Journal of Chromatography, 640 (1993) 231–240
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2675

Determination and identification by high-performance liquid chromatography and spectrofluorimetry of twenty-three aromatic sulphonates in natural waters

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ABSTRACT

A method for detecting and identifying 23 aromatic sulphonates in natural waters was optimized. Samples were pretreated by solid-phase extraction in order to eliminate interferents and to preconcentrate the analytes. Separation of the extracted analytes was accomplished by ion-pair HPLC with fluorimetric detection; the eluent composition and pH were optimized. The detection limits lie in the low $\mu g\ l^{-1}$ range. Some of the investigated compounds were found in the water of the river Bormida (N.W. Italy). Unknown components were identified from their fluorescence spectra.

INTRODUCTION

Aromatic sulphonates and their hydroxy and amino derivatives are used for many purposes, especially as intermediates in dyes manufacture. Their production and use can lead to very pollutant wastes for superficial and underground waters.

These substances are surface active and they can be modified by microorganisms. In fact, it has been observed that a green alga can use 1-naphthalene-sulphonic acid as a sulphur source, even in the presence of sulphate ion, leaving the naphthalene ring undegraded [1]. Other bacteria can produce 5-sulphosalicylic and gentisic acid as intermediates in the biodegradation of 1,6- and 2,6-naphthalene-disulphonic acid [2,3]. Little is known about the possible environmental effects of these substances.

To study these effects adequately, a sensitive and selective method of analysis is required. Further, an ancillary technique for confirmation of unknown components, even at low concentrations, is necessary. In fact, owing to the large number of possible positional isomers of the investigated class of compounds, chromatographic retention time alone cannot support qualitative analysis.

Underground and river waters can contain relatively large amounts of interferents of natural origin (*e.g.*, humic acids) or industrial origin (*e.g.*, non-polar or slightly polar organic substances). Hence there is also a need for pretreatment of the sample in order to remove such interferents.

In previous work, the separation of a limited numbers of isomers was achieved by several techniques: low-pressure ion-exchange chromatography [4], TLC [5], ion-exchange electrokinetic chromatography [6] and ion-pair chromatography [7–9]. All these studies dealt with limited numbers of compounds and the identification of the unknowns was based on the retention times only.

The GC or GC–MS analysis of some aromatic sulphonic acids has been also carried out, after their conversion into volatile derivatives, by means of thionyl chloride and further amidation with an aliphatic amine [10] or by means of phosphorus pentachloride and further esterification with trifluoroethanol [11]. Unfortunately, the substances investigated in this study contain also amino and hydroxy

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groups, which can react with the reactants used for halogenating the sulphonic group. Further, derivatization processes are complex and time consuming.

Anion-exchange liquid chromatography, coupled with UV and particle beam MS detection, has also been proposed for the determination of eight aromatic sulphonic acids [12]. This method seems more suitable for mono- than disulphonic acids; further, the detection limits lie in the nanogram range using UV detection or in the microgram range using particle beam MS detection.

Ion-pair chromatography seemed more promising for the separation of mixtures of large numbers of isomers in the presence of interfering substances. Owing to the intense fluorescence properties of the substances investigated in this study, spectrofluorimetry seemed the most sensitive detection technique, and it also seemed suitable for performing qualitative analysis, even at very low concentrations, owing to the specificity of the shapes of the fluorescence spectra. In this work, the chromatographic technique and the pretreatment of real samples were optimized; the identification of unknown components on the basis of their fluorescence spectra is proposed.

EXPERIMENTAL

Reagents

All aqueous solutions were prepared with ultra-high-quality (UHQ) water obtained by passing deionized water through an Elga-Stat water-purification apparatus. Methanol and acetonitrile, both of HPLC grade with low UV absorption, tetrabutylammonium (TBA) hydroxide and cetyltrimethylammonium bromide (CTMABr) were obtained from Aldrich. The compounds listed in Table I were obtained from Aldrich or Kodak. Citric acid, ammonia solution and magnesium sulphate were obtained from Carlo Erba. Solid-phase extraction (SPE) columns were obtained from Baker and Merck.

Stock solutions of the investigated sulphonates were prepared with UHQ water, kept in dark-glass flasks, stored in a refrigerator (4°C) and were used within 1 week, except those of 4-amino-3-hydroxy-1-naphthalenesulphonate, which were prepared immediately before use.

Apparatus

The HPLC system consisted of a Pye Unicam PU 4015 pump, a Rheodyne valve fitted with a 20-μl loop and a 250 × 4.6 mm I.D. Alltech Adsorbosphere C_8 (5 μm) column. The eluent was degassed by means of a stream of helium.

