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Determination of 19 sulfonamides in environmental water samples by automated on-line solid-phase extraction-liquid chromatography–tandem mass spectrometry (SPE-LC–MS/MS)

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ABSTRACT

The present study describes the development, validation and a practical application of a fully automated analytical method based on on-line solid-phase extraction-liquid chromatography–tandem mass spectrometry (SPE-LC–MS/MS) for the simultaneous determination of 19 sulfonamides, including one acetylated metabolite, in different water matrices. MS/MS detection was carried out in a quadrupole-linear ion trap (QqLIT) mass analyzer. Target compounds were identified in the selected reaction monitoring (SRM) mode, recording two transitions between precursor ions and the two most abundant product ions. The method developed was applied to evaluate the occurrence of the target antibiotics in different water samples: influent and effluent water from waste water treatment plants (WWTP), ground water and surface water. Under optimal conditions, the method detection limits achieved were in the range 0.05–7.84 ng/L for WWTP influent water, 0.01–6.90 ng/L for WWTP effluent water, 0.02–5.13 ng/L for ground water and 0.02–4.52 ng/L for surface water samples. The instrumental repeatability, expressed as RSD, was usually below 10% for the different water matrices. Results showed the wide presence of sulfonamides in the four types of water, including one acetylated metabolite, with maximum concentrations up to 855 ng/L corresponding to sulfapyridine in an influent waste water sample near a densely populated urban area.

1. Introduction

The continuous improvement of analytical methodologies has lowered the limits of detection for a wide array of trace xenobiotics, including pharmaceuticals, in environmental matrices, making their presence, despite at low concentrations, evident during the last few years [1].

Several tonnes of human and veterinary pharmaceuticals are used in Europe every year. Together with their metabolites, remnants of the active compounds (which have not been fully assimilated within the organism) end up in rivers and other natural systems. Waste water treatment plants (WWTPs) can be considered as main contributors to the presence of these substances in the environment, since all the residues from the different pharmaceutical discharges may gather in sewage waters. Besides, some of these compounds show low removal during waste water treatment processes, unspecific for these kind of molecules, and therefore are able to reach surface waters and other environmental matrices afterwards [2].

Since their discovery, antibiotics have been widely used in both human and veterinary medicine, farming and aquaculture, being the estimated total antibiotic market consumption world-wide between 100 000 tonnes and 200 000 tonnes [3]. Sulfonamides represent one of the most commonly used families of antibiotics in veterinary medicine. Although they were frequently applied as human medicines to treat many kinds of infection, nowadays much higher quantities are applied to treat and prevent infectious diseases in livestock and intensive cattle farming. The increase in the number of these confined animal feeding operations, which often lack proper waste management practices, is becoming a serious environmental problem as it constitutes one of the main release sources of these antibiotics in the natural media [4]. Previous studies have demonstrated that residues of sulfonamides (i.e., sulfathiazole and sulfamethazine) were present in manure in levels up to 12.4 mg/kg [5,6]. The excretion of faeces and urine from the medicated animals and the subsequent application of the contaminated manure as fertilizer into agricultural lands are among the major routes through which sulfonamides enter the environment. As they are weak acids

