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Analysis of sulfophthalimide and some of its derivatives by liquid chromatography–electrospray ionization tandem mass spectrometry

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Abstract

A system was developed for the separation of sulfophthalimide (SPI), sulfophthalamide (SPAM), sulfophthalamic acid (SPAA) and sulfophthalic acid (SPA) by ion-pair liquid chromatography and their detection by electrospray ionization tandem mass spectrometry (ESI-MS–MS). Except for SPAM, the 3- and 4-sulfo-isomers of the analytes were separated by HPLC using volatile tributylamine as ion-pairing agent. Initial fragmentations of the analytes in the negative mode involve losses of CO_2 or HNCO or condensation via H_2O or NH_3 elimination. *ortho*-Effects of the sulfonate group were recognized in the fragmentation of the respective 3-sulfo-isomers and allowed us to assign the order of elution of the SPAA isomers. Quantitative analysis of these sulfonated aromatic compounds with MRM detection was elaborated and resulted in detection limits ranging from 1 pg for SPA to 13 pg for SPAA isomers and in limits of quantification of 2–10 μg/L for 5 μL volumes of injected tap water, municipal wastewater or industrial effluents up to salt contents of 0.5–1 g/L. The method was applied to study the isomer-specific chemical and microbial transformations of SPI, which was previously shown to be formed by white-rot fungi from sulfophthalocyanine textile dyes.

Keywords: Dyes; Sulfophthalimide; Sulfophthalamide; Sulfophthalamic acid; Sulfophthalic acid

1. Introduction

Aromatic sulfonated compounds are widely used in and discharged from industrial production processes and households. Among these compounds, sulfonated dyes, linear alkylbenzenesulfonates (LAS) and naphthalenesulfonates have been widely studied. Recently, attention has been drawn to sulfonated naphthalene–formaldehyde condensates (SNFC) [1]. However, the list of sulfonated compounds being discharged into the aquatic environment is still incomplete.

The biodegradation of sulfonated compounds [2–5] and their removal from wastewater by biological means [6–9] has been studied. Biodegradability differs widely, with LAS being well degradable [6] and SNFC being removed only by abiotic processes [1]. Microbial degradation of sulfonated aromatic compounds can be highly selective and may be strongly influenced by the position of substituents as in the case of naphthalenesulfonates [3,7].

Moreover, polar and comparatively stable sulfonated compounds may be generated in natural processes, for example in the metabolism of alachlor

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[10]. It was recently shown that sulfophthalimide (SPI) is formed as a major metabolite when sulfophthalocyanine dyes are oxidized with white-rot fungi [11]. Besides intentionally leading to decolourization, this process generates new sulfonated compounds of lower molecular mass and higher polarity which need to be analyzed.

Ion-pair chromatography with tetrabutylammonium (TBA) cations and either UV or fluorescence detection is a well established analytical method for various aromatic sulfonates (reviewed in Ref. [12]). However, coupling with atmospheric pressure ionization mass spectrometry, which is highly desirable in terms of selectivity and qualitative analysis of novel sulfonates [13], demands volatile ion-pairing agents. The use of ammonium acetate or even triethylamine as mobile phase additives suffers from the limited retention of disulfonated and polysulfonated compounds [13,14]. By using tributylamine (TrBA), a versatile system that allows the detection of polysulfonated aromatic compounds under conditions well suited for MS analysis was established [15].

This ion-pair chromatography–electrospray ionization tandem mass spectrometry approach (LC–ESI-MS–MS) is used here to analyze aromatic sulfocarboxylates that originate from the degradation of sulfophthalocyanine dyes by white-rot fungi [11]. Special attention is drawn to the chromatographic separation and mass spectrometric distinction of positional isomers. The method illustrates both the separation power of ion-pair chromatography with TrBA and the potential of ESI-MS–MS as a selective detector as well as an instrument of high diagnostic value.