The detector was an Hitachi F-4000 computerized spectrofluorimeter, equipped with an 18-μl flow cell and an analogue output. The same instrument was used to run and memorize the fluorescence spectra. Quantitative calculations were performed with a Shimadzu C-R3A data processor.

RESULTS

Optimization of chromatographic conditions

After some preliminary chromatograms, five substances were chosen to test the influence on capacity factors of pH, organic content of mobile phase, ion-pair reagent and buffer concentration. Unless specified otherwise, the composition of the mobile phase was acetonitrile–water (58:42) containing 5 g l^{-1} of CTMABr and 1 g l^{-1} of citric acid (pH 7.0).

Effect of pH. Fig. 1A shows the influence of the pH of the mobile phase on the capacity factors of the five compounds. The ratio of acetonitrile to water in the mobile phase was 60:40 for 1,5- and 2,7-naphthalenedisulphonate and 46:54 for 6-amino-4-hydroxy-2-naphthalenesulphonate and 4-amino-1- and 5-amino-2-naphthalenesulphonate.

The pH of the eluent had a great effect on the capacity factors. Those of 1,5- and 2,7-naphthalenedisulphonate decreased as the pH of the eluent was increased, whereas those of the amino and/or hydroxy derivatives exhibited a more complex trend. Some of them showed a maximum around pH 4.

Ion-pairing reagent concentration. The results for the five selected compounds are shown in Fig. 1B. Their chromatographic behaviour is typical of a reversed-phase system in that an increase in ion-pairing reagent concentration increased the retention times. TBA phosphate was also tried as an ion-pairing reagent, but its performance did not seem better than that of CTMABr, so the development of the chromatographic method was done using the latter reagent.

Organic content of the mobile phase. Fig. 1C shows the results obtained with eluents whose with acetonitrile to water ratios ranging from 50:50 to 58:42.

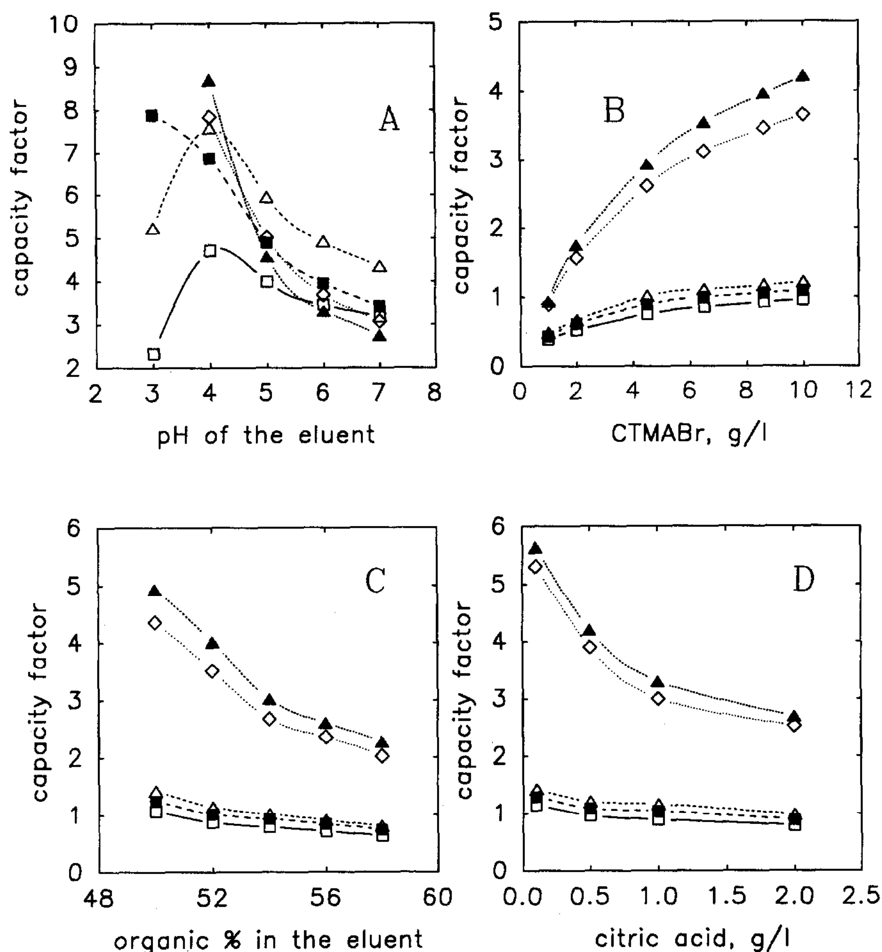

Fig. 1. Effects on capacity factors of (A) pH, (B) CTMABr concentration, (C) percentage of organic component and (D) citric acid concentration in the eluent. □ = 6-Amino-4-hydroxy-2-naphthalenesulphonate; ■ = 4-amino-1-naphthalenesulphonate; △ = 5-amino-2-naphthalenesulphonate; ◇ = 2,7-naphthalenedisulphonate; ▲ = 1,5-naphthalenedisulphonate.

