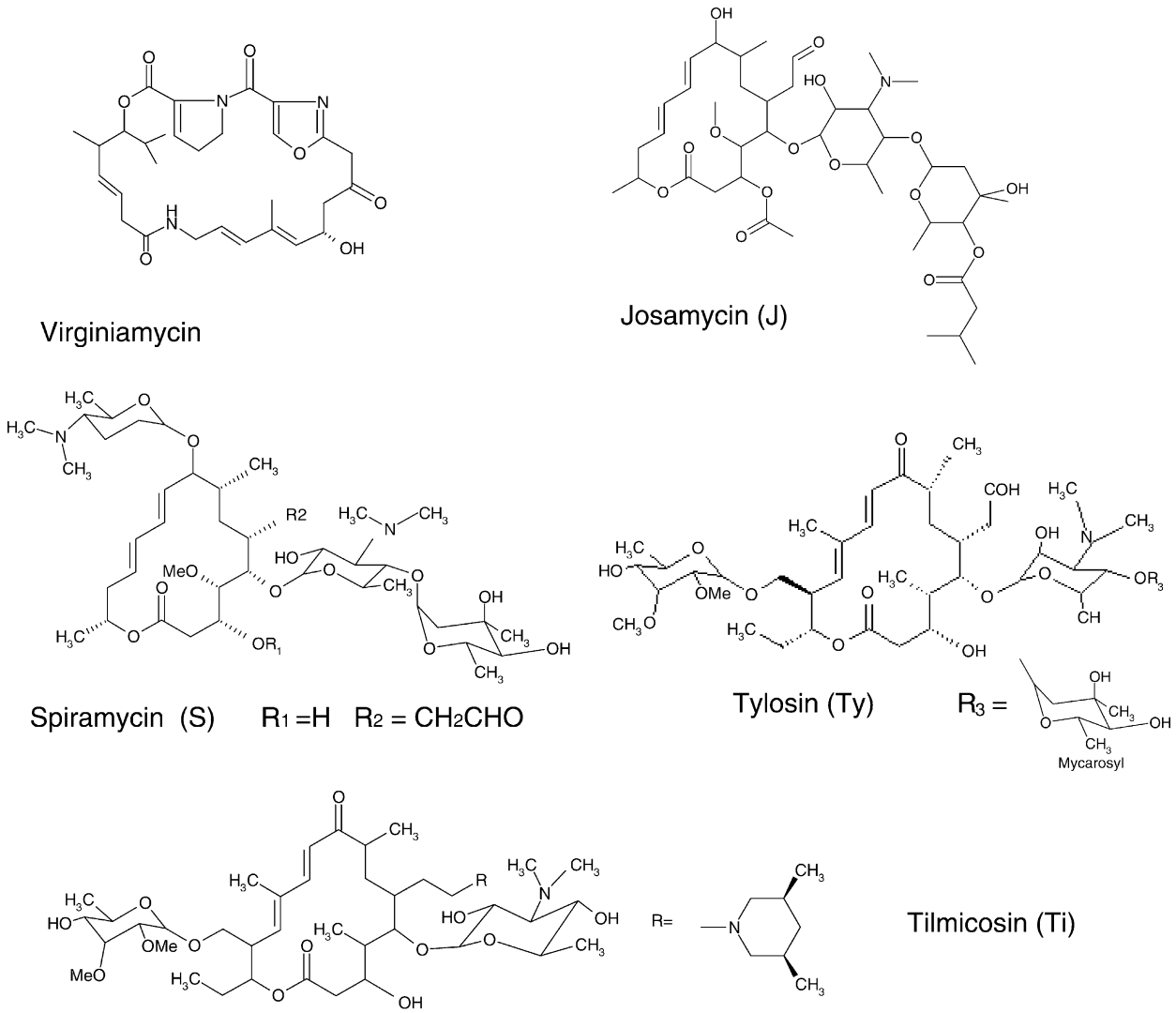

Fig. 7. Structures of the macrolide antibiotics.