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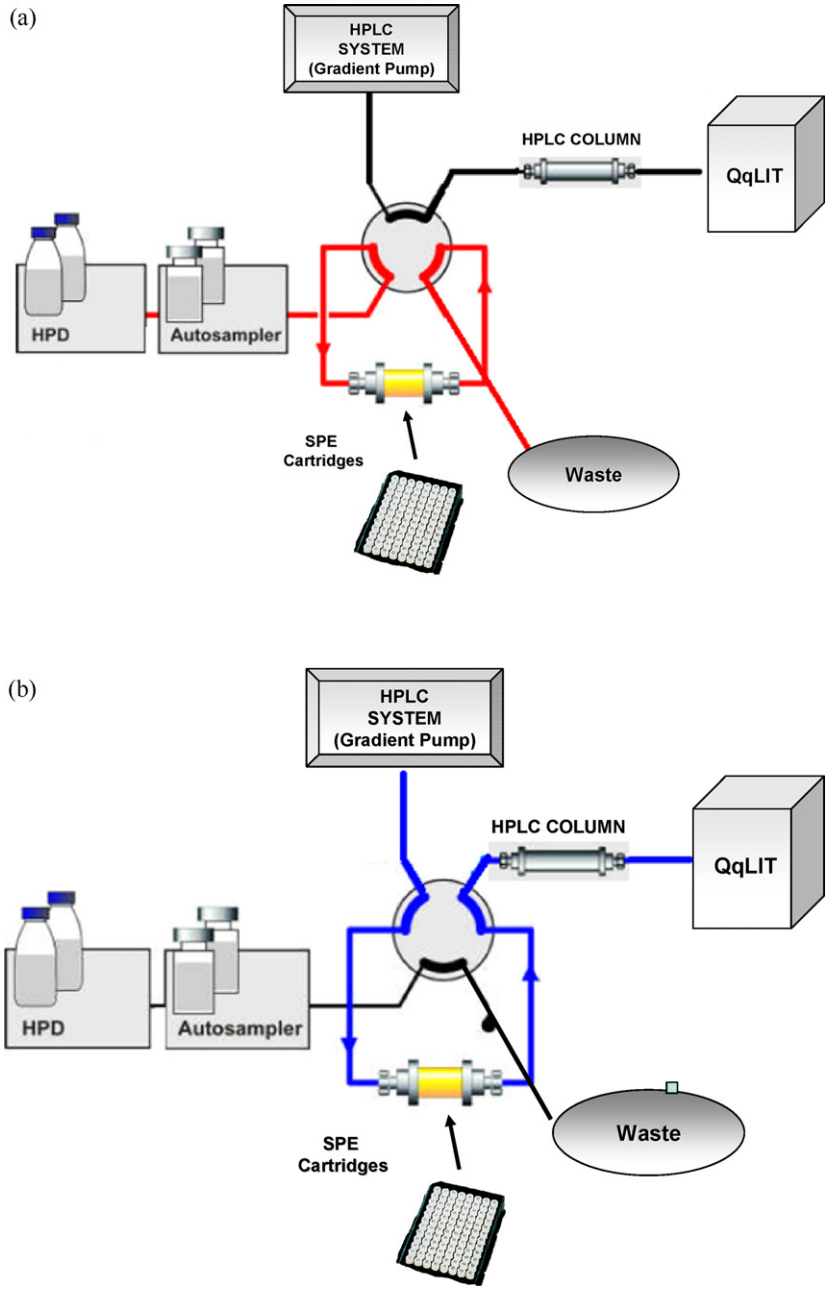

Fig. 1. On-line SPE procedure carried out with Prospekt 2™: (a) extraction of the analytes and (b) elution step.

and both fairly water-soluble and polar compounds, sulfonamides are retained weakly in soil systems, having a high potential for leaching or running off to ground waters and surface waters respectively after their release into the environment [7–11]. Intense rainfall events or the application of manure on irrigation crop lands accelerate these processes and, consequently, the diffuse contamination of ground waters and surface water by these compounds. Similarly, pasture animals may also spread the drugs and their respective metabolites via dung pats or urine into the fields. Aquaculture, hospital effluents, disposal of unused drugs and discharge from WWTPs are other sources to be considered when investigating the origin of the sulfonamides in the environment [12].

Sulfonamide antibiotics have been found in all kinds of water matrices [13]. Despite the low concentrations detected, they are being continuously introduced in the aquatic environment, and concentrations that were previously considered as harmless are leading to the emergence of antibiotic resistant bacteria strains and potential implications for human health and the environment.

Up to now, several studies on the environmental presence and analysis of sulfonamides have been published and different LC–MS/MS analytical methods have been developed for waste waters [14–18], surface water [14–17,19,20] and ground waters [7,9,10] separately or using the same methodology to analyze different matrices [9]. Richter et al. [21] analyzed all the water matrices mentioned before together with drinking water, being four the target sulfonamides and none of them within the scope of this study. Díaz-Cruz et al. [13] analyzed 10 sulfonamides in all the water matrices aforementioned, without affecting the sensitivity and the performance of the method and being equally suitable for all of them. The need to minimize the sample preparation, to improve sample throughput and to reduce analysis cost is a relevant issue, as usually methods for the extraction and quan-

Table 1
Optimized time scheduled SRM transitions used for the LC–MS/MS analysis of the sulfonamides studied (positive ionization mode).