Beyond these limits, the retention times became unacceptably long or short. As expected, an increased acetonitrile content reduced the capacity factors of the analyte compounds.

Buffer concentration. Citric acid was chosen to buffer the pH of the eluent, as it is readily soluble and does not increase the background fluorescence. The results for the investigated compounds are shown in Fig. 1D.

Detector conditions. A computerized spectrofluorimeter was used as a detector, giving high sensitivity, good selectivity and the possibility of characterizing eluted compounds by means of their excitation and emission spectra. The fluorescence emission and excitation spectra of the 23 compounds investigated were examined in the range 220–800 nm; some typical examples are shown in Fig. 2. Spectra were not corrected for the influence of the instrumental system, because the automatic correction range of the spectrofluorimeter covered only wavelengths shorter than 600 nm, whereas the investigated compounds showed characteristic emission also in the range 600–800 nm. The spectrofluorimeter was found to be very stable, and even spectra recorded 3 months apart coincided perfectly.

Amino and hydroxy substituents on the naph-

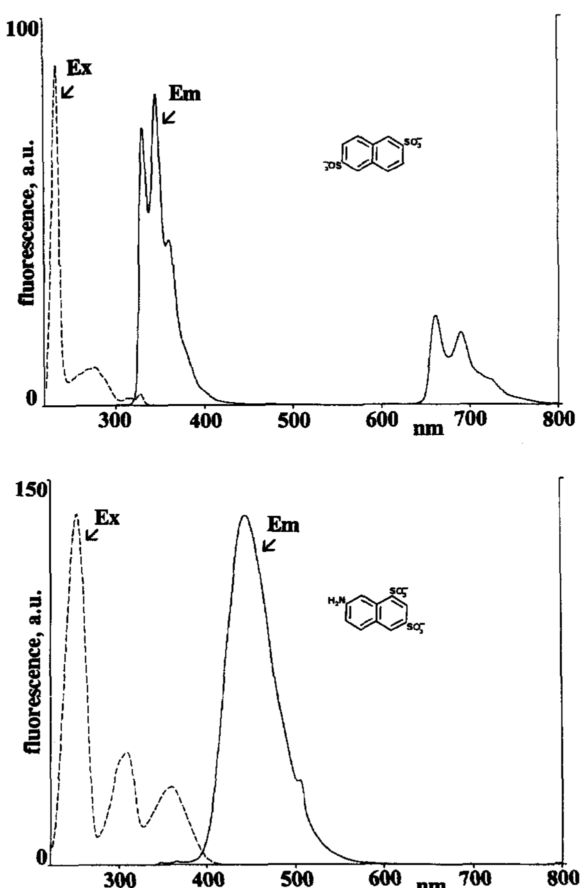

Fig. 2. Typical fluorescence spectra of excitation (dashed line) and emission (solid line) of two investigated compounds.

thalenesulphonate moiety produce a red shift in the excitation and emission maxima with respect to the unsubstituted naphthalenesulphonate. These last compounds and the three positional isomers of aminobenzenesulphonate and of 1-hydroxy-2-naphthalenesulphonate showed a second emission maximum around 660 nm, whereas the other compounds investigated showed no relevant fluorescence between 600 and 800 nm. Therefore, it was possible to separate the 23 compounds investigated into two groups, by detecting chromatograms with excitation and emission wavelengths of 250 and 455 or 240 and 660 nm, respectively. None of the investigated compounds is detected at both pairs of wavelengths. Two injections per sample are required to monitor all the investigated compounds but, with the optimized chromatographic conditions, only 30 min are needed to perform the double chromatographic run.

Quantitative analysis. Quantification was performed by measuring peak heights. The reproducibility of the chromatographic analysis alone (without preconcentration) was tested. Four replicate injections of a standard solution containing nine compounds gave an average relative standard deviation of 8%. Standard solutions of the 23 compounds were progressively diluted with ultrapure water and analysed. The detection limits (without preconcentration, signal-to-noise ratio >6), together with the wavelengths used for their detection, are reported in Table I. The peak heights were found to be linearly proportional to concentration up to the third order of magnitude over the individual detection limits.