2. Materials and methods

2.1. Reference materials

Sulfophthalic acid (SPA) triammonium salt (technical grade, approx. 75% 4-SPA, 25% 3-SPA) was purchased from Aldrich (Milwaukee, USA) and sulfophthalimide (SPI) was synthesized by heating sulfophthalic acid triammonium salt [16] and purified by recrystallization as described elsewhere [11]. Sulfophthalamic acid (SPAA) and sulfophthalamide (SPAM) were prepared from the SPI mixture according to instructions for synthesizing the non-sulfonated homologues: SPAM was prepared by adding 25% ammonium hydroxide solution at room temperature [17,18], while SPAA was formed after adding 10% KOH within 30 min [19,20]. The solutions were neutralized, filled to a predefined volume and used without any isolation of the products.

Standard solutions at 100 mg/L concentration were prepared in ultrapure water, from which dilution was performed in ultrapure water, in tap water, in tertiary treated municipal effluent and an industrial wastewater diluted 1:10 with ultrapure water. Separate solutions for SPI were prepared with all these waters acidified to pH 3 as SPI rapidly hydrolyzes under neutral conditions [21].

2.2. Mass spectrometry

LC–MS–MS analyses were performed with a Quattro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray interface and an electrospray probe. A HP 1100 (Hewlett-Packard) liquid chromatographic system consisting of membrane degasser, binary high-pressure gradient pump, autosampler and column thermostat was coupled to the mass spectrometer. Nitrogen was provided by a nitrogen generator (Model 75-72; Whatman, Haverhill, USA) and used as drying gas (flow 550 L/h) and nebulizing gas (flow 100 L/h). For MS–MS analyses, argon (purity 5.0, Messer-Griesheim, Krefeld, Germany) was used as collision gas at a pressure of $1.3 \cdot 10^{-3}$ mbar in the collision cell.

The mass spectrometer was operated with the electrospray probe in the negative mode, with the probe capillary voltage set to 3.0 kV and the probe tip held at a temperature of 250°C. The cone voltage was set to 20 V and the source temperature was 120°C. For target analysis in the MS–MS mode (multiple reaction monitoring, MRM) a dwell time of 0.2 s per mass trace was used and a maximum of five masses were recorded in parallel. Here, cone voltages and collision energies were time programmed to ensure the highest sensitivity for each of the transitions to be monitored (see below).

2.3. Chromatography

A 150×2 mm reversed-phase column of Phenylhexyl 3 μm material (Phenomenex, Aschaffenburg, Germany) was used for separation. Eluent A was H_2O–MeOH (80:20) and eluent B was H_2O–MeOH (30:70); 5 m*M* tributylamine (TrBA; puriss+, Fluka, Buchs, Switzerland) and 5 m*M* acetic acid (HPLC quality; Malinckrodt Baker, Deventer, Netherlands) were added to both eluents. Elution was at a flow-rate of 0.2 mL/min and a temperature of 50°C with a linear gradient from 20% B (0 min) to 85% B (13 min). After 1 min of isocratic elution the gradient returned to 20% B (15 min) and the system was ready for injection after 24 min. A 5 μL sample volume was injected onto the column.

3. Results and discussion

3.1. Chromatographic separation

Sulfophthalimide and its derivatives (Fig. 1) are well separated with TrBA as ion-pairing agent under the selected conditions (Fig. 2). The order of elution of the different derivatives largely follows the number of acidic groups that are available for ion-pairing: the sulfophthalamides (SPAM) with only one acidic group elute first, followed by the sulfophthalimides (SPI), which the cyclic imide moiety renders more hydrophobic compared to the open diamide (SPAM). The sulfophthalamic acids (SPAA) with two acidic groups elute much later. Finally, the sulfophthalic acid (SPA), which bears three acidic groups, elutes last.

A major characteristic of these compounds is the occurrence of the 3-sulfo- and 4-sulfo- isomers, which is due to the sulfonation process. Only in the case of SPAM are these two isomers not chromatographically separated under the conditions selected. For SPAA a total of four isomers is found (Fig. 1) and separated (Fig. 2). The isomeric separation was less straightforward when using conventional octadecyl instead of the phenylhexyl stationary phase material. The initial distinction between the 3- and 4-sulfo-isomers was achieved by analyzing pure 3-SPI material, which was obtained from the 3-/4-SPI mixture by recrystallization [11], and its reaction products with KOH (3-SPAA) and NH_4OH (3-SPAM, 3-SPA)

3.2. Collision-induced fragmentation

For all compounds, daughter ion spectra obtained by collision-induced dissociation were recorded and characteristic fragmentations were observed (Fig. 3). The initial fragmentations of all four compounds at moderate collision energies are the loss of CO_2 and the nitrogen analogue (HNCO), respectively, as well as condensation reactions with elimination of H_2O or NH_3. The expulsion of SO_2 is observed in the later stages of fragmentation, in agreement with observations for other aromatic sulfonates [13,15]. Clearly, the fragmentation scheme shown in Fig. 3 can be divided into an ''oxygen side'' and a ''nitrogen side'', with the parallel fragments on either side showing a characteristic mass difference of 1 Da.

