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Detection and identification of sulphonamide drugs in municipal waste water by liquid chromatography coupled with electrospray ionisation tandem mass spectrometry

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Abstract

High-performance liquid chromatography coupled with positive-ion electrospray ionisation tandem mass spectrometry was used for the determination and confirmation of 13 sulphonamide drugs in environmental water samples in the low ng/L-range. Enrichment with concentration factors of 130–670 was performed by solid phase extraction, achieving recoveries of 50 to 90%. After gradient elution HPLC, detection and quantification was performed using selected reaction monitoring (SRM) with limits of detection between 0.2 and 3.7 μg/L. Confirmation was obtained by either SRM transitions of collision induced dissociation reactions or daughter ion mass spectra. Primary and secondary effluents of municipal waste water treatment plants and different surface waters were examined. The compounds sulphamethoxazole and sulphadiazine were detected and confirmed with concentrations ranging between 30–2000 ng/L and 10–100 ng/L, respectively. The compound sulphamethizole was detected in low amounts but could not be positively confirmed.

Keywords: Water analysis; Environmental analysis; Sulphonamides

1. Introduction

Bacteriostatic sulphonamide (sulpha) drugs are used in the treatment of infections in livestock [1] and to a lesser extend in the treatment of human infections [2] such as bronchitis and urinary tract infection. Widespread use of sulpha drugs in factory farming without proper withdrawal periods led to accumulation of sulphonamides in meat, eggs and milk as well as in fish [3–5]. Because of the possible risk of resistance development in humans, the legal concentration limits for sulphonamides were set to 100 μg/kg in edible animal tissue and 10 μg/L in milk (valid for the EU and the USA). The low concentration limits led to widespread activities to develop fast and sensitive methods to screen foodstuffs for sulphonamide drugs.

Sample clean-up and enrichment is mostly done by liquid–liquid extraction (LLE) [6,7], although in some cases solid-phase extraction (SPE) is used [8–10]. In the first half of the 1990s, high-performance liquid chromatography with UV detection (HPLC–UV) was widely applied [3,8,11]. Analysis by gas chromatography–mass spectrometry after derivatisation (GC–MS [12,13], GC–MS–MS [14]), GC with electron-capture detection [14] and thin-layer chromatography [10] have also been used for the determination of sulphonamides.

Henion et al. developed a direct liquid introduction HPLC–MS–MS (atmospheric pressure ioni-

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sation, API) method to detect four sulpha drugs in racehorse urine and plasma as early as 1982 [15]. In 1986 Finlay et al. reported the detection of five sulpha drugs with the use of a moving belt MS–MS technique [16]. However, HPLC–MS and HPLC–MS–MS techniques became favoured only in the last few years [6,7,17–20]. Detection of positive ions is preferred, because the yield is three-fold higher than for negative ions [21]. Instead of HPLC, some authors also used capillary electrophoresis coupled with MS [22], MS–MS and even MS–MS–MS [23].

Depending on the sulpha drug applied, up to 80% is being released from the body with the urine [24]. Drugs used in livestock treatment are dispersed with the sewage on fields and can reach ground water. Sulphonamides applied in human treatment are discharged into the receiving water via municipal waste water treatment plants (STPs). Thus, sulpha drugs are potential organic micropollutants in water. The fate of sulphonamides, among other pharmaceuticals, in different environmental compartments has recently been reviewed by Halling-Sørensen et al. [25]. Screening analysis by HPLC–MS–MS [26] proved the occurrence of sulphamethoxazole, the most commonly used sulphonamide in human treatment in municipal waste water and surface water. To date, concentration levels of sulpha drugs are no issue of governmental regulation in drinking water production.

This report describes the analysis of sulpha drugs in effluents from waste water treatment plants and surface waters by SPE, HPLC–UV and HPLC–MS–MS with electrospray ionisation. The combination of high enrichment factors in the SPE with the very selective detection by selected reaction monitoring (SRM) allows for determination of sulpha drugs from levels of 0.3–30 ng/L. Additionally, structural confirmation was received by daughter ion scans and SRM with supplementary transitions.

2. Experimental

2.1. Chemicals

Sulphonamide standards were obtained from Sigma (Deisenhofen, Germany). Stock solutions of all standards were prepared by dissolving 20 mg of each sulpha drug in 20 mL methanol. Trifluoracetic acid (TFA) was purchased from Fluka (Deisenhofen, Germany). Methanol (gradient grade), hydrochloric acid (analytical-reagent grade) and sodium hydroxide (analytical-reagent grade) were obtained from Merck (Darmstadt, Germany). Formic acid and acetonitrile (Baker ultra gradient grade) were purchased from Fisher (Berlin, Germany). An Elga water purification system (Ubstadt-Weiher, Germany) was used to further purify deionised water. For pH adjustment HCl or NaOH and a WTW microprocessor pH 96 pH-meter (Weilheim, Germany) were used.