Optimization of pretreatment conditions

Sample clean-up. River water samples were eluted on a C_{18} SPE cartridge with the aim of removing non-polar substances potentially dangerous for the chromatographic column. Under the same experimental conditions, the analytes studied were not retained from an aqueous solution. A small volume (5 ml) of aqueous solutions of the compounds listed in Table I, at concentrations 50 times higher than the individual detection limits, were analysed before and after elution through the C_{18} cartridge. No effect on the concentrations of the solutes was observed.

Stationary phase for SPE. The extraction of the 23 analytes from aqueous solutions was tried with various types of solid-phase sorbents. The following procedure was used: 20 ml of standard test mixtures whose components, at the same concentrations as the solutions used for testing the sample clean-up, were eluted through quaternary amine, amino and diamino Baker SPE cartridges. Further tests were conducted using C_8 and C_{18} cartridges, previously loaded with CTMABr.

Chromatographic analysis of the eluted solutions indicated that all the cartridges retained 100% of the solutes. The cartridges were also examined visually by illuminating them with a UV lamp. The adsorbed compounds produced a single and narrow fluorescent band at the top of the stationary phase column.

Elution of the adsorbed aromatic sulphonates. Although the solutes were quantitatively retained, many of them were not quantitatively recovered by elution. Extraction from ion-exchange cartridges

TABLE I

RESULTS OF THE CHROMATOGRAPHIC ANALYSIS AND SOLID-PHASE EXTRACTION OF AROMATIC SULPHONATES

NS = naphthalenesulphonate; NDS = naphthalenedisulphonate; BZS = benzenesulphonate; A = excitation maximum (nm); B = emission maximum (nm); C = retention time (min); D = detector wavelength, ex/em (1 = 240/660 nm, 2 = 250/455 nm); E = detection limit of the chromatographic analysis (ppb); F = mean recovery ± S.D. ($n = 6$) (%); G = concentration in the solution to be extracted (ppb).

Substance	Chromatography					SPE	
	A	B	C	D	E	F	G
1-NS	230	338	7.25	1	25	85 ± 5	1.9
2-NS	230	339	6.97	1	25	87 ± 10	1
1,5-NDS	230	336	11.29	1	100	81 ± 7	4
2,6-NDS	234	345	8.49	1	12	88 ± 15	0.5
2,7-NDS	234	342	10.37	1	50	92 ± 13	2
1-OH-2-NS	240	350	14.64	1	15	96 ± 17	0.6
1-OH-4-NS	237	436	5.06	2	10	87 ± 16	0.3
1-OH-3,6-NDS	245	475	9.76	2	100	94 ± 23	3
2-OH-3,6-NDS	240	462	17.30	2	8	107 ± 19	0.3
2-OH-6,8-NDS	239	468	15.95	2	5	103 ± 16	0.2
2-NH_2-1-NS	246	406	7.53	2	20	86 ± 11	0.1
4-NH_2-1-NS	247	415	4.03	2	5	84 ± 3	0.2
5-NH_2-2-NS	249	455	4.33	2	4	89 ± 3	0.15
8-NS_2-2-NS	249	464	5.41	2	4	88 ± 4	0.15
3-NH_2-2,7-NDS	251	440	10.21	2	1	92 ± 7	0.05
7-NH_2-1,3-NDS	251	444	12.71	2	1	92 ± 5	0.05
4-NH_2-3-OH-1-NS	248	442	4.51	2	2	78 ± 3	0.3
4-NH_2-5-OH-1-NS	346	400	3.64	2	60	77 ± 11	4
6-NH_2-4-OH-2-NS	253	407	3.73	2	10	78 ± 3	0.4
8-NH_2-1-OH-3,6-NDS	240	408	9.03	2	25	79 ± 11	1
2-NH_2-1-BZS	245	366	4.18	1	150	87 ± 12	6
3-NH_2-1-BZS	245	366	3.24	1	120	97 ± 9	5
4-NH_2-1-BZS	253	347	2.95	1	25	98 ± 3	1

was tried with aqueous solutions of pH 1–13, saturated solutions of LiCl and $MgSO_4$, pure methanol, pyridine and 5% HCl in methanol. All these eluents, except the last, gave very poor results. The recoveries obtained with 5% HCl in methanol were only partially satisfactory, because the recovered amounts of six substances were below 50%. Further, in the chromatographic analysis, some closely eluted compounds were resolved worse, even if the HCl was neutralized with NH_3 prior to injection.

More satisfactory results were obtained with reversed-phase SPE cartridges loaded with CTMABr. C_8 and C_{18} stationary phases furnished by Merck and Baker were tested. As the Adsorbex C_{18} type gave slightly better results than the others, it was adopted in subsequent studies.