For SPA, either a two-fold decarboxylation towards the benzenesulfonate anion (*m*/*z* 157) occurs or a condensation to the corresponding anhydride (*m*/*z* 227) takes place. From the anhydride the sequential expulsion of either CO_2 and SO_2 (via *m*/*z* 183) or of SO_2 and CO_2 (via *m*/*z* 163) converge into *m*/*z* 119. The sulfophthalic acid anhydride anion (*m*/*z* 227) is also formed from SPAA, indicating that elimination of NH_3 is more favourable than the loss

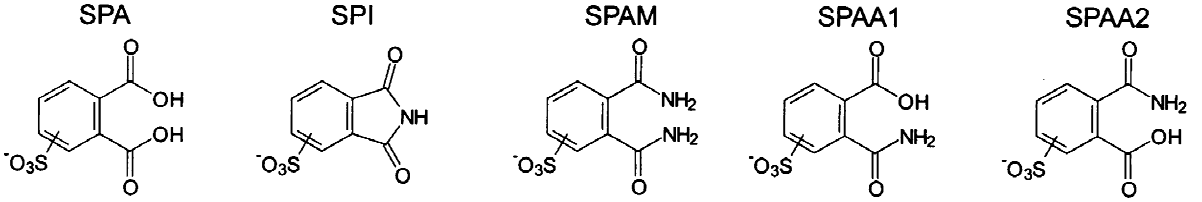

Fig. 1. Structures of the compounds under investigation.

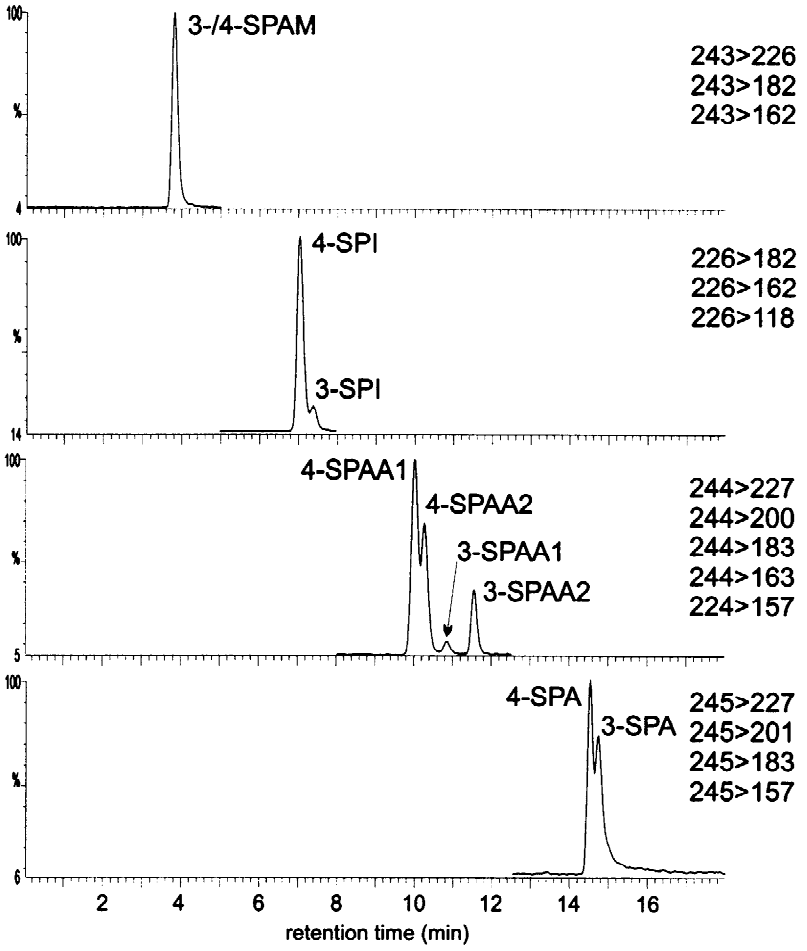

Fig. 2. Separation of sulfophthalimide and its derivatives by ion-pair chromatography and detection by MRM (ESI-MS–MS in negative mode); refer to Table 2 and Fig. 3 for the transitions indicated.

