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Ion-interaction high-performance liquid chromatography and micellar electrokinetic capillary chromatography: two complementary techniques for the separation of aromatic sulfonated compounds

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Abstract

Aromatic sulfonates and their amino and/or hydroxyl derivatives are widespread environmental pollutants. Since they are commonly present as complex mixtures, with the presence of many isomers, their separation is still a critical analytical step. Due to the lack of a standard reference method, recommendations are made to always use two independent methods in order to compare and confirm the results. Due to the ionic nature of these compounds ion-interaction HPLC and micellar electrokinetic capillary chromatography were chosen to solve this separative problem. With the use of both techniques the separation of 22 aromatic sulfonates was optimized, and the effect of several experimental parameters evaluated. The results obtained were discussed in terms of resolution, sensitivity and precision of the methods. An industrial effluent was analyzed employing the proposed procedures.

Keywords: Naphthalenesulfonic acids; Sulfonic acids; Aromatic compounds; Surfactants

1. Introduction

Aromatic sulfonates (ASs) are produced on a large scale in the chemical industry since the end of the 19th century. In particular aromatic sulfonates are widely used in detergent products as fluorescence whitening agents (FWs) and in pharmaceutical industry, amino- and hydroxynaphthalenesulfonates (ANSs; HNSs) are employed as intermediates in dye production, alkylated naphthalene sulfonates are used as suspending and wetting agents, dispersants and stabilizers.

These substances are surface-active and can be modified by microorganisms. Some green alga employ naphthalenesulfonic acids as sulfur source, even in the presence of sulfate ion, leaving the naphthalene ring undegraded [1]. Other bacteria produce 5-sulfosalicylic and gentisic acid as intermediates in the biodegradation of 1,6- and 2,6-naphthalenedisulfonic acid [2].

The limited attention previously paid towards aromatic sulfonates other than linear alkylbenzene sulfonates (LASs) within aquatic environment might be due to their apparently low ecotoxicological potential: the aquatic toxicity of aromatic sulfonates appears to be small, and the risk of bioaccumulation is limited since the octanol–water partition coefficients (pK_{ow} values) typically range below 2. However, low K_{ow} values are conversely indicative of a high mobility within the aquatic system. Contrary to

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LASs, polysulfonated aromates and substituted monosulfonated aromates are reported to be persistent and exhibit only limited biodegradability. Solubility enhancement of hydrophobic xenobiotics deposited within soils and sediments is a side-effect of surfactant release into aquatic systems that is also under discussion.

Many analytical methods have been proposed for aromatic sulfonic acids separation and determination. GC or GC–MS analysis of some ASs is possible after conversion to volatile derivatives by reaction with thionyl chloride and further amidation with an aliphatic amine [3] or with phosphorus pentachloride and esterification with trifluoroethanol [4]. Problems occur in the derivatization of aromatic sulfonic acids also containing amino and hydroxy groups that can react with the derivatizers.

Liquid chromatography is certainly the more adapt and used technique. Sulfonic acids are strong acids, completely dissociated in aqueous or aqueous–organic solutions over a broad pH range, so that diluted acidic buffers cannot be used to suppress their ionization and improve their chromatographic properties. In reversed-phase system with pure aqueous–organic eluents the acids are eluted close to the column dead volume with little separation. Aromatic amino–sulfonic acids are an exception to this rule and their acid–base properties are similar to those of carboxylic acids. Consequently, separation of these acids in buffered aqueous–organic mobile phases is in principle possible [5]. This is not the case with other aromatic sulfonic acids, which can be separated increasing their retention by addition of ionic compounds to the mobile phase.

The separation of a limited number of isomers was achieved by different techniques: TLC [6], ion-exchange electrokinetic chromatography [7], RP-HPLC with buffer elution [8] and anion-exchange chromatography [9]. This last technique has also been coupled with particle-beam MS for the determination of eight sulfonates, this method being more suitable for mono- than for disulfonic compounds, and sensitivity generally being worse than when using spectrophotometric detectors [10].

The separation of sulfonic acids can also be achieved by reversed-phase HPLC with mobile phases containing strong electrolytes (salts) at concentrations of 0.1–1 mol/l. In the presence of salts, ionic interactions in the stationary phase are suppressed, retention is increased and the separation selectivity is enhanced [11,12]. The retention can be controlled by adjusting the concentrations of either the salt or the organic solvent in mixed mobile phases. This technique makes it possible to separate not only 1- and 2-naphthalenesulfonic acids [13,14], but also more than 10 isomeric naphthalene mono- to tetrasulfonic acids [15], which are eluted in the order of decreasing number of sulfonic groups. Substituted amino- and hydroxynaphthalenesulfonic acids [12,16] or isomers of 4,4′-diaminostilbene-2,2′-disulfonic acid [17] can also be separated. Aqueous–organic mobile phases have been used to separate sulfonic acid derivatives of phenol on a β-cyclodextrin bonded phase [18].

The most employed HPLC technique for the separation of polar ASs is the ion-pair [19,20] with UV [21–23], diode-array [24,25] or fluorescence detection [10]. The retention and the separation selectivity depend on the nature of the ion-pairing reagent, on the number of sulfonic groups, on the character of the aromatic ring and on the nature, number and position of the substituents.

The retention can be controlled by adjusting experimental parameters such as the concentration of both the ion-pairing reagent and the organic solvent in the mobile phase [19,26,27]. This technique has been successfully used to separate some unsubstituted naphthalene- and anthraquinonesulfonic acids [19,28] and their hydroxy and amino derivatives [25,29,30]. However, it failed to resolve complex mixtures containing various isomers of naphthalenesulfonic acids with different numbers of sulfonic groups [19].

Ion-pair chromatography is often coupled with off- or on-line [31,32] solid-phase ion-pair extraction on C_8 and C_{18} cartridges with CTMABr [25] or TBA [31,32] as ion-pair reagent, on PLRP-S sorbent [33,34]. Sample clean-up (solid-phase extraction, SPE) [24], to remove interferents both of natural or industrial origin, has also been performed on graphitized carbon black [35].

Although capillary zone electrophoresis (CZE) seems ideally suited for the analysis of aromatic sulfonic acids, little work in this field has been published so far. In addition to electrophoretic separations of acidic azo dyes in industrial products

[36–38] and, coupled with MS, in the environment [39], CZE has been applied to the separation of several substituted benzenesulfonic [40] and anthraquinonesulfonic [28] acids and isomeric 4,4′-diaminostilbene-2,2′-disulfonic acids [17]. CE has been used to separate 21 aromatic sulfonates in two runs and some factors affecting the migration have been studied [41]. Some acidic azo dyes and aromatic sulfonic acids could be separated by micellar electrokinetic capillary chromatography (MEEC) in borate buffers containing cholic acid [42]. More recently a comparison between CZE and MECC [43], shows the larger possibility in terms of selectivity of MECC in the separation of 11 aromatic sulfonates. A few earlier results [28,37,44] indicate that HPLC and capillary electrophoresis complement each other with respect to the resolution of sulfonic acid dyes and dye-intermediates.