2.2. Sampling

Grab samples of primary and secondary effluent from municipal sewage treatment plants in Berlin, Germany, as well as surface water, were filtered over 0.45-μm cellulose nitrate membranes (Sartorius, Göttingen, Germany). If not analysed immediately, the samples were stored in dark at 4°C.

2.3. Sample extraction

Sample extraction was performed on a Visiprep 24 DL extractor (Supelco) using 200 mg LiChrolut EN SPE cartridges (Merck). After conditioning of the cartridges with 5 ml of each methanol, methanol–water (1:1) and water (pH 2.5), the samples, with a pH adjusted to 2.5, were percolated with a flow rate of 200 mL/h. The loaded cartridges were washed with 2–5 mL water (pH 2.5) and then eluted with 3 mL methanol–water (1:1) and 10 mL methanol. The eluates were combined and concentrated to approximately 1.5 mL using a rotating condensor Speedvac Plus SC110A (Savant by Thermoquest, Egelsbach, Germany). 40 μL of diluted (1:10) trifluoroacetic acid and in some cases 300 μl methanol were added to maintain homogeneity. Internal standard (I.S.) sulphaphenazole was added after sample preparation to compensate for variations in volume. Sulphaphenazol is used as standard because it is not used in Germany and is thus not expected in the samples.

Sample volumes of 200–1000 mL were extracted, leading to enrichment factors ranging between 130 and 670. Recovery experiments with sulphonamide concentrations between 1 μg/L and 100 μg/L were

carried out in secondary effluent and ultrapure water. In the case of secondary effluent, recovery values were corrected by blank value subtraction.

Tests to further purify samples were carried out using sequential extraction with 500 mg LiChrolut C18e extraction cartridges (Merck) prior to 200 mg LiChrolut EN SPE cartridges. For the first extraction step, the sample pH was adjusted to 8.5. The filtrate from this step was then treated as described above.

2.4. Liquid chromatography

A liquid chromatograph (Hewlett-Packard series 1100, Waldbronn, Germany) equipped with a vacuum solvent degassing unit, a binary high-pressure gradient pump, an automatic sample injector, and a column thermostat was used for all separations. UV was controlled either by a HP 1100 diode array detection (DAD) system or a HP 1100 variable-wavelength detector set to 260 nm.

The conditions for HPLC–UV analysis were as follows: 2 minutes isocratic 0% B, followed by a gradient of 40% B in 28 minutes. The column was flushed by a further increase to 80% B in 1 minute and reequilibrated at 0% B in 6 minutes. Solvent A was acetonitrile–water (3:97) and solvent B was acetonitrile–water (80:20), both containing 0.05% TFA (w/v). The column employed was a Supelcosil ABZ+ (Supelco) 250×2.1 mm with 5 μm particle size, with a 20×2 mm pre-column of the same material. Column temperature was maintained at 45°C and a flow rate of 0.3 mL/min was employed. Sample volumes of 20 μL were injected.

Separation in the case of MS detection was accomplished using a 250×2 mm Supersphere RP18 endcapped column, with a particle size of 4 μm, (Knauer, Berlin, Germany) operated with a pre-column 20×2 mm containing the same sorbent. Sulphonamides were chromatographed at a column temperature of 45°C and a flow rate of 0.3 mL/min. The gradient was programmed from 0 to 50% B in 30 minutes and then, to clean the column, to 100% B in 2 minutes. Reequilibration time at 0% B was 7 minutes. Solvent A was acetonitrile–water (3:97) and solvent B acetonitrile–water (75:25), both containing 1% formic acid (v/v). Sample volumes of 10 or 20 μL were injected.

2.5. Mass spectrometry

A Quattro LC (quadrupole–hexapole–quadrupole) mass spectrometer with a orthogonal Z-spray-electrospray interface (Micromass, Manchester, UK) was used. Drying gas as well as nebulising gas was nitrogen generated from pressurised air in a Whatman Model 75-72 nitrogen generator (Whatman, Haverhill, USA). The nebuliser gas flow was set to approximately 85 L/h and the desolvation gas flow to 850–950 L/h. Infusion experiments were performed using a Model 11 single syringe pump (Harvard, Holliston, USA), directly connected to the interface. For simultaneous detection by DAD and MS, the outlet of the UV detector was connected to the interface.

For operation in the MS–MS mode, collision gas was argon 5.0 (Messer, Berlin, Germany) with a pressure of $1.3 \cdot 10^{-3}$ mbar in the collision cell. The interface temperature was set to 300°C and the source temperature to 100°C, with the cone voltage (CV) maintained at 40 V for all transitions. Collision energies (CEs) of 15 or 18 eV and dwell times between 0.35 and 0.5 s/scan were chosen. Collision induced dissociation (CID) transitions in SRM mode and scan ranges in daughter ion mode are given in Results and discussion.

3. Results and discussion

3.1. Sample extraction

The structures of the compounds studied are shown in Fig. 1.