of H_2O. Along this fragmentation pathway, SPAA joins the ''oxygen side'' of the fragmentation scheme (Fig. 3). By decarboxylation (to m/z 200) SPAA switches to the ''nitrogen side''.

On the ''nitrogen side'', decarboxylation does not occur. Instead, SPAM expels HNCO and converges with the decarboxylation product of SPAA (m/z 200). This may finally lose HNCO to form the benzenesulfonate anion. The condensation reaction (neutral loss of NH_3) of SPAM leads to SPI, and similar fragments subsequently occur from both compounds. Astonishingly, the fragmentations of SPI are identical to those seen for the anhydride (m/z 227) on the ''oxygen side'', with a sequential removal of CO_2 and SO_2 and vice versa, leading via m/z 182 or m/z 162 to m/z 118. As no information on the fragmentation of any of the compounds studied here was available in the literature, the structures of some of the fragments in Fig. 3 are speculative.

The fragmentation of the non-sulfonated phthalic acid has been studied by several authors. Using fast atom bombardment in the negative-ion mode, single decarboxylation and, to some extent, a second decarboxylation was observed from the molecular anion [22]. Under electrospray ionization, two-fold decarboxylations from the dianion were recorded; elimination of water to form the anhydride anion

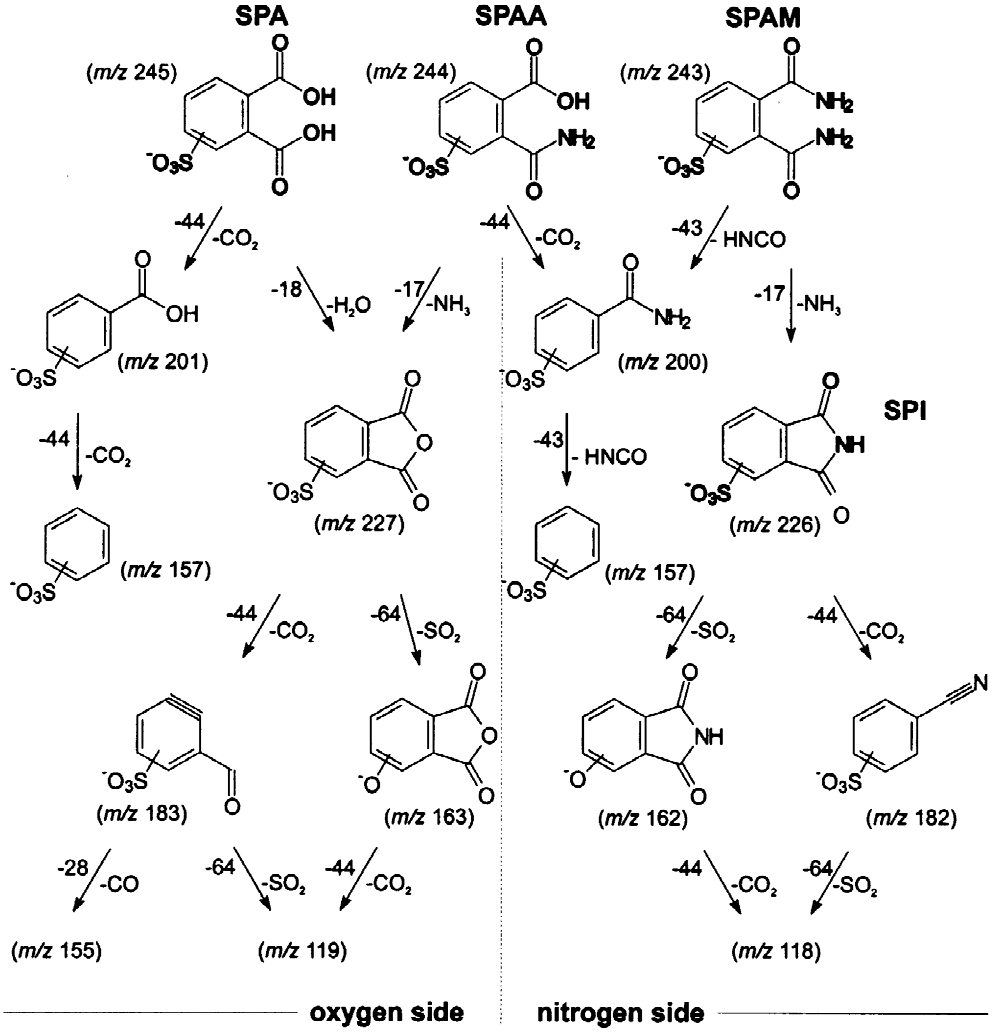

Fig. 3. Fragmentations of the molecular anions of SPA, SPAA, SPAM and SPI obtained by CID using ESI-MS–MS in the negative mode, and the suggested fragment structures.

cannot occur from the dianion [23]. However, the elimination of water dominated in a very early study using API-MS [24]. Although these fragmentations correspond to those observed for SPA in collision-induced dissociation (CID) it should be noted that the fragmentations here likely evolve from the fully protonated dicarboxylic acid, as the single charge of the SPA monoanion will be located at the most acidic group, i.e. the sulfonate moiety. Loss of NH_3, CO_2 and CO was observed in the electron-impact mass spectra of non-sulfonated phthalamic acid [25]. This, again, parallels the fragmentations in our CID mass spectra (Fig. 3).