Compounds	$[M+H]^+$	SRM transitions	RT	DP	CE	CXP	SRM Ratio ± STD
Sulfacetamide	215	215/156	3.2	46	21	10	1.40 ± 0.31
		215/92		46	35	6	
Sulfisomidin	279	279/124	3.3	76	33	8	2.02 ± 0.11
		279/186		76	23	14	
Succinyl-sulfathiazole	356	356/256	4.2	71	25	16	1.58 ± 0.22
		356/192		71	33	16	
Sulfathiazole	256	256/156	4.3	40	25	14	5 ± 0.26
		256/92		40	25	10	
d_4-Sulfathiazole	260	260/160	4.3	71	25	6	3.53 ± 0.27
		260/96		71	25	6	
Sulfaguanidine	215	215/156	4.3	56	13	10	2.33 ± 0.42
		215/92		56	31	4	
Sulfadiazine	251	251/156	4.5	46	27	10	1.30 ± 0.13
		251/108		46	30	8	
N^4-acetylsulfamethazine	321	321/134	4.6	86	35	4	1.45 ± 0.21
		321/124		86	35	4	
Sulfapyridine	250	250/156	4.7	51	28	12	1.17 ± 0.02
		250/92		51	31	6	
Sulfamerazine	265	265/92	5.4	61	47	6	1.30 ± 0.15
		265/156		61	27	8	
Sulfamethazine	279	279/156	6	26	30	10	1.48 ± 0.08
		279/124		26	35	10	
Sulfamethizole	271	271/156	6.3	36	23	12	6.85 ± 0.48
		271/108		36	23	8	
Sulfamethoxypyridazine	281	281/156	6.3	66	27	14	2.01 ± 0.09
		281/126		66	27	12	
Sulfadoxine	311	311/156	10.4	46	29	12	2.25 ± 0.40
		311/92		46	45	4	
Sulfamethoxazole	254	254/156	11.4	56	25	10	2.08 ± 0.24
		254/108		56	27	10	
Sulfisoxazole	268	268/156	12	71	21	10	1.45 ± 0.23
		268/113		71	21	8	
Sulfaquinoxaline	301	301/156	13	76	25	10	1.78 ± 0.14
		301/92		76	47	12	
Sulfabenzamide	277	277/156	13	56	17	10	1.71 ± 0.35
		277/92		56	41	6	
Sulfadimethoxine	311	311/156	13	76	31	8	4.37 ± 0.20
		311/92		76	31	6	
Sulfanitran	336	336/156	14.7	66	17	12	2.05 ± 0.25
		336/198		66	29	14	

RT: retention time (min); Compound dependent parameters: CE: collision energy (eV); DP: declustering potential (V); CXP: collision cell exit potential (eV). SRM ratio given with the standard deviation.