The pK_a values of the examined sulpha drugs vary between 5.5 (sulphaquinoxaline, No. 14) and 10.4 (sulphanilamide, No. 1) [27]. To achieve high recoveries on the polymeric extraction material, the samples were acidified to pH 2.5 to obtain the undissociated forms. Recoveries were determined using ultrapure water and secondary effluent spiked with sulphonamides (Table 1). As expected, recoveries are much better for high than for low concentrations. Effects of matrix composition and volume were tested at low concentrations: recoveries of 1 μg per compound in 1000 mL ultrapure water were higher than under the same conditions in

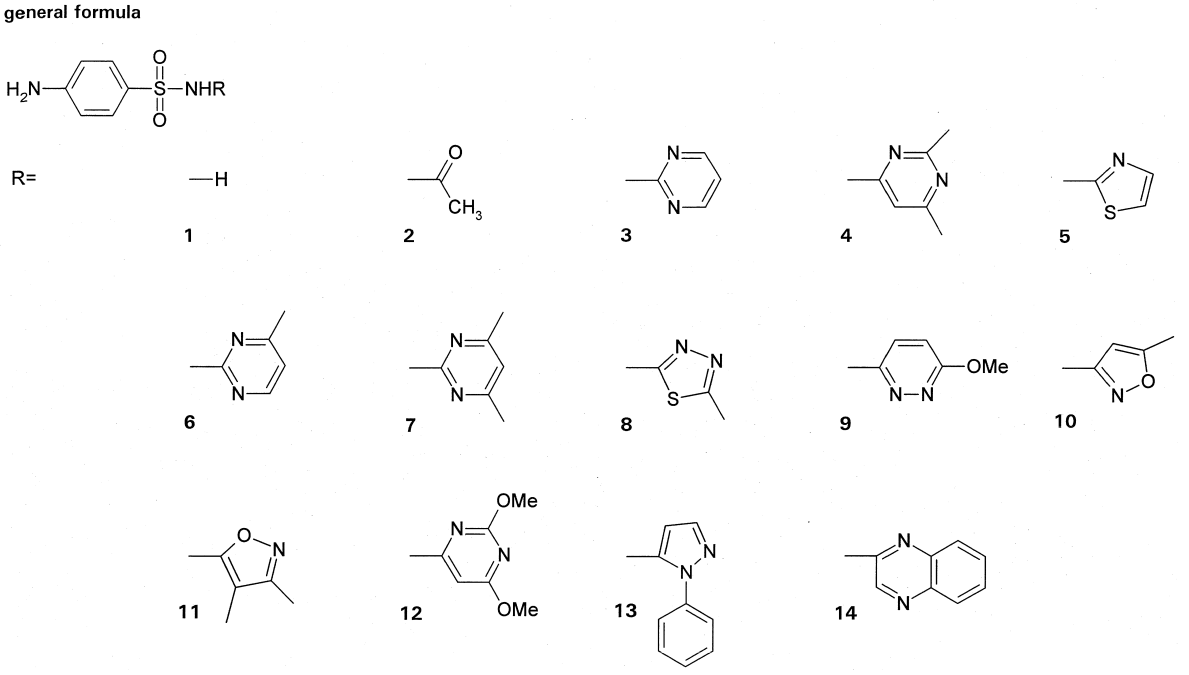

Fig. 1. Structures of the studied compounds. For compound names, refer to Table 1.

Table 1
Studied compounds: recoveries in ultrapure water and secondary effluent

No.	Compound	Recovery±R.S.D.[a]				
		30 μg/800 mL; 37.5 μg/L ultrapure water[b]	30 μg/800 mL; 37.5 μg/L secondary effluent[b]	1 μg/1 L; 1 μg/L ultrapure water[c]	1 μg/1 L; 1 μg/L secondary effluent[c]	1 μg/200 mL; 5 μg/L secondary effluent[c]
1	Sulphanilamide	32±2	18±3	29±1	40±9	34±3
2	Sulphacetamide	99±4	79±12	87±1	32±4	40±3
3	Sulphadiazine[d]	92±4	94±13	93±5	49±6	61±3
4	Sulphisomidine	87±5	89±13	100±3	71±8	72±3
5	Sulphathiazole	81±4	83±13	93±7	70±13	76±5
6	Sulphamerazine	97±5	101±16	90±7	60±8	62±6
7	Sulphamethazine	89±5	85±13	88±7	68±7	75±4
8	Sulphamethizole	80±4	75±12	77±3	48±5	58±3
9	Sulphamethoxypyridazine	82±5	84±13	82±2	64±7	72±4
10	Sulphamethoxazole[d]	98±5	96±13	99±2	81±12	77±4
11	Sulphisoxazole	94±4	93±11	94±1	71±8	74±4
12	Sulphadimethoxine	80±5	82±12	88±2	93±11	90±5
13	Sulphaphenazole (I.S.)					
14	Sulphaquinoxaline	85±5	72±9	78±2	71±8	82±5

[a] $n=5$.
[b] Quantified using UV detection.
[c] Quantified using SRM detection.
[d] Since it was not possible to obtain uncontaminated secondary effluent, recovery values had to be corrected by blank value subtraction.

secondary effluent. Enrichment of 1 μg in 200 mL secondary effluent showed only slightly better rates when compared to enrichment of 1 μg out of 1000 mL. Thus, when examining environmental samples 1000 ml were enriched to ensure higher absolute amounts for confirmation purposes.