pH was excluded following the manufacturer's recommendation, borate buffer (pH 9.3) led to severe peak distortion in LC on the X-Terra RP18® stationary phase (probably due to the complexation of the glycosidic functions of the macrolides with the borate ions), the biological buffers such as CAPS and CAPSO absorbed in the low UV range so that the ammonia buffer was the only buffer, among those tested, that could be used for this analysis. As illustrated in Fig. 8, all the compounds of interest were separated at pH 10, a pH value at which it has been verified in CZE (through the measurement of the electrophoretic mobilities) that the compounds of interest did not possess any charge. In such conditions, five macrolides were separated, with symmetrical peak shapes. As verified in LC, these compounds could not at all be separated with the more classical C_{18} Hypersil stationary phase (with or without SDS) owing to their great interactions with the residual silanol groups that have a disastrous effect on peak symmetry and efficiency. For these compounds, the efficiencies calculated in CEC on the X-Terra stationary phase, depended on the nature of the solute (from 16 000 plates/m for Virginiamycin to 96 000 plates/m for Josiamycin and Tilmicosin) and were low when compared to those measured for the non-basic solutes (amphenicol antibiotics). The lower diffusion coefficient and the higher molecular weight of the macrolides may account for such differences between the simple molecules and the macrolides.

3.2.3. Separation of basic test solutes

Clozapine, diphenhydramine, protriptyline and imipramine, usually used as basic test solutes in LC were also studied in CEC with the X-Terra RP18® material. Owing to the pK_a decrease of organic bases in hydro-organic solutions [28] all these solutes were neutral in the experimental conditions used throughout this study (pH 10, percentage acetonitrile >30%) except for protriptylin that remained cationic even at this high pH value as verified in CZE ($\mu_{ep} \cong 1.6 \times 10^{-4}$ cm^2 s^{-1} V^{-1}) in (acetonitrile/ammonia buffer 10 mM pH 10, 30/70, v/v). When injecting these solutes in CEC just

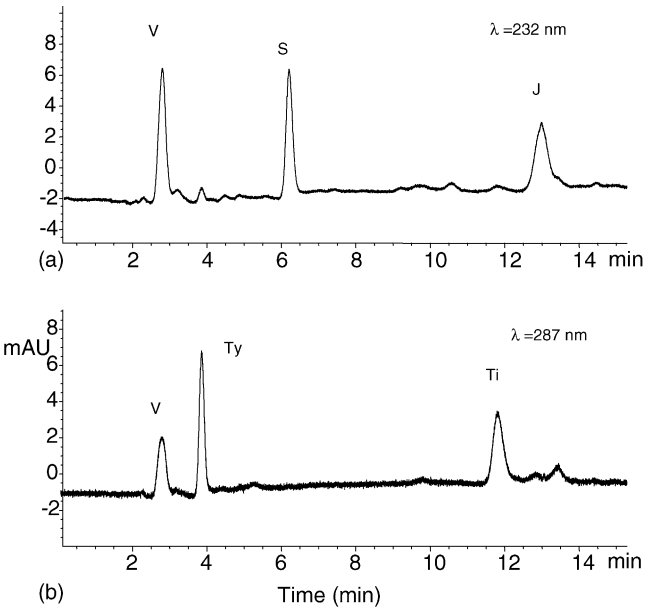

Fig. 8. Separation of macrolide antibiotics in CEC with the X-Terra stationary phase. Column: L = 32 cm, l = 8.5 cm and 75 μm in i.d. (short-end injection mode). Stationary phase: X-Terra RP18® (d_p = 3.5 μm), V = 15 kV, injection 2.5 kV, 2 s. Mobile phase: acetonitrile/ammonia buffer (45/55, 10 mM, pH 10), SDS 10 mM. V: Virginiamycin; Ty: Tylosin; S: Spiramycin; Ti: Tilmicosin and J: Josamycin.

after the capillary has been hydrodynamically preconditioned with the mobile phase (ΔP = 200 bars) (Fig. 9a) the retention factors calculated for the compounds were in good agreement with those determined in LC in the same experimental conditions. Only protriptylin that remained cationic at this pH exhibited an apparent retention factor ($k_{app} = (t_r/t_{eo} - 1)$ = 3.9) lower than the retention factor determined in LC (k = 6.61) in the same experimental conditions, indicating that the electromigration of this compound (directed towards the detection extremity) significantly decreased its migration time. It was also verified in liquid chromatography that, if the addition of SDS only slightly decreased the retention factors of the neutral species, it significantly increased the retention of the cationic ones (such as protriptylin) owing to electrostatic interactions (attractive ones) with the anionic surfactant adsorbed onto the stationary phase: $k_{protriptylin}$ increased from 4.16 to 6.61 when a 10 mM SDS concentration was added to the mobile phase. Efficiencies ranging from 125 000 to 145 000 plates/m were reached for these basic compounds at a linear velocity of about 0.1 cm s^{-1} and no peak tailing was observed. These efficiencies were lower than those obtained for non-basic compounds (amphenicol antibiotics) but at least twice higher than efficiencies obtained in conventional LC in the same mobile phase conditions with an X-Terra RP18® commercial column.