It is noteworthy that the sulfonate isomers prefer different fragmentation pathways. As a rule of thumb, the 4-isomers of SPA, SPAA and SPAM are preferentially subjected to decarboxylation or elimination of isocyanic acid, whereas fragmentation of the 3-isomers starts with a condensation reaction. This condensation is likely due to an *ortho*-effect of the sulfonate group: the negatively charged oxygen of the sulfonate group may act as a nucleophile that,

in a five-member cyclic transition state, assists in releasing the hydroxy or amino group from the carboxylate moiety. If the sulfonate group is located in the 4-position, no *ortho*-effect occurs and sulfophthalic acid behaves like phthalic acid and decarboxylates [22,23].

3.3. Multiple reaction monitoring

From these fragmentation processes, transitions were selected that allow a sensitive detection of all the analytes (Table 1). Additionally, the relative fragment intensities allow us to distinguish between the sulfo-isomers, thereby complementing the chromatographic separation (Fig. 4). For SPI, the 4-isomer (Fig. 4a) shows intensive m/z 162 and m/z 118 fragments with m/z 182 being nearly absent, whereas 3-SPI exhibits all three fragments in comparable intensity (Fig. 4b). The pattern is more complex for the four SPAA isomers (Fig. 4c): the first 4-SPAA shows m/z 227, 200 and 163 as fragments, while the second 4-SPAA shows m/z 200 and m/z 157. The first 3-SPAA isomer shows up in the m/z 227 and m/z 183 trace and m/z 200 only is detected for the second eluting 3-SPAA isomer. In the case of SPA (Fig. 4d), the 4-isomer shows m/z 201 and 157 and the 3-isomer can be recorded at m/z 227 and 183.

The fragments observed for the two 3-SPAA isomers allow elucidation of their structure in more detail: since the first eluting 3-SPAA expels NH_3 to form sulfophthalic acid anhydride (m/z 227; Fig. 3) and since we assume the condensation reaction to be supported by an *ortho*-effect of the sulfonate group, this isomer is very much likely to be benzene-3-sulfo-2-carboxylamide-1-carboxylic acid. In the second eluting 3-SPAA, the *ortho*-effect induces decarboxylation (to m/z 200; Fig. 3), showing that now the carboxylic acid group is adjacent to the sulfonate (benzene-3-sulfo-2-carboxyl-1-carboxamide). It is then inferred that the same elution order is found for the 4-SPAA isomers. Indeed, the fragmentation parallels that for the 3-SPAAs in that the second eluting 4-SPAA decarboxylates to m/z 200, while the first eluting 4-SPAA also shows elimination of NH_3 to m/z 227 and subsequent expulsion of SO_2 (to m/z 163) (Table 1; Fig. 4).