The recoveries were generally high with exception of sulphanilamide (No. 1) and sulphacetamide (No. 2). For sulphanilamide, breakthrough was tested by using two stacked EN cartridges. A solution of 60 μg in 1000 ml secondary effluent was extracted. Sulphanilamide (No. 1) was found in the lower cartridge in a higher concentration (12 μg).

No experiments testing the recoveries in surface water were performed. The dissolved organic carbon (DOC) in secondary effluent is at least twice as high as in surface water. Given that the main influence on recovery rates is competitive adsorption, recoveries are not likely to be lower in surface water than in secondary effluent.

The prepurification step to reduce the amount of chemical noise in the UV detection (see Experimental for details) exhibited recoveries in the same range. However, the UV chromatograms of the two-step extraction did not did not differ from those obtained by the one-step extraction, proving that neutral and basic compounds do not contribute to the background matrix. Therefore, no further experiments were conducted in this direction.

3.2. HPLC–UV

Samples were acidified to avoid precipitation and occlusion of analytes.

For UV detection external calibration was applied. Linear calibration curves of standards in ultrapure water with concentrations of 0.1, 0.5, 1, 5, 10 and 50 mg/L were obtained. Regression coefficients were above $r^2=0.9998$. Limits of detection (LODs) and limits of quantification (LOQs) were calculated according to the German norm DIN 32645 [28]. LODs ranged between 0.2 mg/L and 0.6 mg/L and LOQs between 0.6 mg/L and 1.7 mg/L, both determined in ultrapure water (Table 2). For enriched secondary effluent, the LOD, together with the LOQ, increased five- to 10-fold, not allowing for quantification in the μg region. Water samples were therefore analysed by HPLC–MS–MS.

Although better separation was achieved with TFA as modifier, formic acid was used with MS detection, as TFA was found to reduce the sensitivity of the MS detection by a factor of 5. The accompanying change in pH led in spite of a change of column material to coelution of a pair of sulphonamides (Nos. 4 and 5) (Fig. 2).

3.3. MS: detection and quantification

Sulphonamide standards were used to obtain mass

Table 2
Retention times, mass spectral data, LOD and LOQ values for UV and SRM detection

No.	Compound	t_R (min)		$[M+H]^+$	$[B+H]^+$	UV		SRM	
		UV	MS			LOD (mg/L)	LOQ (mg/L)	LOD (μg/L)	LOQ (μg/L)
1	Sulphanilamide	4.10	3.75	173	–	0.4	1.1	3.7	10.2
2	Sulphacetamide	8.35	8.35	215	60	0.2	0.6	3.5	9.6
3	Sulphadiazine	9.74	9.94	251	96	0.3	0.8	0.3	0.8
4	Sulphisomidine	10.50	11.42	279	124	0.3	0.8	3.2	8.8
5	Sulphathiazole	12.35	11.55	256	101	0.4	1.1	1.3	3.6
6	Sulphamerazine	12.72	12.70	265	110	0.4	1.1	0.6	1.7
7	Sulphamethazine	15.17	15.09	279	124	0.5	1.4	1.3	3.6
8	Sulphamethizole	17.50	15.91	271	116	0.4	1.1	0.2	0.6
9	Sulphamethoxypyridazine	16.95	16.31	281	126	0.2	0.6	1.0	2.8
10	Sulphamethoxazole	22.10	20.46	254	99	0.2	0.6	0.9	2.5
11	Sulphisoxazole	24.51	22.22	268	113	0.4	1.1	1.3	3.6
12	Sulphadimethoxine	28.02	25.73	311	156	0.6	1.7	1.0	2.8
13	Sulphaphenazole (I.S.)	28.87	25.87	315	160	0.3	0.8	1.2	3.3
14	Sulphaquinoxaline	29.30	26.24	301	146	0.6	1.7	0.8	2.2