An exhaustive review including references of the works done up to 1995 has been written by Reemtsma [45].

In the present work are presented: (i) a more exhaustive investigation of the ion-pair (here named ion-interaction) chromatographic system for the separation of polar ASs, with the effect of the different experimental factors involved in the retention simultaneously studied by chemometric treatment of experimental design; (ii) the optimization of an ion-interaction HPLC method for the analysis of ASs; (iii) the study of a separation of ASs under MECC conditions and the effect of an alkylamine as additive; and (iv) a comparison of the two methods and their application to the analysis of an industrial effluent.

2. Experimental

2.1. Chemicals and reagents

The 22 aromatic sulfonates considered: 1-naphthalenesulfonic acid (1-NS), 2-naphthalenesulfonic acid (2-NS), 2-amino-1-naphthalenesulfonic acid (2-A-1-NS), 5-amino-2-naphthalenesulfonic acid (5-A-2-NS), 8-amino-2-naphthalenesulfonic acid (8-A-2-NS), 1-amino-5-naphthalenesulfonic acid (1-A-5-NS), 4-amino-1-naphthalenesulfonic acid (4-A-1-NS), 6-hydroxy-2-naphthalensulfonic acid (6-H-2-NS), 4-hydroxy-1-naphthalenesulfonic acid (4-H-1-NS), 4-amino-3-hydroxy-1-naphthalenesulfonic acid (4-A-3-H-1-NS), 6-amino-4-hydroxy-2-naphthalenesulfonic acid (6-A-4-H-2-NS), 6-amino-1-hydroxy-3-naphthalenesulfonic acid (6-A-1-H-3-NS), 3-amino-2,7-naphthalenedisulfonic acid (3-A-2,7-NdS), 7-amino-1,3-naphthalenedisulfonic acid (7-A1,3-NdS), 2-amino-1,5-naphthalenedisulfonic acid (2-A-1,5-NdS), 1-hydroxy-3,6-naphthalenedisulfonic acid (1-H-3,6-NdS), 2-hydroxy-3,6-naphthalenedisulfonic acid (2-H-3,6-NdS), 3-nitrobenzenesulfonic acid (3-NBS), 4-phenolsulfonic acid (4-PS), 4-hydroxy-3-nitrobenzenesulfonic acid (4-H-3-NB), 1,2-benzenedisulfonic acid (1,2-BdS), 2,6-anthraquinonedisulfonic acid (2,6-AntdS) were purchased from Aldrich (St. Louis, MO, USA). The molecular structures of the analytes are reported in Fig. 1.

All solvents were HPLC grade from Merck (Darmstadt, Germany). Nonylamine, octylamine and heptylamine were from Fluka (St. Louis, MO, USA) reagents, phosphoric acid and sodium tetraborate were purchased by Carlo Erba (Milan, Italy). Sodium dodecylsulfate (SDS) was supplied by Merck and polyossiethylen-23-lauryl ether (Brij 35) was supplied by Sigma (St. Louis, MO, USA).

Bidistilled water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Apparatus

The ion-interaction HPLC (IIR-HPLC) analyses were carried out on a chromatographic system Consta Metric 3200 Fluid Metric Pump interfaced with a spectrophotometric UV Spectro Monitor 3200 detector with variable wavelength.

The CZE and MECC analysis have been carried out on a electrophoresis apparatus Eureka 2100 model (Kontron) equipped with a diode array (190–500 nm) detector. A fused-silica capillary with 62.5 cm (50 cm to the detection window)×0.050 mm I.D. was used (Supelco Celect).

A Metrohom 654 pH meter (Herisau, Switzerland), equipped with a combined glass-calomel electrode was employed for pH measurements. Absorbance measurements were performed on a Unicam 8700 spectrophotometer. CE buffers and sam-

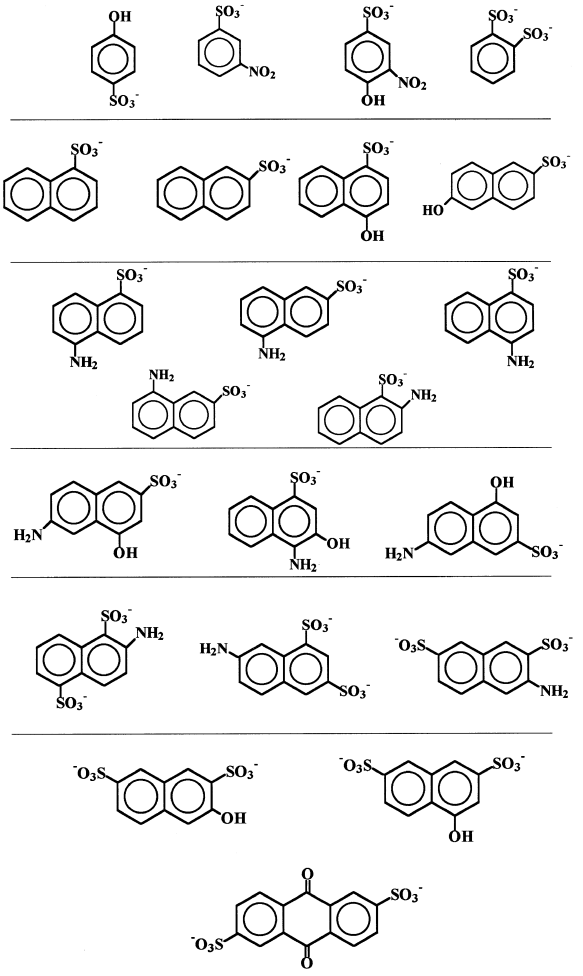

Fig. 1. Molecular structures of the 22 analytes.

ples were degased by a Branson 2200 ultrasonic bath.

2.3. Real sample characteristics

The analyzed effluent, provided by a factory based in Turin, has the following composition: sulfonates compounds 30%, variously substituted aromatic organic compounds 18% (equivalents to 82.25 g/l of organic carbon), water and inorganic ions. The density is 1.31 g/ml and the total carbon is 130.8 g/l of which 129 g/l is organic carbon. The dry residue is 600 g/l (46% in mass: 0.6% of N, 22.8% of C and 1.6% of H). The approximate concentrations, as given by the industry, of the sulfonated aromatic present in the wastewater are: 22.0 g/l 1-NS, 27.0 g/l 2-NS, 2-H-3,6-NdS 19.5 g/l.

For the analysis the sample was diluted, 1/10 000 (v/v) for IIR-HPLC analysis and 1/1000 (v/v) for MECC analysis, and filtered on cellulose acetate filters 0.45 μm (Millipore) and then 0.22 μm.