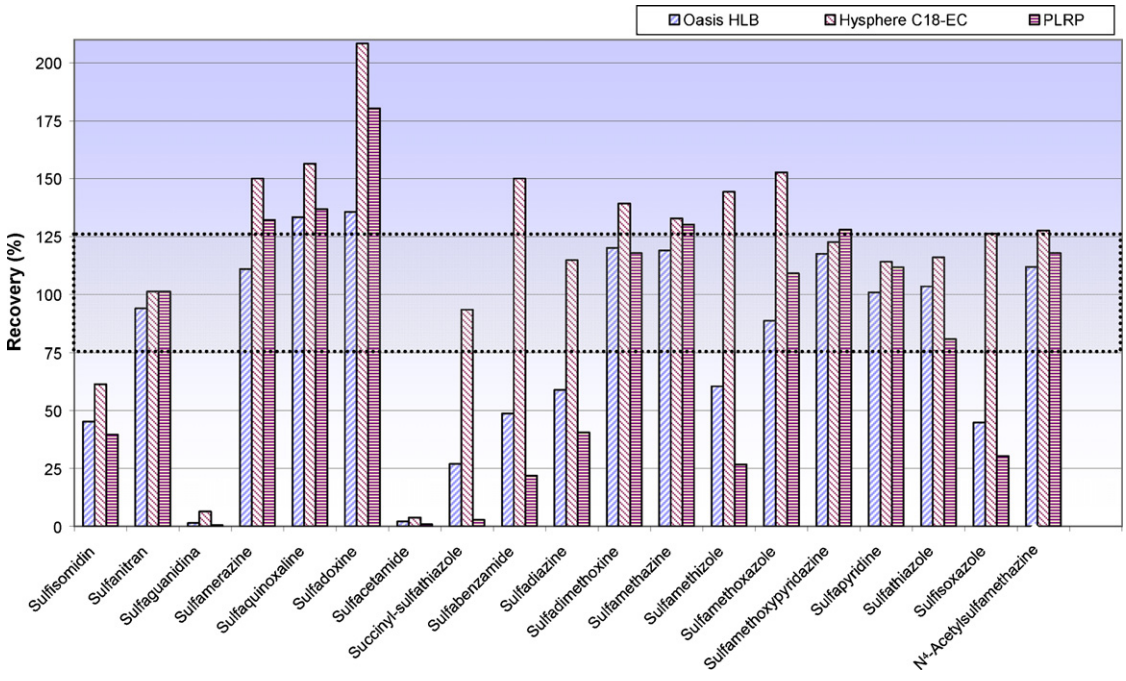

Fig. 2. Recovery values obtained from the evaluation of HLB Oasis, Hysphere C18 EC and PRLPs cartridges.