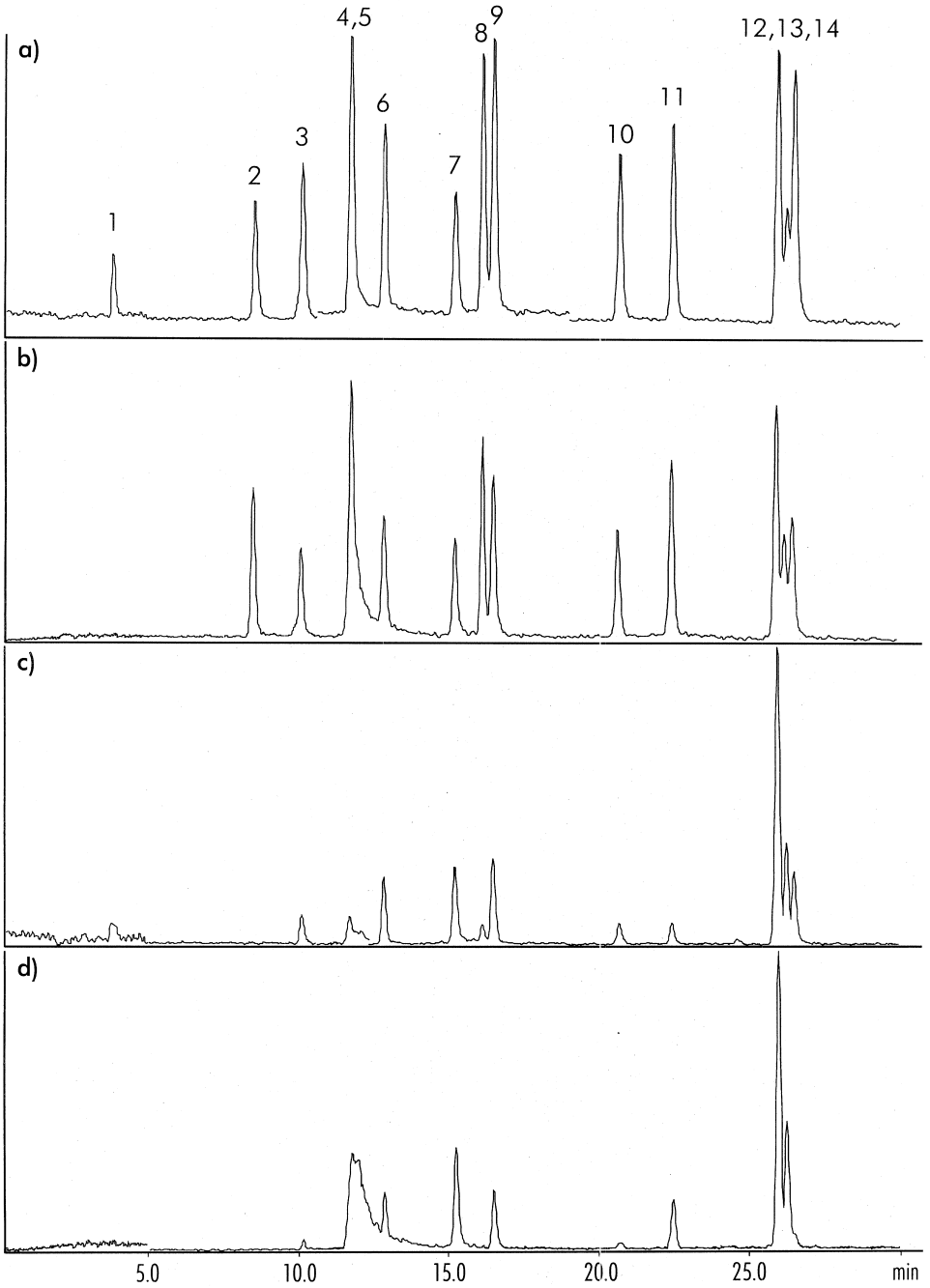

Fig. 2. Separation of a 5 μg/L standard mixture of 14 sulphonamides in ultrapure water with SRM detection. (a) Total ion chromatogram of SRM fragmentations $[M+H]^+ \rightarrow m/z$ 156 and m/z 156→m/z 92 for detection and quantification. (b) Total ion chromatogram of SRM fragmentations $[M+H]^+ \rightarrow [B+H]^+$ and m/z 156→m/z 92. (c) SRM traces $[M+H]^+ \rightarrow m/z$ 156 of (a) for confirmation purposes. (d) SRM traces $[M+H]^+ \rightarrow [B+H]^+$ of (b) for confirmation purposes. For peak assignment, refer to Fig. 1 and Table 1.

spectra and to optimise MS parameters by infusion experiments. The examined sulphonamides differ in their heterocyclic base, but they all exhibit the same sulphonamide unit (see Fig. 1). Consequently, the CID mass spectra are characterised by the same fragmentation pattern throughout this compound class, which results in very simple and easily interpretable spectra [6,7]: they show class specific ions at m/z 92, 108, and 156 plus a base specific fragment $[B+H]^+=[M+H]^+-m/z$ 156 which corresponds to the heterocyclic base moiety of the compounds (Fig. 3). An exception is sulphanilamide (No. 1), which does not contain a $[B+H]^+$ fragment.