2.4. Ion-interaction RP-HPLC analysis conditions

The stationary phase was a Merck LiChrospher 100 RP 18 (250×4.00 mm) column fully end-capped (5 μm), used together with a guard pre-column Merck LiChrospher RP-18 (5 μm). The stationary phase is dynamically modified, in isocratic conditions, by an alkylammonium salt present in the mobile phase.

The experiments planned by the experimental design required a number of eluents prepared with different combinations of the values of the four variables considered: N, alkyl-chain length, eluent pH; C_M, organic modifier percentage; and C_{IIR}, IIR (alkylammonium phosphate) concentration in the mobile phase. The table of experiments (Table 1) guarantees for the randomization of the experiments.

The optimized eluent was a 5.9 mM heptylamine water–acetonitrile (ACN) (77.5:22.5, v/v) solution, brought at an operational pH 6.25 by *ortho*-phosphoric acid.

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline was obtained; about 40 min at 1 ml/min was necessary. After use the column was washed by

Table 1
Table of experiments of the (a) fractional factorial design, and (b) factor experimental range

Experiment	N	C_{IIR} (mM)	C_M (%)	pH
(a) Fractional factorial design				
1	−	−	−	−
2	+	−	−	+
3	−	+	−	+
4	+	+	−	−
5	−	−	+	+
6	+	−	+	−
7	−	+	+	−
8	+	+	+	+
(b) Factor experimental range				
−	7	2	20	6
+	9	6	30	8
0	8	4	25	7

flowing water (0.50 ml/min for 15 min), a water–ACN (50:50, v/v) mixture (0.50 ml/min for 20 min) and finally 100% ACN (0.5 ml/min for 5 min). The injection volume was 100 μl and the spectrophotometric detection was performed at 230 nm.

2.5. Capillary electrophoresis conditions

The employed running buffers were: (i) 25 m*M* sodium tetraborate (pH 9.0±0.1); (ii) 25 m*M* sodium tetraborate (pH 9.0±0.1)+5 m*M* octylamine; (iii) 25 m*M* sodium tetraborate (pH 9.0±0.1)+75 m*M* Brij 35; (iv) 25 m*M* sodium tetraborate (pH 9.0±0.1)+5 m*M* octylamine and 75 m*M* Brij 35; (v) 25 m*M* sodium phosphate (pH 7.0±0.1); (vi) 25 m*M* sodium phosphate (pH 7.0±0.1)+5 m*M* octylamine; (vii) 25 m*M* sodium phosphate (pH 7.0±0.1)+75 m*M* Brij 35; and (viii) 25 m*M* sodium phosphate (pH 7.0±0.1)+5 m*M* octylamine and 75 m*M* Brij 35.

All buffers and samples were filtered (cellulose acetate 0.22 μm) and degased for 3 min using an ultrasonic bath before being injected. When a new capillary was used, it was washed with Milli-Q water for 10 min, activated with 0.1 *M* NaOH for 10 min and rinsed with Milli-Q water for 10 min. Then it was equilibrated with the running buffer for 15 min at 20 kV. The same treatment was applied daily before starting the analysis and each time the buffer composition was changed. Between every two runs the capillary was rinsed for 3 min with the buffer. The separations were run at 20 kV keeping the capillary temperature constant at 25±0.1°C. The sample was introduced by applying a negative pressure for 4 s. The diode-array detection was performed in the 200–350-nm wavelength range, allowing the identification of the examined compounds by comparing their spectra with those of pure standards.

3. Results and discussion

3.1. Relevant factors in IIR-HPLC retention: chemometric investigation and optimization

In spite of the relative large use of the ion-pair HPLC technique, still there is not a unique accepted theory concerning the retention in this mode. There are two current theories, i.e., the ion-exchange model and the hydrophobic interaction after ion-pair formation model. It is not just the authors' opinion that these models represent the theoretical extremes of the actual situation [45–48], and throughout this paper we will refer to this chromatographic technique as HPLC with ion-interaction reagent or briefly IIR-HPLC.

IIR-HPLC is influenced by a large number of experimental variables and many papers deal with the effects on aromatic sulfonates retention of factors as eluent pH, kind of alkylammonium cation (the IIR), ion-interaction reagent concentration, organic modifier amount, etc. These studies [19,20,25,33] have been performed always by a one-variable at a time approach (OVAT), so not taking into account the possible and probable interaction among the different factors. Here we have chosen to apply a chemometric treatment of experimental design, since the results obtained in this way can give more reliable information on the actual retention mechanism for the different kind of aromatic sulfonic compounds.

Since in recent years in our laboratories a number of IIR-HPLC methods employing alkylammonium phosphate as the ion-interaction reagent have been developed with satisfactory results, the same kind of IIR was tested in the separation of polar aromatic sulfonates too.

In order to screen the factor importance, a fractional factorial design [49–52] requiring eight experiments (i.e., mobile phase preparations) was performed. This extremely reduced design allows the estimation of the principal effects confounded with the second order interactions. The starting conditions were chosen on the basis of previous studies. The four parameters studied were: alkyl chain length (N) of the IIR, eluent pH, concentration of the IIR (C_{IIR}) and percentage of organic modifier in the mobile phase (C_M). Table 1a reports the table of the experiments and Table 1b the maximum (+) and minimum level (−) of each factor considered. In order to achieve an estimation of the experimental precision, two experiments in the central conditions (0) (Table 1b) were performed too.

The results of this experimental design allow, through the simple calculation of the algorithm of Yates, to estimate the effects of the principal factors and of their second order interactions, reported in

Table 2
The effects of the factors and of their relevant interactions calculated for the 22 analytes from the fractional factorial design

	N	C_{IIR}	$N \times C_{IIR}$	C_M	$N \times C_M$	$C_{IIR} \times C_M$	pH
4-FS	1.93	—	—	−1.58	—	—	—
6-A-4-I-2-NS	5.08	—	2.33	−4.71	−2.00	−2.68	−4.50
1-A-5-NS	5.00	—	2.16	−4.39	−1.62	−2.54	−4.54
4-A-1-NS	5.65	—	2.50	−5.21	−1.96	−2.79	−5.05
6-A-1-I-3-NS	5.21	—	3.68	−5.12	−1.83	−2.34	−4.77
6-I-2-NS	7.75	2.38	4.25	−7.88	−3.83	−4.50	−7.27
4-I-3-NBS	17.77	3.92	6.29	−14.39	−9.77	−13.16	−17.06
5-A-2-NS	9.83	3.15	5.62	−10.57	−5.17	−5.76	−9.32
4-A-3-I-1-NS	13.00	7.13	10.01	−16.42	−9.09	−12.76	−16.58
1-I-4-NS	14.31	5.50	8.43	−18.10	−10.85	−10.58	−15.24
1,2-BdS	27.71	−6.91	−2.89	−8.17	—	−17.98	−24.03
3-NBS	13.99	4.20	8.22	−14.52	−9.063	−11.43	−17.13
8-A-2-NS	14.22	—	7.14	−18.50	−9.183	−8.57	−15.48
2-A-1-NS	10.12	—	2.24	−19.73	−6.095	—	−4.46
1-I-3,6-NdS	23.99	−10.90	—	−12.89	—	−4.69	−10.90
2-A-1,5-NdS	21.53	−10.82	15.25	−20.48	−6.61	—	−14.07
3-A-2,7-NdS	21.65	−10.74	18.50	−12.20	3.32	−9.74	−29.72
7-A-1,3-NdS	17.20	−17.07	−4.43	−8.50	7.87	−3.28	−20.77
1-NS	16.72	−8.12	2.33	−27.30	−3.54	—	−12.87
2-NS	16.73	−11.76	—	−31.00	−2.63	3.10	−12.55
2-I-3,6-NdS	23.93	−19.59	−12.34	−22.50	2.09	2.25	−20.46
Ant-2,6-dS	15.52	−11.21	10.32	−28.21	—	6.40	−19.73