However, and as illustrated in Fig. 9a–e, when successive injections of the test solutes were implemented, dramatic variations in retention and selectivities were observed whereas the EOF velocity remained quite constant. First it was verified that no degradation of the solutes occurred and

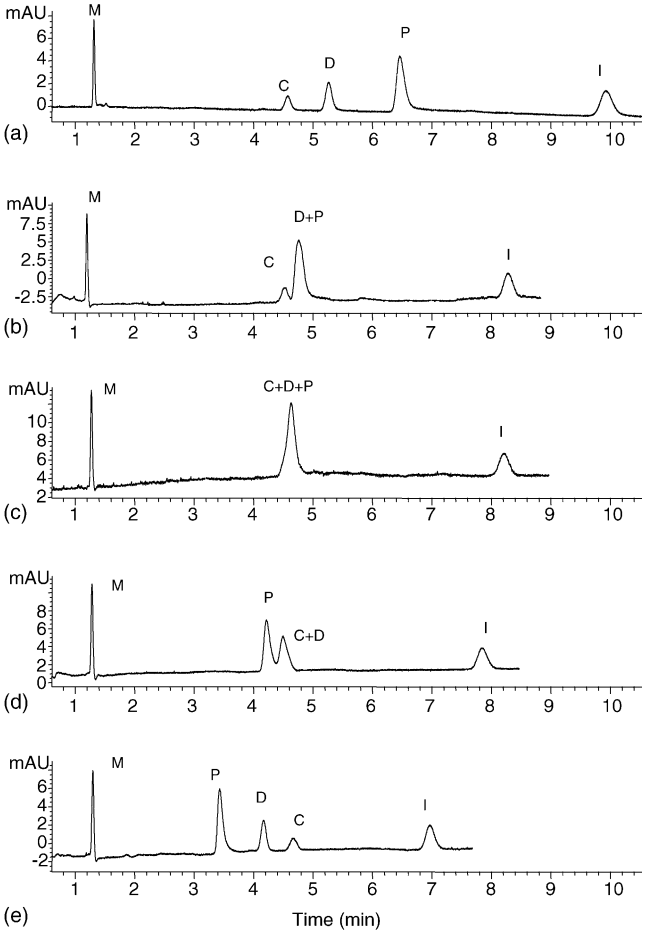

Fig. 9. Separation of four basic test solutes in CEC with the X-Terra packing material. Column: L = 32 cm, l = 8.5 cm and 75 μm in i.d. (short-end injection mode). Stationary phase: X-Terra RP18® (d_p = 3.5 μm) V = 15 kV, injection 2.5 kV, 2 s. Mobile phase: acetonitrile/ammonia buffer (50/50, 10 mM, pH 10), SDS 10 mM. M: EOF marker; C: clozapin; D: diphenhydramin; P: protriptylin; I: imipramin; 10a: first injection after an hydrodynamic conditioning; 10b–e: successive injections without any renewal of the mobile phase.

then it was demonstrated that a progressive degradation of the mobile phase was responsible for these variations: when the column was flushed (under a pressure of about 200 bars) with fresh mobile phase, the first injection after the column conditioning led to the expected retention factors and efficiencies. The evaporation of the organic solvent was not responsible of the observed effect since the retention factors of clozapin (pK_a = 7.5) and of another neutral retained compound (naphthalene) were not affected whereas the retention factors of protriptylin and diphenhydramin dramatically decreased. It was attributed to the electrolysis of the mobile phase at the anodic injection, leading to the oxidation of the hydroxide ions OH^- and to a decrease of the mobile phase pH in the inlet vial, according to the following equation:

$$2OH^- \rightarrow H_2O + \tfrac{1}{2}O_2 + 2e^-$$

This assumption was verified in CZE where protriptylin, the solute being the more affected by the mobile phase