Time scheduled SRM windows contained up to four transitions with dwell times of 0.35 s/scan in the case of four and 0.5 s/scan when using fewer transitions. Each detection window contained the m/z 156→m/z 92 reaction plus up to three $[M+H]^+$→m/z 156 reactions. Since the CEs within groups of reactions turned out to be similar they were not optimised for every individual transition but merely for groups of reactions: the CEs of the $[M+H]^+$→m/z 156 reactions were set to 18 eV and the CE of the m/z 156→m/z 92 reaction to 15 eV. Although CEs could be further optimised for individual transitions [6], the obtained sensitivity was found to be sufficient for most examined sulphonamides. The lower sensitivities observed for sulphanilamide (No. 1), sulphacetamide (No. 2) and sulphadiazine (No. 3) was attributed to suppressed ionisation due to the high water content at the beginning of the gradient [29]. To enhance sensitivity further, the resolution of the second quadrupole was reduced to less than single mass resolution ($R=m/\Delta m=98$ as measured for the m/z 156 fragment of sulphamethoxazole).

Quantification was performed using external calibration. Calibration curves were obtained by diluting standards of 1, 5, 10, 50, 100, 500 and 1000 μg/L in ultrapure water. Curves were second-order with regression coefficients of $r^2>0.996$ to $r=0.998$. LODs and LOQs [28] ranged from 0.2 μg/L to 3.7 μg/L and from 0.6 μg/L to 10.2 μg/L, respectively, measured in ultrapure water (Table 2). Day-to-day changes in sensitivity were equalised by comparison of I.S. solutions. Daily precision was determined with six consecutive injections of a standard mixture. Standard deviation ranged from 1 to 5%.

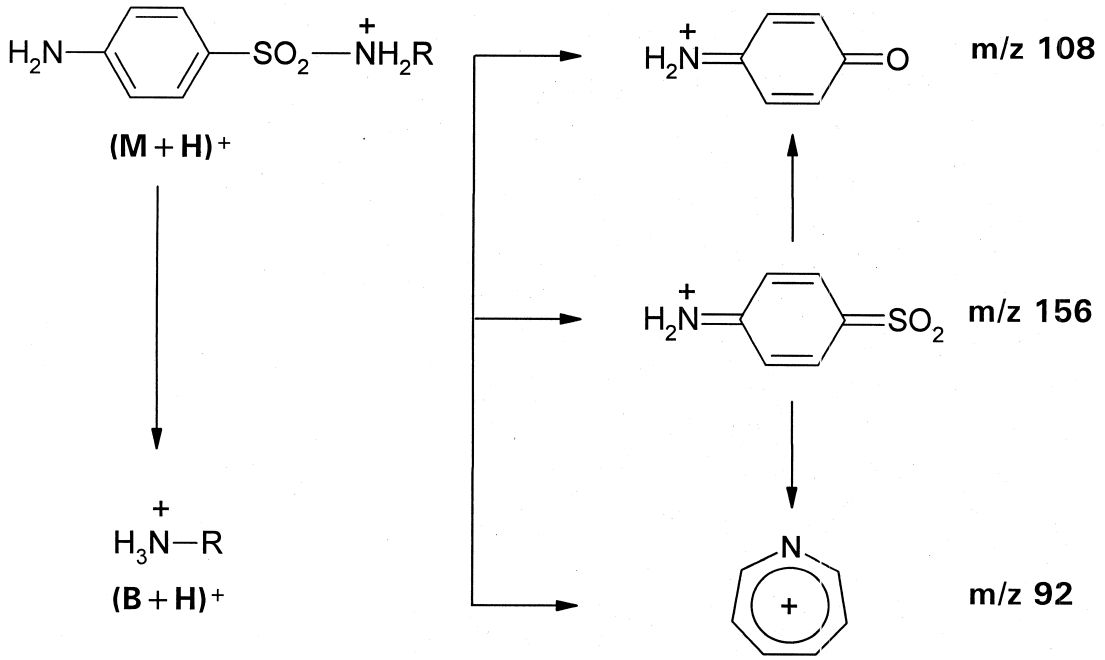

Fig. 3. Common CID fragmentation reactions for sulphonamide drugs.

3.4. MS: confirmation

3.4.1. Daughter ion scans

The most conclusive way of structural confirmation with HPLC–MS–MS is the recording of time scheduled product ion spectra and comparison to standard spectra obtained under the same conditions. For this experiment, the first quadrupole was set to $[M+H]^+$, while the second quadrupole scanned a mass range from m/z 50 to the mass of the precursor ion. This set-up however leads to a reduced sensitivity as compared to SRM, thus, small amounts of detected compounds cannot be confirmed with certainty. An example for positive identification is shown in Fig. 4.

3.4.2. SRM

For confirmation at a lower concentration level, time scheduled SRM with transitions $[M+H]^+ \rightarrow [B+H]^+$ was applied. Presence of CID reaction $[M+H]^+ \rightarrow [B+H]^+$ in connection to the m/z 156$\rightarrow m/z$ 92 fragmentation was examined. The CE of the m/z 156$\rightarrow m/z$ 92 reaction was set as above, while the CEs of the $[M+H]^+ \rightarrow [B+H]^+$ reactions were set to 18 eV. Positive confirmation was assumed if ratios of peak areas corresponded to ratios obtained by standards. Although the detection sensitivity of the $[M+H]^+ \rightarrow [B+H]^+$ transitions is somewhat lower than the sensitivity of the $[M+H]^+ \rightarrow m/z$ 156 transitions, confirmation is still possible at the low μg/L level, as shown in the next section.