Table 2. The information here contained results quite easy to read since the effect of each factor is expressed by a numeric value and the sign gives the direction of the effect. It can be noted that factors and factor interactions have different weights, and more rarely opposite effect direction, in determining the retention of the different analytes. It would be possible to find different relations between the kind of compound and factor effects.

In particular the analysis of the coefficients (Table 2) confirms that the AS behaviour, when changing the experimental parameters, generally follows the trend evidenced in previous studies for the anions. Thus, AS retention as chain length increases, likely because higher lipophilicity favours the formation of a larger number of active sites on the column surface. Retention decrease at higher pH can be explained with a decrease of the fraction of protonated amine forming the first layer onto the stationary phase. As concerns the dependence on C_{IIR} usually the retention times increase when C_{IIR} increase, being greater the quantity of moiety adsorbed onto the ODS (or the number of ion-pairs). On the other hand in a few cases it was revealed an opposite behaviour, and a non linear C_{IIR}/t_R dependence has been evidenced: depending on the C_{IIR} range considered the retention can decrease or increase, increasing C_{IIR} itself. In this particular case mono- and disulfonates show opposite behaviour: monosulfonates retention increase when increasing C_{IIR}, while disulfonates retention decrease. This is a quite unexpected result: the retention of disulfonates was expected to increase more than that of monosulfonates when increasing C_{IIR}, due to stronger electrostatic interaction. Maybe this effect can be explained as an effect of the ionic strength, that also increase at higher C_{IIR}. At higher ionic strength a decrease in the retention is observed, due to competition and shielding effects exerted by the electrolyte (in this case the *ortho*-phosphate added to adjust the pH of the octylamine solution). This is a general effect, but is much more significant for more hydrophilic and charged analytes. It must be noted that in some cases the coefficients (N, C_{IIR}) of 1-NS and 2-NS are much closer to those of di- than to those of monosulfonates.

By the Ward hierarchical clustering method a dendrogram, based on the correlation coefficient of

Pearson [52], was built: the clusters formed contain the analytes whose retention respond in a similar way when changing the factors. The dendrogram, reported in Fig. 2, visualizes as expected the separation of the analytes in the two main groups of mono- and disulfonated, in spite of the naphthalene or benzene aromatic nucleus. Close to the cluster containing the disulfonates a small cluster containing 1-NS, 2-NS and 2-A-1-NS is present. Another large group—with subdivisions—contains all the amino- and/or hydroxy-substituted monosulfates, 3-NBS, 4-H-3-NBS and 4-PS. Probably a clusterization of these molecules would require an investigation at higher pH (not allowed by a silica-based stationary phase), since in the range of pH 6–8 the amino groups are always uncharged and also the hydroxyls likely do not give a significative contribution to the total molecular charge. In some cases, more than the nature of the substituents (NH_2^- or OH^- or both) the relative positions of the groups seem important in determining the behaviour of the molecular retention.

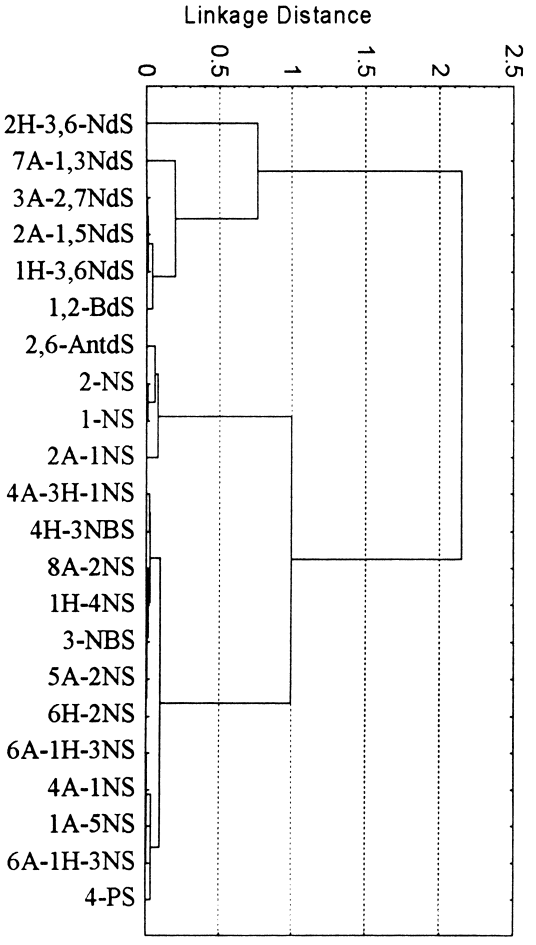

Fig. 2. Dendrogram of the 22 analytes (Ward's method).

In spite of the fact that to build models with strong predictive ability would require more experimental work, including a fold-over design and a star design (some non-linear behaviours were evidenced), an attempt of optimizing the separation was performed. As relevant factor interactions were chosen ($N\times C_{IIR}$), ($N\times C_M$), ($C_{IIR}\times C_M$)—instead of ($C_M\times$pH), ($C_{IIR}\times$pH) and ($N\times$pH).

A grid search algorithm was employed, determining the conditions for the maximum t_R interval between the nearest peaks in the predicted chromatograms which leads to an optimization of the peak resolution. The grid search algorithm, though considered less efficient than other optimization methods, nevertheless allows the introduction of constraint on the response values. Moreover, it allows the achievement of the desired accuracy by iterating the procedure with progressively shorter search steps. During the optimization a further condition concerning the longest retention times, to have a total analysis time was imposed, in order to have the total elution within 70 min.

It was not possible to find out conditions for the resolution of all the analytes. In the optimized conditions: 5.9 m*M* heptylamine *ortho*-phosphate in ACN–water (22.5:77.5, v/v), at pH 6.25, the separation of 20 analytes occurred in 64 min, with just the coelution between 4-A-3-H-1-NS and 3-A-2,7-NdS. The chromatogram obtained is reported in Fig. 3.