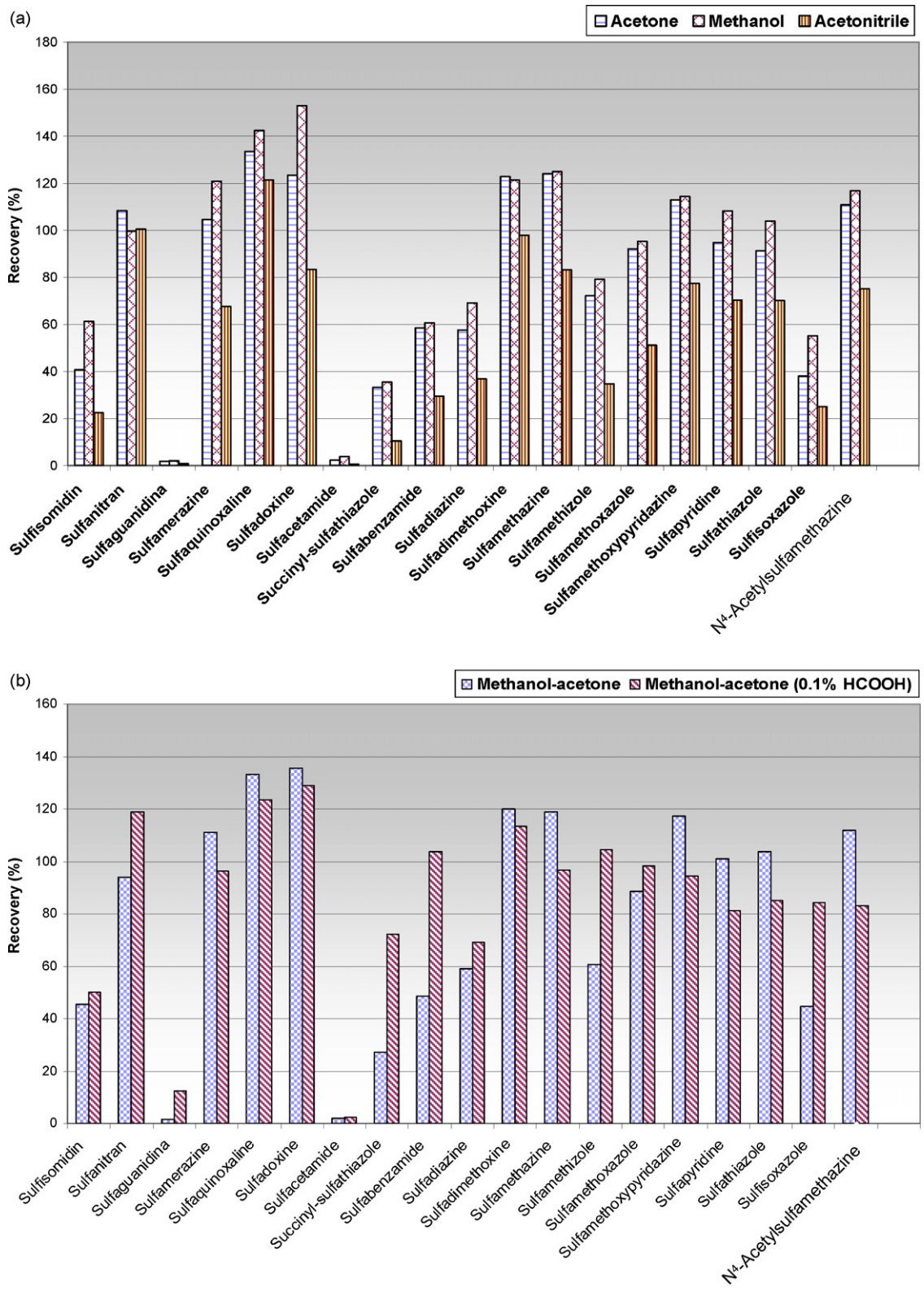

Fig. 3. Recovery values obtained from the evaluation of the recoveries using different equilibration solvents. (a) Acetonitrile, acetone and methanol. (b) Mixtures of methanol and acetone.

tification of antibiotics and pharmaceuticals in general in water matrices are time consuming and involve several steps (filtering and homogenization, clean-up of the sample and preconcentration and final analysis). Given the need of analytical methodology capable of detection at environmental levels (low picogram per liter level), and taking the environmental and analytical concerns mentioned into account, the aim of this work is to develop an automated multi-residue analytical method, based on on-line solid-phase extraction-liquid chromatography–tandem mass spectrometry (SPE-LC–MS/MS), for the simultaneous determination

of 19 selected sulfonamides in natural waters. This new method integrates LC–MS/MS analysis with on-line SPE, which is one of the most suitable sample preparation approaches available. Minimum sample manipulation, sample volume, time and solvents savings, and improved throughput are among the main advantages provided by this technique. Previous works account for the many advantages of this on-line SPE procedure [22–24] but, to the authors' knowledge, only one publication deals with on-line