3.5. Analysis of municipal secondary effluents and surface waters

Analysis of municipal secondary effluent and surface water samples from different sources revealed the suitability of the above described method.

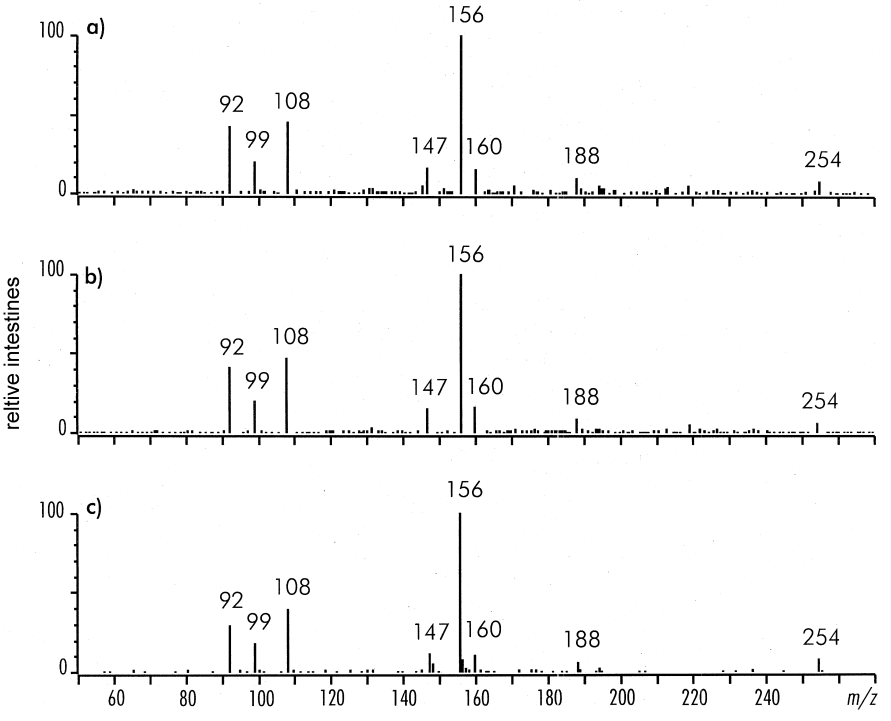

Fig. 4. Confirmational mass spectra of sulphamethoxazole (No. 10), obtained by time scheduled daughter ion scans. (a) 1.5 μg/L sulphamethoxazole in a secondary effluent sample (1 L, enriched by factor 530). (a) One litre enriched secondary effluent, spiked with 1 μg/L sulphamethoxazole. (c) 500 μg/L sulphamethoxazole standard in ultrapure water.