3.2. Separation optimization in MECC conditions

Preliminary CZE analyses were run using classical electrophoretic buffers, borate and phosphate at pH 9.0 and 7.0, respectively, varying their concentration, the temperature and the applied voltage. Because of the presence in the examined mixture of a large numbers of isomers having a charge/mass ratio equal or very similar, no satisfactory separation was obtained. In the optimized conditions (borate 25 m*M*, pH 9.0, 25±1°C, 20 kV), in about 40 min analysis,

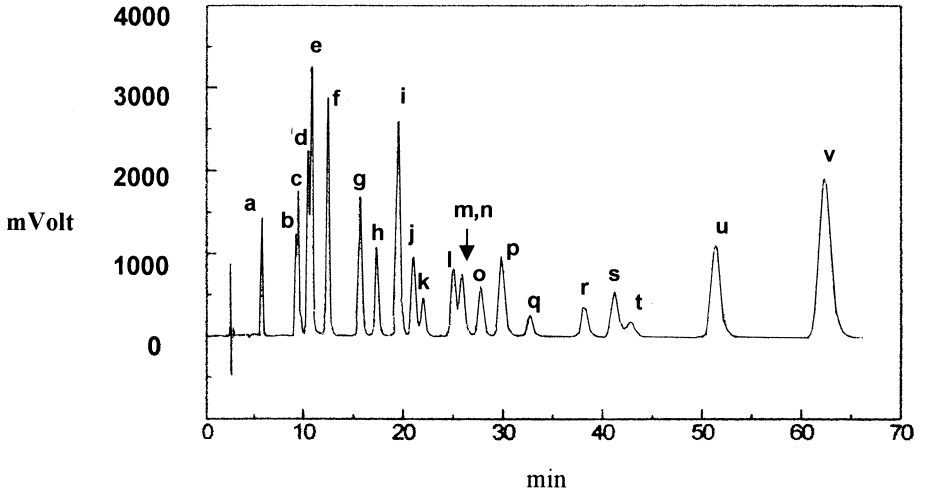

Fig. 3. IIR-HPLC chromatogram obtained in the optimised conditions. Mobile phase: 5.9 m*M* heptylammonium phosphate in ACN–water (22.5:77.5, v/v), pH 6.25, flow-rate 1.0 ml/min. UV detection at 230 nm. Stationary phase: LiChrospher 100 RP-18, (5 μm, end-capped). Injected volume: 100 μl. Analytes concentration: 4.5 mg/l each. Peak identification: (a) 4-PS, (b) 6-A-4-H-2-NS, (c) 1-A-5-NS, (d) 6-A-1-H-3-NS, (e) 4-A-1-NS, (f) 6-H-2 NS, (g) 5-A-2-NS; (h) 4-H-3-NBS, (i) 4-H-1 NS; (j) 3-NBS, (k) 2-A-1,5-NdS, (l) 1-H-3,6-NdS, (m) 4-A-3-H-1-NS, (n) 3-A-2,7-NdS, (o) 7-A-1,3-NdS, (p) 8-A-2 NS, (q) 1,2-BdS, (r) 2-H-3,6 NdS, (s) 2-A-1-NS, (t) 2,6-AntdS, (u) 1-NS, (v) 2-NS.

10 peaks were eluted for the 22 sample components with only four peaks corresponding to a single compound. These operating conditions were just taken as reference for the electroosmotic velocity. Before optimizing the separation, three neutral molecules (acetone, aniline and a butyl derivative of pyridylazonaphtole, PAN-C_4) were analyzed with all the examined buffers in order to characterize the electroosmotic flow and to identify the time range for the elution window, which in MECC falls between the electroosmotic flow and the micellar migration. The results are summarized in Table 3.

Acetone can always be considered the marker for the electroosmotic flow, because it is neutral and not solubilized in the micelles. In the absence of surfactant, its migration time is constant and increases only at pH 7.0, in the presence of octylamine; in this case it is reasonable to assume an interaction of the protonated octylamine with the silanol groups on the capillary walls, thus reducing the electroosmotic

Table 3
Effect of different buffer composition on the migration of neutral molecules

Buffer	Migration time (min)		
	Acetone	Aniline	PAN-C_4
Borate, pH 9.0	4.49	4.54	—
Borate, pH 9.0+SDS	5.40	7.01	25.64
Borate, pH 9.0+Brij 35	5.69	5.72	5.88
Borate, pH 9.0+octylamine	4.58	4.55	—
Borate, pH 9.0+Brij 35+octylamine	5.90	5.22	5.71
Phosphate, pH 7.0	4.56	4.50	—
Phosphate, pH 7.0+SDS	5.45	7.10	24.98
Phosphate, pH 7.0+Brij 35	5.77	5.70	5.82
Phosphate, pH 7.0+octylamine	5.30	5.18	—
Phosphate, pH 7.0+Brij 35+octylamine	5.68	5.59	5.96

Buffer 25 m*M*; surfactant 75 m*M*, octylamine 5 m*M*; *T* in the cartridge, 25±1°C; applied voltage, 20 kV.

flow. The same increasing in migration time is obtained when surfactants are added; even in the absence of a specific electrostatic interaction, such molecules can adsorb on the capillary walls with the same effect of octylamine at pH 7.0.

The PAN-C_4 was injected as micellar marker only in the presence of surfactants, being insoluble in water; it was chosen instead of the mostly used Sudan III because of its stronger interaction with the micelles, thus resulting in a more precise evaluation of the micellar velocity (A. Bianco Prevot et al., unpublished results).

The same elution time obtained in the presence of Brij 35 for acetone, aniline and PAN-C_4 shows that, for neutral molecules, only the different partition coefficients does not influence the migration if the micelles are not charged because the micellar pseudo-phase also moves upon the electroosmotic stream as the bulk solution. This hypothesis is confirmed by the data obtained with SDS, where aniline, slightly partitioned with the SDS, elutes later than acetone, and PAN-C_4, representative of the micellar movement, migrates very slowly, because of its high negative charge and electrophoretic mobility directed against the electroosmotic flow.

It has been extensively documented in literature that the introduction of micellized surfactants in the running buffer could improve the separation efficiency in the case of neutral compounds, moderately hydrophobic, having different partition coefficient with charged micelles [53,54]. The permanent charge of our target compounds will decrease their hydrophobicity and interaction with the micelles but, in spite of that, a certain effect can still be present and exploited. In previous papers other sulfonates have been examined in the presence of both ionic (SDS) and non ionic (Brij 35) aggregates (A. Bianco Prevot et al., unpublished results, [55,56]), and based on the promising results, these two surfactants were employed also in this study. In addition, the effect of octylamine, already used in IIR-HPLC, was studied.