Methanol gave satisfactory results for the extraction of the substances adsorbed on the C_{18} SPE cartridge. In order to find the optimum overall volume of eluent, during elution fractions of 0.5 ml were collected and analysed. Those corresponding to an overall volume larger than 2 ml contained negligible amounts of the analytes, hence that volume was adopted to elute the SPE cartridge.

Recoveries from standard solutions of the analytes, at the reported concentrations, are given in Table I, together with the standard deviations calculated, for a 95% confidence interval, from the data for six replicate extractions.

Influence of pH and inorganic salts. The influence of inorganic salts and pH on the extraction yields was also investigated. The pH of the standard

solutions was adjusted from 4 to 9 with either HCl or NaOH. Further tests were conducted with solutions with 30 g l^{-1} NaCl or 2 g l^{-1} Na_2SO_4 added.

The recoveries of all the compounds decreased; in particular 30 g l^{-1} of NaCl reduced the recoveries of orthanilic, methanilic and sulphanilic acid below 30%, whereas 2 g l^{-1} of Na_2SO_4 and an acidic pH reduced the recoveries of methanilic and sulphanilic acid below 50%. A basic pH had only a slight influence on the extraction yields. The recoveries of the other substances listed in Table 1 were always >70%.

Conditioning of the SPE cartridges. Conditioning of the cartridges was found to be very useful in increasing the recoveries; it consisted of eluting with 2 ml of methanol, two aliquots of UHQ water, 2 ml of 1% CTMABr solution and two aliquots of 5 ml of UHQ water. This procedure was carried out three times before passing the water sample through the cartridge. A pretreatment consisting of a single series of elutions resulted in poorer recoveries, particularly for those compounds that have longer retention times. No increase in recoveries was observed if the cartridge were pretreated with more than three series of elutions.

DISCUSSION

The chromatographic separation of the analytes was performed with an isocratic eluent delivered at a flow-rate of 1.5 ml min^{-1}. Gradient elution was tried, but isocratic conditions were preferred in order to increase the sensitivity (due to the better stability of the baseline), to obtain more reproducible retention times and to shorten the analysis time (because no column conditioning was required after each analysis). Further, isocratic conditions allowed the background fluorescence to be subtracted efficiently from the spectra of the analytes; therefore, it was also possible to characterize unknown analytes by means of their fluorescence spectra even at very low concentrations.

The optimized mobile phase had a pH of 7.0, an acetonitrile–water ratio of 58:42 and contained 7 g l^{-1} of CTMABr and 1 g l^{-1} of citric acid. The pH was buffered to 7.0, as lower pH values produced poorer peak shapes for many compounds and lengthened the retention times; at that pH, all the compounds were eluted within 18 min with acceptable resolution. An acetonitrile-to-water ratio of 58:42 was considered to be the most favourable because none of the 23 compounds investigated was non-retained and they were all eluted within 18 min with good resolution of adjacent peaks. A 7 g l^{-1} concentration of CTMABr seemed the most favourable, as lower concentrations resulted in poorer peak shapes for some compounds, and all the peaks were well resolved in an acceptable time.

The excitation/emission monochromators of the detector were set alternatively at 250/455 or 240/660 nm and their band widths were 20/40 nm, respectively. Two replicate injections of each sample were made. Good separations of the analytes and short analysis times were obtained. Typical chromatograms are shown in Fig. 3.

Before the analysis, the water samples were filtered through a 0.45-μm cellulose acetate filter and then eluted through a 400-mg C_{18} Merck SPE column in order to retain non-polar interferents.

Analytes were extracted from water by passing a suitable amount of sample through another C_{18} SPE cartridge at a flow-rate of 8 ml/min. That cartridge was previously conditioned as described above. Volumes of sample exceeding 100 ml resulted in an excessive increase in the flow resistance across the cartridge, and consequently in an increase in the analysis time. The retained compounds were further eluted with 2 ml of methanol and analysed by HPLC.

A further injection was done in order to obtain fluorescence spectra of the eluted substances. A few seconds before a peak appeared, the eluent flow was gradually slowed and then stopped on the top of the peak. The band widths of the excitation and emission monochromators were set at 5 nm and a first pair of spectra of unknown analyte were obtained and memorized. The flow of the eluent was then started again, and it was subsequently re-stopped on the decreasing portion of chromatographic peak; a second pair of spectra were memorized, under the same instrumental conditions. The latter spectra were subtracted from the corresponding former spectra, in order to avoid eluent and matrix disturbances, and the resultants were compared with those of the standards. It was observed that the spectra of the unknown peaks were identifiable if their maximum intensity was greater than twice the background fluorescence.