3.4. Quantitative analysis

For all analytes except SPAM, calibration was

Table 1
Determination of SPI and its derivatives by ESI-MS–MS: molecular anions (M − H), fragments used for MRM detection (F1–F3), and fragment ratios to verify peak assignment

Subst.	M − H; CV[a] (m/z; V)	F1; CE[b] (m/z; eV)	F2; CE[b] (m/z; eV)	F3; CE[b] (m/z; eV)	F1/F2	F1/F3	F2/F3
3-SPAM		226; 20	182; 25	162; 15	[c]	[c]	
	243						
4-SPAM		226; 20	200; 20	162; 15	[c]	[c]	
4 -SPI			162; 20	118; 30			1.7
	226; 36						
3-SPI		182; 20	162; 20	118; 30	2.7	1.8	0.6
4-SPAA1		227; 15	200; 15	163; 23	3.2	1.7	0.5
4-SPAA2			200; 15	157; 18			0.7
	244; 29						
3-SPAA1		277; 15		183; 20		1.7	
3- SPAA2			200; 15				
4-SPA		201; 13	157; 20		1.4		
	245; 26						
3-SPA		227; 13	183; 15		3.9		

[a] CV, cone voltage (V).
[b] CE, collision energy (eV).
[c] Isomers not separated.

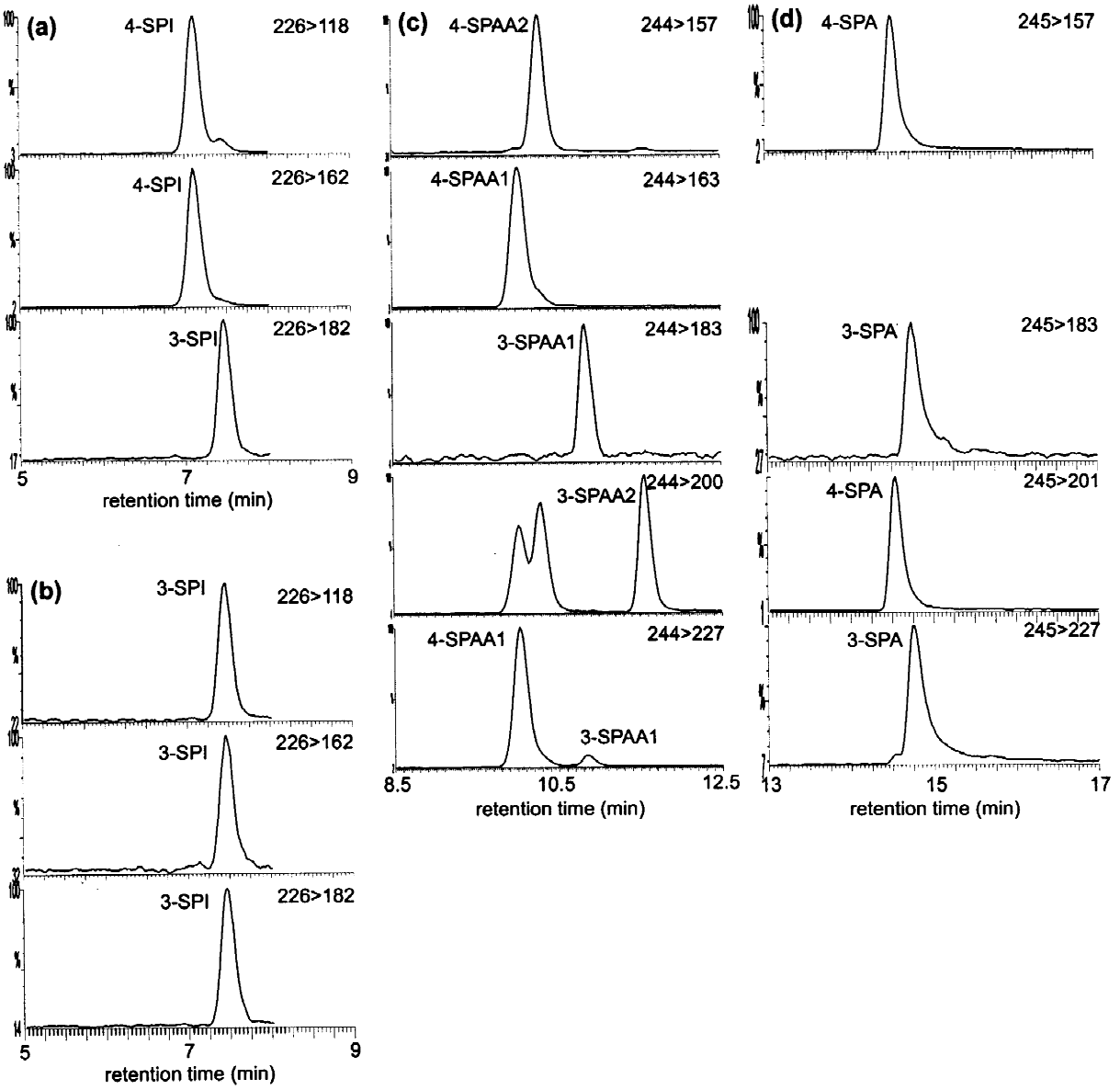

Fig. 4. Expanded chromatogram sections showing MRM detection of the isomers: (a) 3-/4-SPI; (b) pure 3-SPI; (c) SPAA and (d) SPA.

performed in the range from 0.5 mg/L to 0.05 μg/L. The SPAM obtained through synthesis was not sufficiently pure to be used for quantification and SPAM turned out to be irrelevant for the transformation processes studied later. For this reason, it was excluded from quantitative analysis. The portion of each isomer of SPI, SPAA and SPA in the respective standard mixtures was assessed by HPLC–UV analysis and detection at 232 nm, assuming that all isomers exhibit the same absorbance in the UV.

The calibration graphs obtained were linear over the concentration range tested. The limits of detection (LOD) and other results of the calibration are shown in Table 2. Detection limits ($S/N > 3$) range from 1 pg of 3- or 4-SPA injected onto the column to 13 pg for two of the SPAAs (Table 2). For sample