The addition of surfactants significantly improved the separation of the examined sulfonates; adding 75 m*M* of SDS to the 25 m*M* borate buffer at pH 9.0, 12 compounds were identified within 57 min analysis. As it clearly appears in Fig. 4 these results are

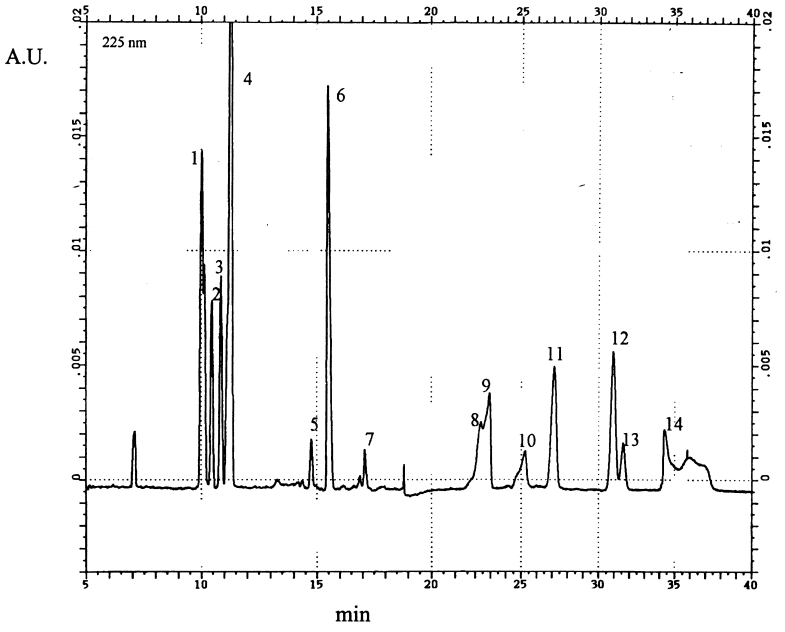

Fig. 4. Pherogram of the 22 analytes. Buffer composition: 25 m*M* sodium tetraborate, pH 9.0, 75 m*M* SDS. 20 kV, 25°C, negative injection for 4 s; detection wavelength 220 nm. Analytes concentration: 15 mg/l each. Peak identification: (1) 5-A-2-NS, (2) 8-A-2-N, (3) 6-H-2-NS, (4) 1-NS +2-NS, (5) coelution, (6) coelution, (7) coelution, (8) 2,6-AntdS, (9) 1-A-5-NS+6-A-4-H-2-NS, (10) 4-PS, (11) 3-a-2,7-NdS, (12) 7-A-1,3-NdS, (13) 2-A-1,5-NdS, (14) 1,2-BdS.

not yet satisfactory; besides the lack in resolution, the peaks are often broadened and not well resolved, many compounds coelute and the analysis time is too high. Both analytes and micelles are negatively charged so the partition with the micelles does not increase the velocity; moreover, the electroosmotic flow being lower than in the pure buffer. The addition of Brij 35 allows to obtain better results: due to its neutral charge, there is no increasing in the generated current, unlike the SDS, thus improving the peak sharpness and the resolution. With phosphate buffer at pH 7.0, 13 peaks can be assigned; at pH 9.0, with borate buffer, 14 compounds were identified while the other eight coelute under four peaks (Fig. 5a,b). Moreover, with the non-ionic surfactant, the absence of electrostatic repulsion substrate-micelle favourites the partitioning with the aggregates, which migrate with the electroosmotic flow, thus strongly reducing the elution time to 18 min at pH 7.0 and to 27 min at pH 9.0.

The addition of octylamine further affects the electrophoretic behaviour. The protonated form of this molecule (pH<8.0) can in principle form an ion-pair with the anionic analytes, reducing their net charge, thus affecting their hydrophobicity and mobility. In the absence of surfactants the octylamine does not introduce any change in the relative mobility of the isomers. Moreover, its interaction with the capillary wall reduces the electroosmotic velocity; this compensates the increasing in migration rate that could take place in the case of formation of neutral adducts and gives at the end a separation similar to that obtained in the pure buffer; an analogous behaviour is observed at pH 9.0 with the borate buffer. On the contrary, in the presence of Brij 35, with phosphate buffer a reduction in the analysis time is observed, even if there is no improvement in the peaks resolution. The best results are obtained with borate buffer and Brij 35: the addition of 5 m*M* octylamine allows the resolution of 20 compounds with the coelution of the two remaining under one peak (Fig. 6). In this case the formations of ion-pairs octylammonium-sulfonates cannot be invoked, the protonated fraction of octylamine at this pH being very small or null. Its presence can just slightly vary the micellar environment, reflecting on a small change in the migration, enough to allow a good separation. These optimized conditions were then compared with the IIR-HPLC results.

3.3. Methods comparison

3.3.1. Efficiency

The resulting peak shape is symmetrical in both the CE and the HPLC mode, but CE offers a better efficiency as higher number of theoretical plates, *N*.

Defining for MECC: $N=5.54\ (l/W_{1/2})^2$ where l is the length to the detector and $W_{1/2}$ is the peak width at half height. For HPLC: $N=5.54(t_R/W_{1/2})^2$ where t_R is the retention time and $W_{1/2}$ is the peak width at half height, CE efficiencies are in the range 5.5×10^6–2.0×10^5 theoretical plates compared to HPLC efficiencies of 9×10^3–1.5×10^4 theoretical plates.

3.3.2. Linearity and LOD

Calibration plots were built in the range of concentration between the limit of determination and 30 mg/l for all the analytes finding satisfactory linearity with correlation coefficients always >0.998 for both techniques. Limits of detection (LODs) were in the range of 0.8–5.2 μg/l in IIR-HPLC conditions and 0.5–3.2 mg/l in the MECC mode, with LOD values taken with the ratio of signal/noise equal to 3. As usually observed comparing HPLC and CE, CE mass detection limit is better than for HPLC, but due to the differences of injection volume and flow-cell optical path length the concentration detection limit in CE are about 1000 times lower in HPLC than in CE.

3.3.3. Precision

In the optimized IIR-HPLC conditions 15 injections of the analytes at a concentration of 10 μg/l were made and the precision was evaluated. The relative standard deviation (R.S.D.) in peak area was found to range between 0.5 and 1.0% for all the analytes. Repeatability of retention times is also satisfactory being between 1.3 and 2.5%.

In the optimized MECC conditions 15 injections of the analytes at a concentration of 2 mg/l gave a R.S.D. in the peak area in the range of 1–5% and a repeatability in the retention time between 0.6 and 1.5%.