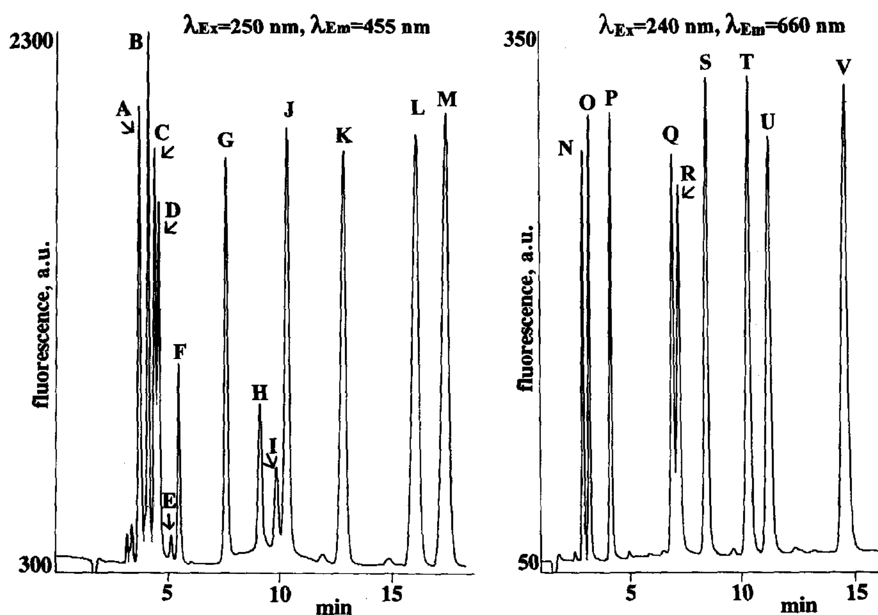

Fig. 3. Chromatograms of a standard mixture of 22 compounds (NS = naphthalenesulphonate, NDS = naphthalenedisulphonate, BZS = benzenesulphonate). A = 4-amino-5-hydroxy-1-NS, 4 ppm; B = 4-amino-1-NS, 0.25 ppm; C = 5-amino-2-NS, 0.15 ppm; D = 4-amino-3-hydroxy-1-NS, 0.3 ppm; E = 1-hydroxy-4-NS, 0.3 ppm; F = 8-amino-2-NS, 0.06 ppm; G = 2-amino-1-NS, 1.2 ppm; H = 8-amino-1-hydroxy-3,6-NDS, 2 ppm; I = 1-hydroxy-3,6-NDS, 5 ppm; J = 3-amino-2,7-NDS, 0.1 ppm; K = 7-amino-1,3-NDS, 0.12 ppm; L = 2-hydroxy-6,8-NDS, 0.8 ppm; M = 2-hydroxy-3,6-NDS, 1.2 ppm; N = 4-amino-1-BZS (sulphanilate), 0.5 ppm; O = 3-amino-1-BZS (methanilate), 3 ppm; P = 2-amino-1-BZS (orthanilate), 3 ppm; Q = 2-NS, 1 ppm; R = 1-NS, 1.9 ppm; S = 2,6-NDS, 0.5 ppm; T = 2,7-NDS, 1.5 ppm; U = 1,5-NDS, 5 ppm; V = 1-hydroxy-2-NS, 1 ppm.

The analyte concentration changes with time, due to diffusion inside or outside the flow cell, were examined by observing the drift of the fluorescence after stopping the flow. The variation in fluorescence intensity was $<1\%$ min^{-1}; distortion of the shape of the spectra was avoided by using the fastest scan speed available (600 nm min^{-1}).

Reference spectra were measured using standard sulphonic acids dissolved in the optimized chromatographic eluent, and were memorized on a floppy disk, with the aim of comparing them with the spectra of unknown components. Standard solutions had a concentration of 1 mg l^{-1}, and no influence of concentration on spectral shapes was observed in the range 0.01–1 mg l^{-1}.

To take into account possible variations of the recoveries of the SPE, an internal standard was added to real samples prior to preconcentration. After the analysis, a factor was calculated by dividing the expected concentration (accounting for the yield in Table I) by the observed value. The yields in Table I for the remaining analytes were multiplied by this factor. Orthanilic acid was found to be absent in all the real samples analysed, hence it was used as the internal standard.

Analysis of river water

The method described was used to analyse water samples from the Bormida, a river situated in the N.W. Italy. The chromatograms of the extract of a sample are reported in Fig. 4; Table II gives the data for the analytes identified in four different samples. As can be seen, their concentrations range from fractions of 1 μg l^{-1} to several thousand mg l^{-1}. The concentration factors adopted for the analysis are given in Table II; the most concentrated sample was diluted 400-fold prior to the analysis.