Table 2
Calibration of LC–ESI-MS analyses and limits of quantification for tap water, tertiary treated municipal effluent and industrial wastewater (diluted 1:10) (for MS–MS conditions, refer to Table 1)

	RT (min)	MRM traces (m/z)	LOD[a]		LOQ[b]		
			(pg)	(μg/L)	Tap (μg/L)	Tert. effl. (μg/L)	Industr. (μg/L)
4-SPI	7.1	162+118	3	0.5	2	5	10
3-SPI	7.5	182+162+118	3	0.8	2	2	5
4-SPAA1	10.0	227+200+163	11	2.1	4	4	5
4-SPAA2	10.3	200+157	13	2.6	4	4	3
3-SPAA1	10.9	227+183	13	2.6	3	3	3
3-SPAA2	11.6	200	5	0.9	2	5	2
4-SPA	14.5	201+157	1	0.2	5	5	10
3-SPA	14.8	227+183	1	0.2	5	5	6

[a] LOD, limit of detection with $S/N > 3$.
[b] LOQ, deviation between nominal and calculated concentration below 20%, with 5 μL injection volume.

volumes of 5 μL directly injected, this corresponds to LODs of 0.2 to 2.6 μg/L. These detection limits correspond to what is usually achieved in LC–MS analysis of aromatic sulfonates with volatile ion-pairing agents and MRM detection [15]. However, when detection has to be performed with single-MS, and SIM instead of MRM [13,14] has to be used, the detection limits can be considerably higher.

The robustness of the method was examined by calibration in tap water, tertiary treated municipal wastewater, and treated industrial effluent. The latter was diluted 1:10 to reduce its salt content to about 0.6 g/L of chloride and sulfate. In all three matrices the chromatography was identical and the limits of quantification (LOQ) vary only slightly with no significant trend. These results show that the method is also applicable to highly loaded industrial effluents. At much higher salt concentrations, such as in undiluted industrial effluent (3 g/L chloride, 3 g/L sulfate), both the chromatography and the MS detection became corrupted.