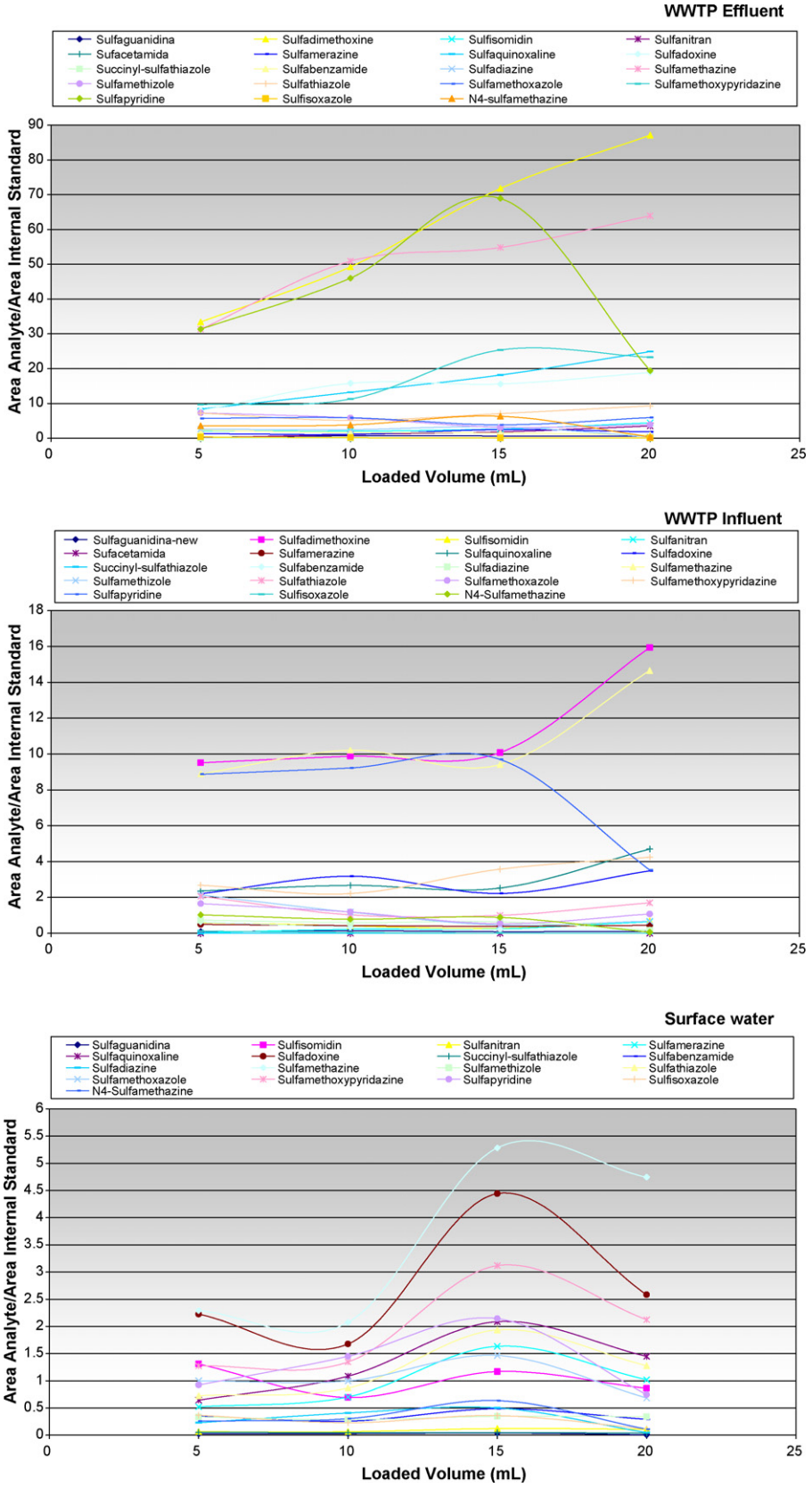

Fig. 4. Breakthrough curve representations for the different water matrices studied.

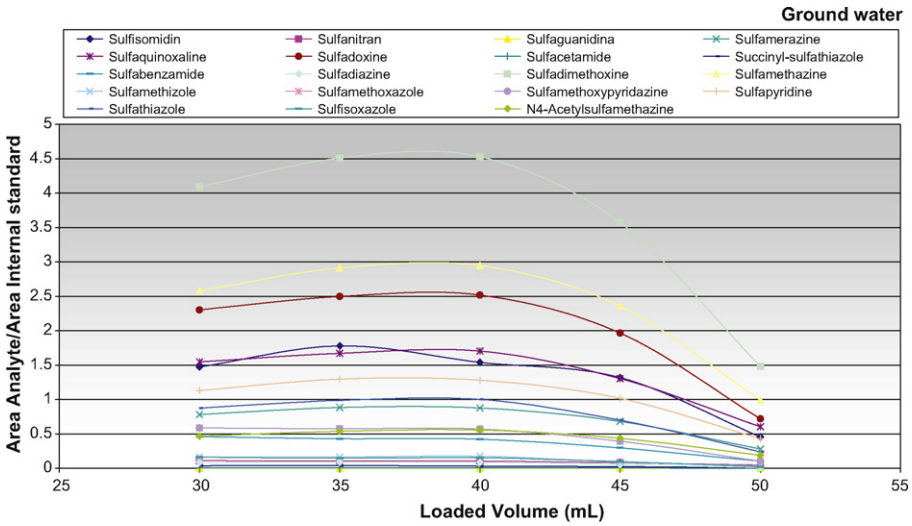

Fig. 4. (*Continued*).

SPE-LC–MS/MS analysis of sulfonamides [19], in which five sulfonamides and their corresponding acetylated metabolites were studied in surface waters.

The last part of our study covers the application of the new methodology to assess the occurrence and fate of 19 selected sulfonamides, including one of their metabolites, in WWTP influent and effluent water, surface water and ground water samples, taken all of them in the region of Catalonia and along the Ebro River Basin.