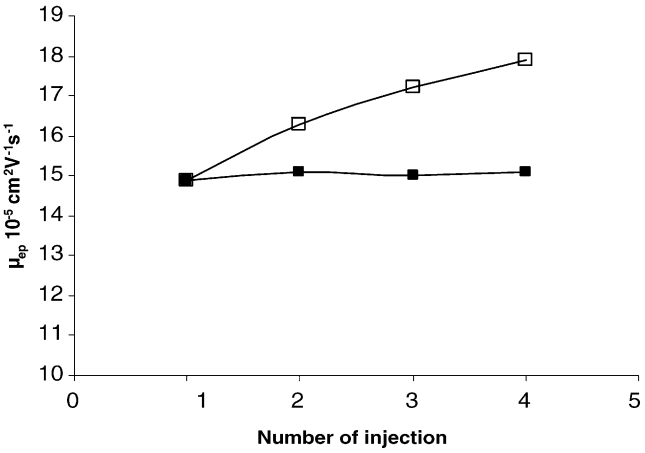

Fig. 10. Effect of the mobile phase electrolysis on the electrophoretic mobility of protriptylin. Fused silica capillary: L = 32 cm, l = 23.5 cm, 370 μm o.d., 50 μm i.d. Mobile phase: acetonitrile/ammonia buffer (50/50, 10 mM, pH 10), SDS 10 mM. V = 25 kV, hydrodynamic injection: 25 mbar, 2 s. (■) With mobile phase renewal between each injection (i = 14 μA); (□) without any renewal of the mobile phase between successive injections (running time for each injection: 10 min).

degradation, was injected several times without changing the mobile phase buffer reservoir and the mobile phase content of the capillary between the successive runs. At it can be seen Fig. 10, the electrophoretic mobility (i.e. ionization degree) of protriptylin progressively increased during the successive runs, whereas it remained constant when the mobile phase buffer inlet reservoir and capillary content were hydrodynamically renewed between each run. Concomitantly a slight increase of the separation current was observed (from 14 to 15.2 μA) when the mobile phase was not renewed between each analysis thus confirming its partial electrolysis. The modification of the dissociation degree consecutive to the pH decrease, affected the solutes having a pK_a value close to the working pH thus modifying the relative contributions of the different mechanisms responsible for the separation of the solutes, i.e. the electromigration of the protonated compounds towards the cathode, the hydrophobic interactions with the reversed phase and the electrostatic interactions of the cationic solutes with the anionic surfactant adsorbed onto the stationary phase. The net effect (on the retention time of the solute) depends on the relative contributions of these three phenomena. Despite many attempts to avoid the disastrous effect of the electrolysis on the repeatability of the analysis (such as an increase the buffer ionic strength from 10 to 25 mM or the replacement of the ammonia buffer by a CAPS or a CAPSO buffer (at the same pH and concentration) no significant improvement was obtained. Although this phenomenon of mobile phase electrolysis has already been mentioned in the literature (especially in capillary zone electrophoresis [29]) it was demonstrated that it can be limited in CZE by changing the buffer reservoir content between each analysis, by hydrodynamically renewing the buffer capillary content prior each analysis and by adjusting the position of the capillary extremity. In CEC, although the extremity of the capillary column was right positioned (at least 4 mm below the electrode extremity) in order to prevent zones of changed pH generated from hydrolysis from entering the capillary it was not possible to suppress the disastrous effect of electrolysis on repeatability.

4. Conclusion

In this study, we demonstrate that the X-Terra RP18® material specially designed for the analysis of basic compounds in liquid chromatography may be used in CEC and in large pH range. Although the X-Terra RP18® material does not allow obtaining a sustainable EOF with classical hydroorganic mobile phases, it is possible to increase the EOF by adding a charged surfactant (anionic or cationic one) in the mobile phase that adsorbs onto the surface material increasing its charge density. The charge of the surfactant (cationic or anionic), its concentration, its alkyl chain length and the percentage of the organic modifier in the mobile phase allow controlling the EOF in direction (cathodic or anodic EOF) and in intensity. Moreover, it is demonstrated that in presence of a surfactant the EOF becomes independent of the pH of the mobile phase. It was also verified that whereas the addition of a surfactant does not modify significantly the retention of neutral solutes it increases the retention of species having an opposite charge owing the electrostatic interactions with the adsorbed surfactant. High efficiencies and symmetric peak shapes are attainable with basic solutes even at high linear velocities. However, it was demonstrated that the hydrolysis of buffered hydro-organic mobile phases (50% of aqueous fraction at a 10 or a 25 mM concentration) leads to a lack of repeatability for solutes having a pK_a close to the pH of the mobile phase, owing to the variations of the dissociation degree of the solutes that impair both electromigration and retention phenomena.

Acknowledgments

The authors gratefully acknowledge Dr. Uwe Neue from Waters Corporation (Milford, MA, USA) for providing the X-Terra RP18® stationary phase.

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