As an example of qualitative identification, Fig. 5 reports the four spectra (excitation and emission) obtained for the unknown peak F, together with those of 2,7-naphthalenedisulphonic acid; as can be seen, they coincide well. Good agreement was also

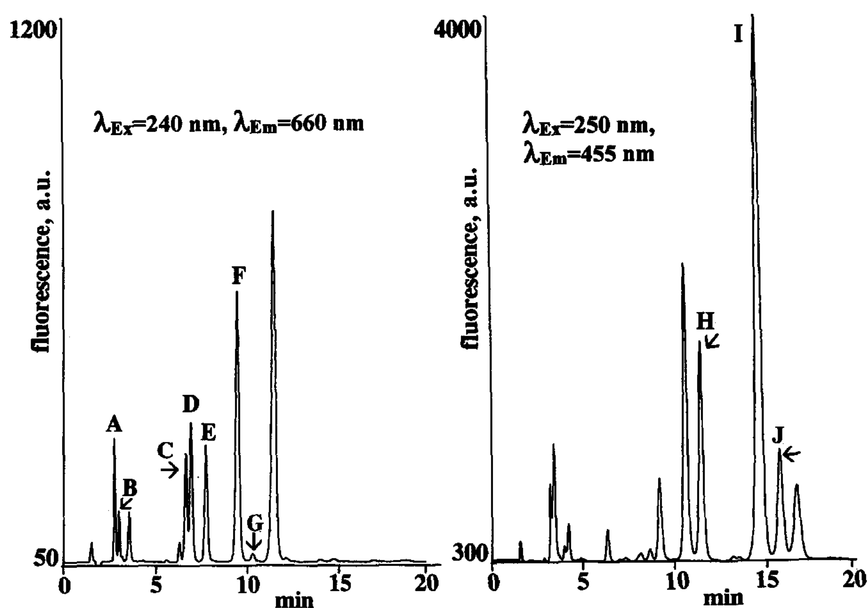

Fig. 4. Chromatograms obtained from the sample D in Table II. The spectra of the unidentified peaks did not coincide with any of those of the standards. A = 4-amino-1-BZS; B = 3-amino-1-BZS; C = 2-NS; D = 1-NS; E = 2,6-NDS; F = 2,7-NDS; G = 1,5-NDS; H = 7-amino-1,3-NDS; I = 2-hydroxy-6,8-NDS; J = 2-hydroxy-3,6-NDS (abbreviations as in Fig. 3).

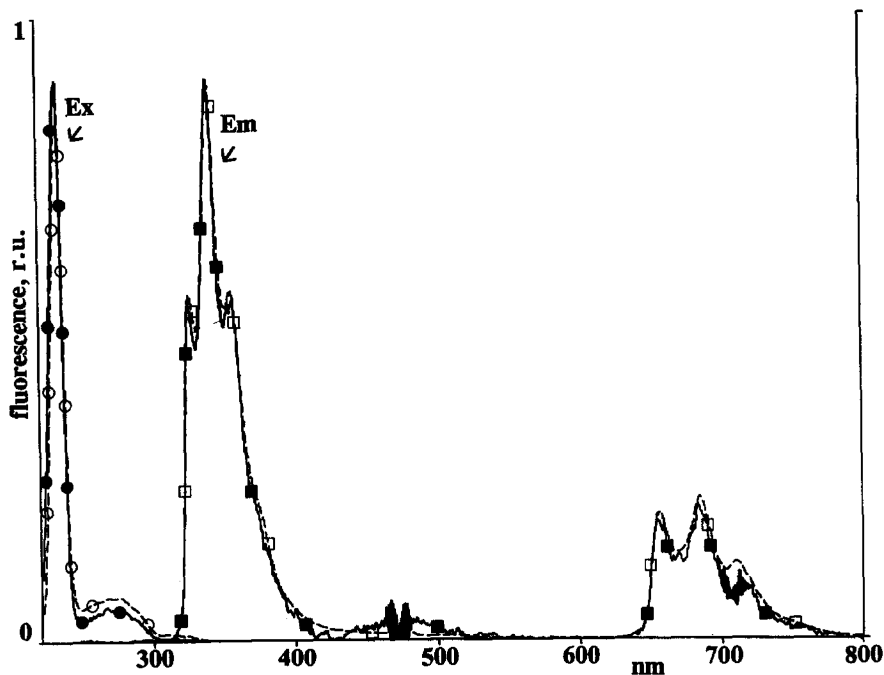

Fig. 5. Superimposed excitation and emission spectra of the unknown peak F (● = excitation, ■ = emission) and of 2,7-naphthalenedisulphonate (○ = excitation, □ = emission). The bands of stronger noise around 470 and 700 nm are due to the Rayleigh scattering of the incident light; r.u. = relative units.