2. Experimental

2.1. Reagents and chemicals

HPLC-grade solvents (water, methanol, acetone and acetonitrile) and formic acid (98–100%) were supplied by Merck (Darmstadt, Germany).

High purity standards (>99%) of the 19 selected sulfonamides were purchased from Sigma (St. Louis, MO, USA). Stock standard solutions for each one of the analytes were prepared in methanol (MeOH) at 1 mg/mL and stored in the dark at −2 °C. Standard solutions of the mixtures of all compounds at concentrations ranging between 1 ng/mL and 500 μg/mL were prepared by appropriate dilution of the stock solutions in MeOH. The standard mixtures were used as spiking solutions for preparation of the aqueous calibration standards and in the recovery studies. Aqueous standard solutions contained <0.1% of MeOH.

Internal standard d_4-sulfathiazole (99.9%) was purchased from Toronto Research Chemicals (Ontario, Canada). Stock solutions were also prepared in methanol and stored at −2 °C until use.

2.2. Sample collection

Twenty-four hours-integrated samples of WWTP influent or effluent waters were taken in four different WWTPs. Surface water and ground water samples within agricultural areas were taken simultaneously. All the water matrices were collected in amber polyethylene terephthalate (PET) bottles and transported to the laboratory under cooled conditions (4 °C). Upon reception, samples were filtered through 0.45 μm Nylon filters (Whatman, Maidstone, UK) to eliminate suspended solid matter and then stored at 4 °C in the dark until analysis which was always carried out within 48 h of collection to avoid degradation.

2.3. Method development

2.3.1. On-line solid-phase extraction

Fully automated on-line preconcentration and purification of samples, aqueous standards and operational blanks was performed using an automated on-line SPE sample processor Prospekt-2™ (Spark Holland, Emmen, The Netherlands). This system consists of an automated cartridge exchange (ACE) module, which holds two trays of 96 extraction cartridges each, and a high pressure dispenser module (HPD) for handling of solvents by a 2 mL high pressure syringe. SPE solvents for conditioning, equilibration, sample application and clean up are provided by the HPD. The ACE module has two clamps and two high pressure valves. An aliquot of the raw sample is introduced by the autosampler and, when the SPE is completed, the cartridge is transferred to the elution clamp where the analytes will be eluted from the SPE cartridge directly onto the LC column by the HPLC. A scheme of the apparatus is represented in Fig. 1. The whole eluted volume gets to the chromatographic system instead of a final reconstituted extract as in off-line procedures, where usually volumes of 200 mL or bigger are reduced to approximately 0.5 mL and only around 20 μL will be injected in the mass analyzer [2,13]. During LC–MS/MS analysis, the extraction of the next sample is carried out on a new cartridge on the other clamp. Therefore, SPE is carried out entirely in parallel with the LC–MS/MS run. This configuration shortens the cycle times (in this case 23 min of sample analysis plus the conditioning and equilibration times only for the first sample). The Prospekt-2™ is controlled by means of the Sparklink software version 3.0 (Spark Holland).

2.3.2. LC–MS/MS analysis

LC-tandem MS analyses were carried out in a system consisting of an HP 1100 chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbospray electrospray (ESI) interface. The chromatographic separation was performed using an Atlantis C18 (Waters, 150 mm × 2.1 mm, 3 μm of particle size) LC-column preceded by a guard column with the same packing material. Sulfonamides were analyzed in the positive ionization mode (PI). The flow rate was set to 0.2 mL/min, being eluent A HPLC grade water slightly acidified with 0.1% of formic acid, and eluent B acetonitrile with 0.1% formic acid. The elution gradient started with 25% of eluent B, increasing to 80% in 10 min and 100% in 11 min. During the further 2 min the column was cleaned and readjusted to the initial conditions in 3 min, and equilibrated for 7 min.

Table 2
Quality parameters of the SPE-LC–MS/MS analytical method for all the water matrices studied: linear correlation coefficients (r^2). iLOD: instrumental limit of detection. iLOQ: instrumental limit of quantification. RSD: intraday repeatability expressed as relative standard deviation.