3.5. Application

We used this method to study the fate of SPI which is formed by white-rot fungi from sulfophthalocyanine dyes [11]. Characteristic chromatograms obtained by MRM detection are shown in Fig. 5: 3-SPI and 4-SPI were detected from a solution of Reactive Blue 15 incubated with purified enzymes of a white-rot fungus (Fig. 5b) [11], while only traces were found in the initial solution of the technical dye (Fig. 5a); these traces may be by-products or breakdown products. When a technical mixture of SPI isomers was incubated with aerobic bacteria for 5 weeks, the major portion of the organic carbon was mineralized [21], but some 3-SPA and, above all, 3-SPAA together with traces of 3-SPA remained in the solution (Fig. 5c).

These results indicate an isomer-specific microbial degradation of 4-SPI and an abiotic hydrolysis of 3-SPI to form 3-SPAA [21]. These isomer-specific conversions could only be detected due to the method presented here.

4. Conclusions

Ion-pair LC–ESI-MS–MS with TrBA as ion-pairing agent has again proven to be a powerful tool for the analysis of aromatic compounds with multiple acidic groups. For three of the four compounds investigated, full separation of the positional isomers was obtained in a reasonable analysis time. MRM detection provides both a higher sensitivity and a higher selectivity as compared to SIR detection. The complete method is robust enough to allow for direct injection of high-strength industrial wastewaters with salt contents of 0.5–1 g/L. At higher concentrations, however, dilution of the samples or SPE is required to maintain chromatographic separation and mass spectrometric detection.

The fragmentation cascades of sulfophthalic acid derivatives at moderate collision energies involve decarboxylation, expulsion of HNCO (from the carboxamide moiety) and condensation by H_2O and

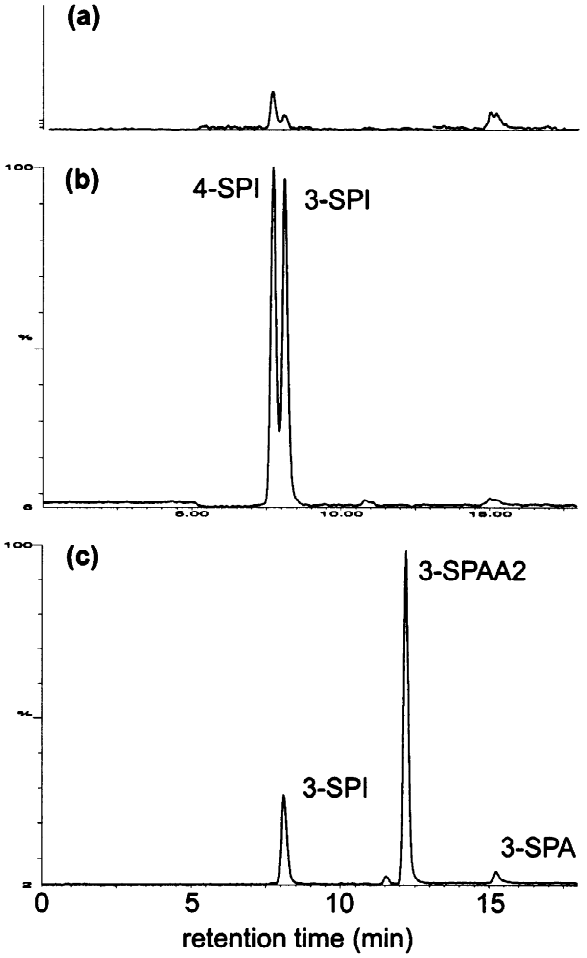

Fig. 5. MRM chromatograms of the sulfophthalocyanine textile dye Reactive Blue 15 (a) before and (b) after incubation with purified enzymes from a white-rot fungi [11] (scaled) and (c) of a solution of 3-/4-SPI after biodegradation over 35 days [21].

NH_3 elimination, respectively. The preferential fragmentations of the various isomers could be interpreted by taking into account the *ortho*-effect of the 3-sulfo-group. This allowed us to assign all positional isomers of SPAA. On this basis, isomer-specific MRM traces could be chosen. Despite this specificity of MRM detection, a chromatographic separation of isomers is advisable for reliable quantitative analysis.

This method allows us to follow the isomer-specific transformation processes of a technical SPI mixture.

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