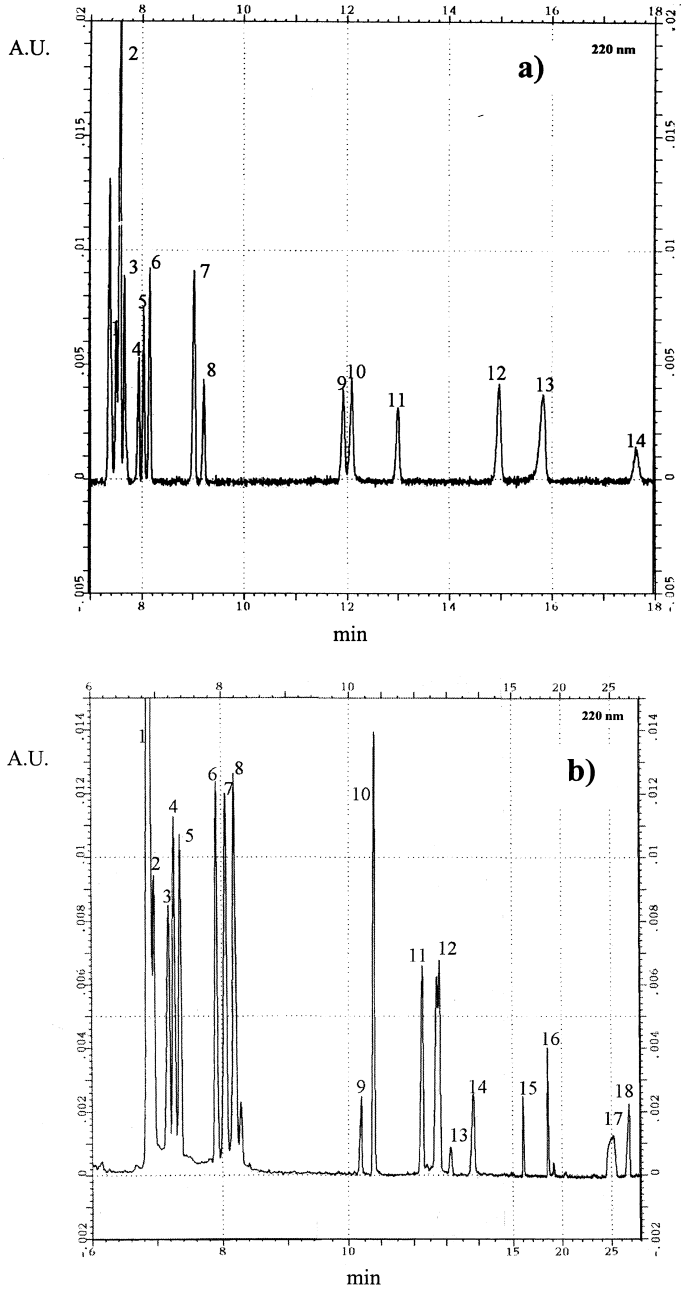

Fig. 5. Pherogram of the 22 analytes. Buffer composition: (a) 25 m*M* sodium phosphate, pH 7.0, 75 m*M* Brij 35. (b) 25 m*M* sodium tetraborate, pH 9.0, 75 mM Brij 35. Analytes concentration 15 mg/l each. Other conditions as in Fig. 4. (**a**) Peak identification: (1) 6-H-2NS, (2) 2-NS, (3) 8-A-2-NS, (4) 2-A-1-NS, (5) 1-NS, (6) 5-A-2-NS, (7) 4-A-5-NS, (8) 1-A-5-NS, (9) coelution, (10) 2,6-AntdS, (11) 2-H-3,6-NdS, (12) 3-A-2,7-NdS, (13) 7-A-1,3-NdS, (14) 2-A-1,5-NdS. (**b**) Peak identification: (1) 2-NS, (2) 5-A-2-NS+8-A-2-NS, (3) 2-A-1-NS, (4) 1-NS, (5) 6-A-4-H-2-NS+1-A-5-NS, (6) 6-H-2-NS, (7) 4-A-1-NS+6-A-1-H-3-NS, (8) 4-A-3-H-1-NS+3-NBS (9) 2,6-AntdS, (10) 4-H-1-NS, (11) 3-A-2,7-NdS, (12) 7-A-1,3-NdS, (13) 2-H-3,6-NdS, (14) 2-A-1,5-NdS, (15) 4-PS, (16) 1-H-3,6-NdS, (17) 4-H-3-NBS, (18) 1,2-BdS.