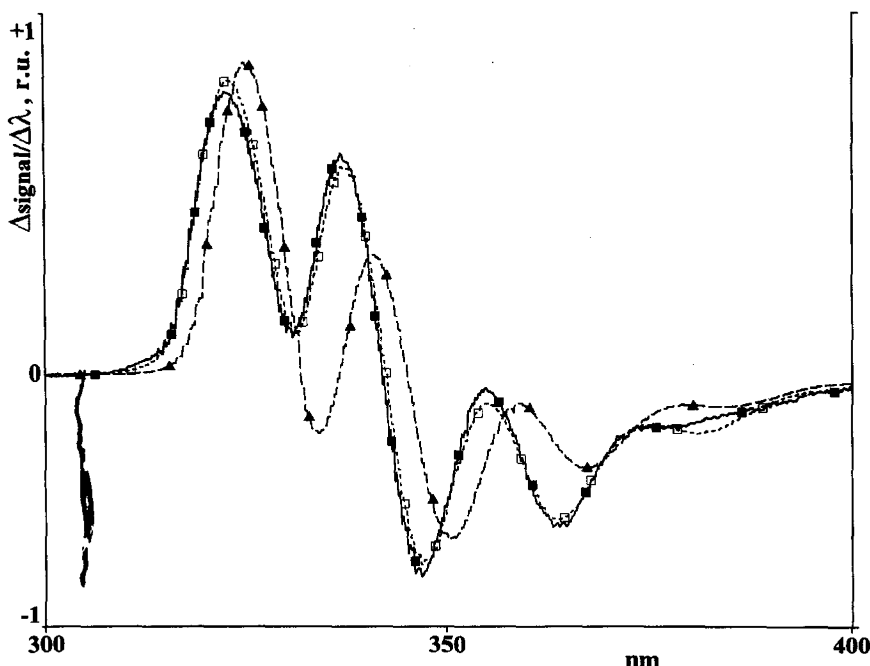

Fig. 6. First-order derivative of the emission spectra in the range 300–400 nm. ■ = Unknown peak F; □ = 2,7-naphthalenedisulphonate; ▲ = 2,6-naphthalenedisulphonate; r.u. = relative units.

TABLE II

CONCENTRATIONS OF THE IDENTIFIED COMPOUNDS IN FOUR RIVER WATER SAMPLES

NS = naphthalenesulphonate; NDS = naphthalenedisulphonate; BZS = benzenesulphonate.

Substance	Concentration (mg l^{-1})			
	A[a]	B[a]	C[a]	D[b]
7-NH_2-1,3-NDS	0.0002	0.0017	0.0027	76
2-OH-6,8-NDS	0.0015	0.0092	0.0065	1667
2-OH-3,6-NDS	0.0011	0.024	0.035	371
4-NH_2-1-BZS	0.0045	0.0026	0.0015	408
3-NH_2-1-BZS	NF[c]	0.015	0.0076	943
2-NS	NF[c]	0.0083	0.0017	558
1-NS	0.0065	0.016	0.031	1569
2,6-NDS	0.0016	0.0030	0.0013	245
2,7-NDS	0.010	0.015	0.0093	1658
1,5-NDS	NF[c]	0.16	0.27	195

[a] The concentration factor of the pretreatment was 30:1.
[b] The sample was diluted 400-fold with UHQ water; the analysis was performed on 2 ml of the diluted solution.
[c] NF = Not found.

observed with the spectra of the 2,6-isomer, which precedes the 2,7-isomer in the elution order. In order to discriminate between these standards, the first-order derivatives of the emission spectra were also taken into account. Fig. 6 shows the first-order derivatives, in the range 300–400 nm, of the emission spectra of the unknown analyte F and of the two cited isomers. It can be seen that the derivative of the spectrum of the 2,7-isomer coincides very well with that of the unknown, whereas that of the 2,6-isomer not. Similar conclusions were reached also for the other compounds identified. It was observed that the retention times, together with the fluorescene excitation and emission spectra, can furnish specific indications about the nature of the unknown analytes found in river water.

CONCLUSIONS

An HPLC separation and a characterization method based on spectrofluorimetry was optimized for the identification of aromatic sulphonic acids

derivatives in natural waters. The method is very sensitive, it allows the spectroscopic identification of unknown compounds and it is able to discriminate even positional isomers having very similar chromatographic and spectroscopic characteristics.

In the future, further investigations will be carried out in order to study whether the compounds considered in this work can be modified in the aquatic environment.

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