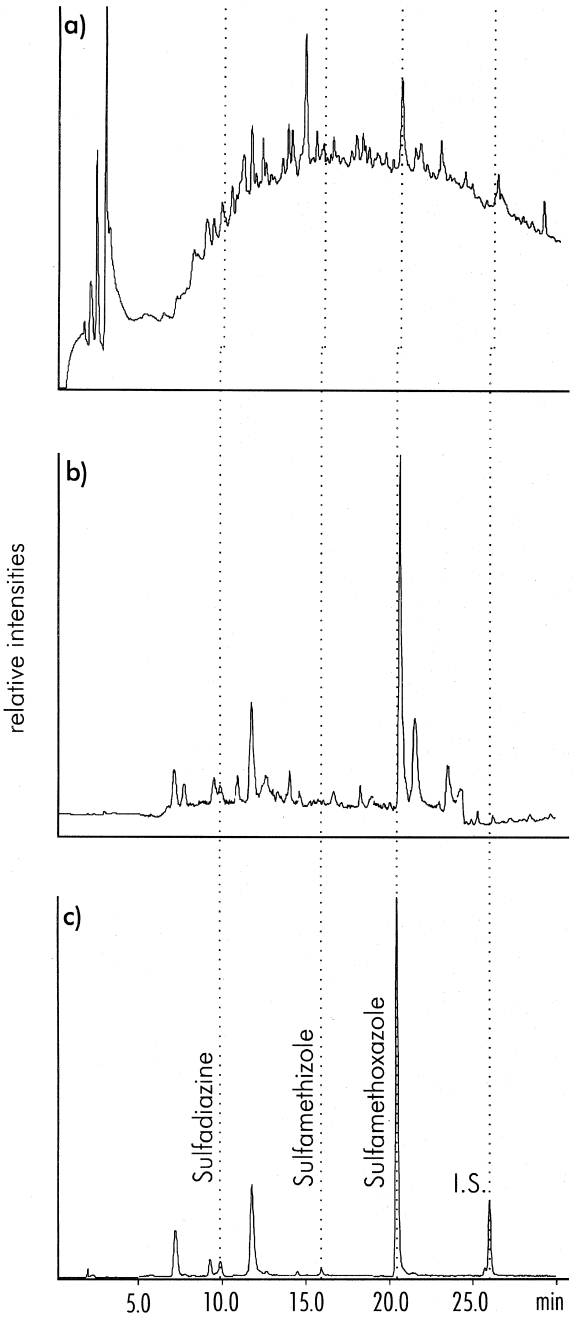

Fig. 5. Comparison of detection modes and detection of three sulpha drugs in secondary effluent of a STP in Berlin, Germany (1 L, enriched by factor 530). (a) 260 nm trace of DAD. (b) Total ion chromatogram of time scheduled SIM with $[M+H]^+$ and m/z 156 traces, CV set to 40 V and dwell times to 0.4 s/scan. Detection windows were adopted from the SRM method. (c) Time scheduled SRM (total ion chromatogram as in Fig. 2a). For peak assignment, refer to Fig. 1 and Table 1.

Comparison of the different detection methods UV, single ion monitoring (SIM) with HPLC–MS and SRM for secondary effluent highlights the differences in sensitivity and selectivity (Fig. 5). The enriched matrix is too complex for UV detection. While SIM, which is used by some authors [19], is one-order of magnitude more sensitive than SRM, it is not as selective. In most examined samples, sulphamethoxazole (No. 10) and sulphadiazine (No. 3) could be unambiguously identified (Table 3). In the case of sulphamethoxazole, concentrations were sufficient to obtain daughter ion mass spectra (Fig. 4), while for sulphadiazine, SRM of the $[M+H]^+ \rightarrow [B+H]^+$ transition was utilised (Fig. 6).

Sulphamethizole (No. 8) was detected in amounts near to the detection limit in one STP (Fig. 5c). No daughter ion mass spectra could be recorded and the sensitivity of the $[M+H]^+ \rightarrow [B+H]^+$ fragment of this compound proved to be too low for positive confirmation.

While the first two compounds are known to be applied in human medication, sulphamethizole is not available as a single agent drug in Germany. Therefore it remains unclear, whether sulphamethizole is

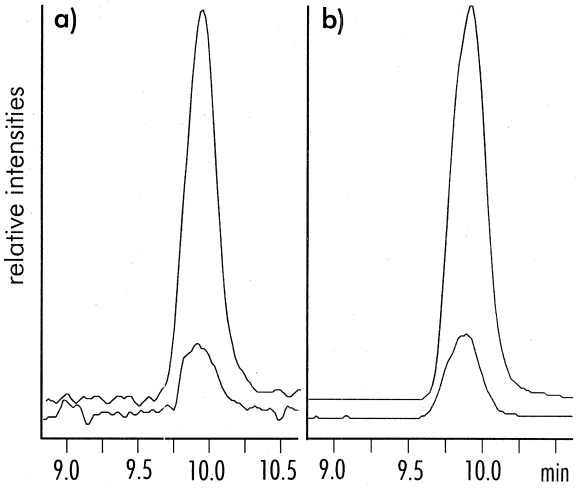

Fig. 6. Confirmation at low concentration levels: comparison of peak area ratios for transitions $[M+H]^+ \rightarrow m/z$ 156 (large peaks) and $[M+H]^+ \rightarrow [B+H]^+$ (small peaks) for sulphadiazine (No. 3) in secondary effluent. (a) 80 ng/L sulphadiazine in secondary effluent (200 mL, enrichment factor 110). (b) 1 L enriched secondary effluent, spiked with 1 μg/L sulphadiazine.

Table 3
Examined water samples from rivers and waste water treatment plants

Matrix	Location	Sulphamethizole (ng/L)	Sulphadiazine (ng/L)	Sulphamethoxazole (ng/L)
Surface water[a]	Elbe/Stade	–	–	30
	Elbe/Brunsbüttel	–	–	30
	Oder/Schwedt	–	–	60
	Tegeler Fliess/Berlin	–	7	85
	Panke/Berlin	–	–	–
Secondary effluent	STP I/Berlin[b]	–	26±2	300±12
	STP II/Berlin[c]	6±4	81±48	1500±320
Primary effluent	STP II/Berlin[b]	5±15	100±85	2460±500

[a] Single determination.
[b] 90% confidence interval, $n=2$.
[c] 90% confidence interval, $n=3$.

applied in sufficient quantity to explain the detected amounts.

4. Conclusions

The occurrence of sulphamethoxazole and sulphadiazine in secondary effluents and surface waters together with sulphamethizole in one secondary effluent shows the importance of sulpha drugs as organic micropollutants. The findings of this study prove the suitability of the established method to determine small amounts of sulpha drugs from highly complex environmental matrices. SPE enrichment factors of more than 600 can be achieved without major losses in recovery. HPLC–MS–MS in the SRM and daughter ion scan mode provides a powerful tool to confident determination and confirmation, allowing for analysis at concentration levels between 0.2 and 3.7 μg/L. Extension of the described method to other environmental matrices like waste waters from treated livestock should be easily possible.

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