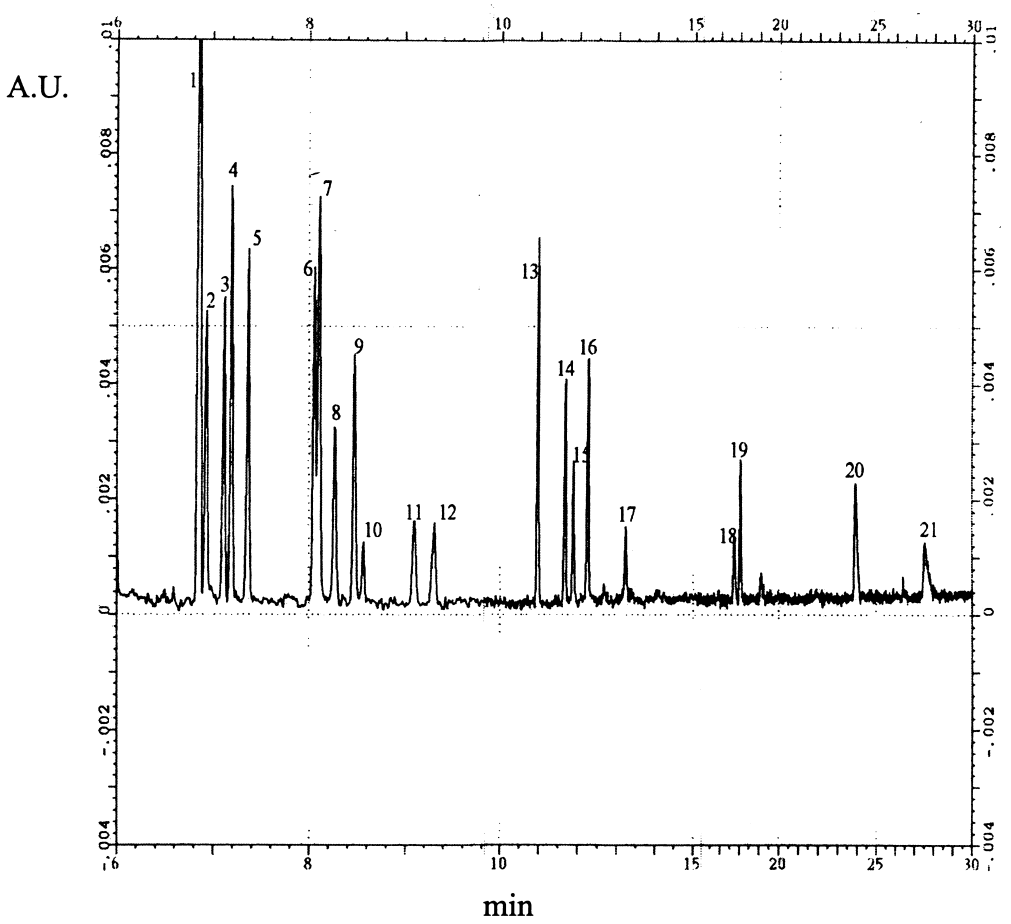

Fig. 6. Pherogram of the 22 analytes. Buffer composition: 25 m*M* sodium tetraborate, pH 9.0, 75 m*M* Brij 35, 5 m*M* octylamine. Other conditions as in Fig. 4. Analytes concentration 15 mg/l each. Peak identification: (1) 2-NS, (2) 8-A-2-NS, (3) 2-A-1-NS, (4) 1-NS, (5) 5-A-2-NS, (6) 4-A-1-NS, (7) 6-H-2-NS, (8) 1-A-5-NS, (9) 6-A-H-I-3-NS, (10) 6-A-4-H-2-NS+4-A-3-H-1-NS, (11) 2,6-AntdS, (12) 3-NBS, (13) 4-H-1-NS, (14) 3-A-2,7-NdS, (15) 2-H-3,6-NdS, (16) 7-A-1,3-NdS, (17) 2-A-1,5-NdS, (18) 4-PS, (19) 1-H-3,6-NdS, (20) 4-H-3-NBS, (21) 1,2-BdS.

3.4. Application to an industrial waste

An industrial effluent containing polar aromatic sulfonates together with other aromatic compounds were analyzed by the two complementary methods developed. Due to the high concentrations no sample preconcentration was necessary, on the contrary a dilution with distilled water (1/10 000, v/v, for IIR-HPLC analysis and 1/1000, v/v, for MECC analysis) was necessary. Both IIR-HPLC chromatogram and MECC electropherogram obtained after such dilution present a good baseline and allows the identification of the three aromatic sulfonates present. Quantification by calibration plot and standard addition, respectively, gave the results reported in Table 4, while Fig. 7a,b reports the chromatogram and the pherogram obtained.

In conclusion the separation of 20 of the 22

Table 4
Application to an industrial wastewater: comparison of the results obtained by IIR-HPLC and MECC

	2-I-3,6-NdS	1-NS	2-NS
Given concentration (g/l)	19.5	22.0	27.0
IIR-HPLC	20.4	23.6	26.1
MECC	20.9	24.4	25.8

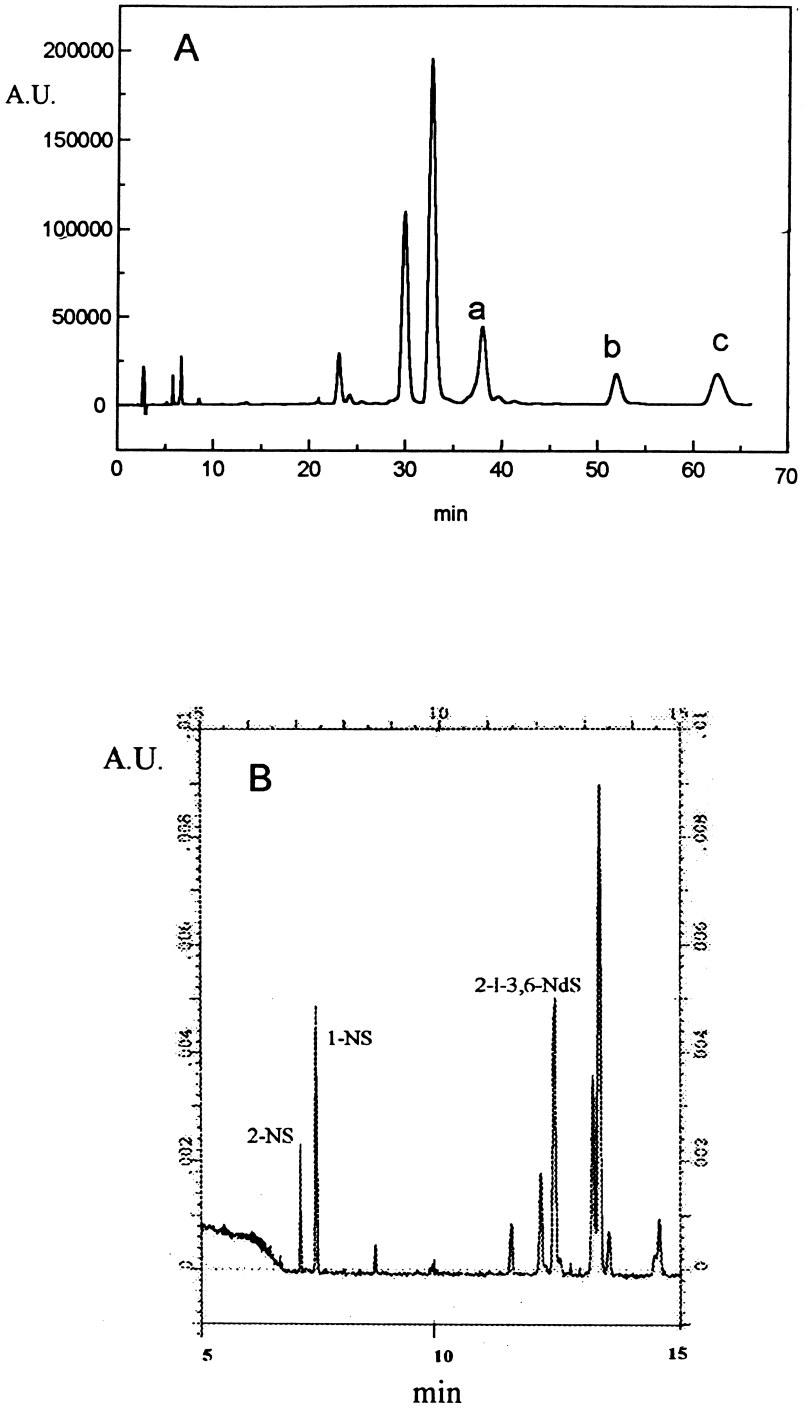

Fig. 7. Analysis of an industrial wastewater: (A) IIR-HPLC: (a) 2-I-3,6-NdS, (b) 1-NS, (c) 2-NS. Conditions as in Fig. 3. (B) MECC. Conditions as in Fig. 6.

compounds considered is possible with both the developed methods and their complementary use allows the identification and quantification of the coeluted analytes. Moreover the application of two different techniques is needed to confirm the presence and the identity of analytes present in complex mixtures.

As expected MECC leads to better peak shapes, higher efficiency and lower analysis time, whereas IIR-HPLC is superior in its sensitivity. As far as accuracy and precision are concerned both the methods gave satisfactory results.

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