Compounds	WWTP influent			WWTP effluent			Ground water			Surface water		
	$(r^2)^a$	iLOD (pg injected)	RSD[b] (%)	$(r^2)^a$	iLOD (pg injected)	RSD[b] (%)	$(r^2)^a$	iLOD (pg injected)	RSD[b] (%)	$(r^2)^a$	iLOD (pg injected)	RSD[b] (%)
Sulfisomidin	0.9998	0.00104	3.23	0.9980	0.00056	2.01	0.9997	0.00083	3.23	0.9999	0.00059	4.37
Sulfanitran	0.9998	0.00418	2.59	>0.9999	0.00097	2.00	0.9996	0.00041	2.59	0.9996	0.00091	3.32
Sulfaguanidina	0.9998	0.11900	21.6	0.9948	0.07900	9.61	0.9986	0.09450	14.8	0.9999	0.01350	25.95
Sulfamerazine	0.9998	0.00296	1.77	>0.9999	0.00047	2.88	0.9998	0.00196	1.77	>0.9999	0.00086	2.06
Sulfaquinoxaline	0.9998	0.00049	0.51	0.9998	0.00049	0.59	0.9998	0.00030	0.51	0.9991	0.00024	1.43
Sulfadoxine	0.9948	0.00083	1.50	0.9988	0.00016	3.30	0.9999	0.00040	1.50	0.9999	0.00027	2.93
Sulfacetamide	0.9990	0.09530	8.23	0.9855	0.64300	7.24	0.9982	0.37700	11.0	0.9994	0.01910	8.08
Succinyl-sulfathiazole	0.9978	0.00430	9.53	0.9849	0.01490	12.9	0.9962	0.36600	9.53	0.9998	0.02730	2.39
Sulfabenzamide	0.9998	0.00277	4.79	>0.9999	0.00068	5.05	>0.9999	0.00244	4.79	0.9998	0.00079	0.07
Sulfadiazine	0.9998	0.00204	4.66	0.9996	0.00094	6.39	0.9998	0.00140	4.66	0.9998	0.00164	3.38
Sulfadimethoxine	>0.9999	0.00050	1.46	0.9998	0.00012	1.16	0.9998	0.00006	1.46	>0.9999	0.00027	2.78
Sulfamethazine	0.9998	0.00110	1.83	0.9994	0.00046	1.86	0.9996	0.00037	1.89	>0.9999	0.00018	2.88
Sulfamethizole	>0.9999	0.00933	3.84	0.9990	0.00364	5.65	0.9999	0.01780	6.61	0.9995	0.01040	5.13
Sulfamethoxazole	0.9996	0.00440	1.55	0.9996	0.00095	2.83	0.9998	0.00259	1.55	0.9998	0.00142	1.16
Sulfamethoxypyridazine	>0.9999	0.00070	1.54	>0.9999	0.00031	2.00	0.9999	0.00033	1.54	0.9998	0.00025	3.82
Sulfapyridine	0.9998	0.00190	3.04	>0.9999	0.00074	4.22	0.9997	0.00034	3.04	0.9999	0.00033	1.90
Sulfathiazole	>0.9999	0.00160	10.3	0.9998	0.00132	1.75	>0.9999	0.00210	10.3	>0.9999	0.00132	1.31
Sulfisoxazole	0.9998	0.00160	6.70	0.9982	0.00108	4.24	0.9998	0.01590	6.70	0.9999	0.00443	5.25
N^4-acetylsulfamethazine	0.9998	0.00280	4.42	0.9996	0.00074	3.85	0.9998	0.00112	4.42	0.9997	0.00009	1.00

[a] Calibration range between 0.05 ng/L and 1000 ng/L.
[b] Relative standard deviation ($n = 5$, spike concentration 100 ng/L).

For increased sensitivity and selectivity, MS/MS data acquisition was performed in the selected reaction monitoring (SRM) mode. For each analyte, two transitions between precursor ions and the two most abundant product ions were monitored; the more abundant one was used for quantitation and the other one for confirmation. Table 1 shows the optimized LC–MS/MS conditions used for the analysis of the target analytes. The optimization of the MS/MS experimental conditions was performed in a previous study [13] and were as follows: capillary voltage, 3.5 kV; source temperature, 700 °C; desolvation temperature, 450 °C; extractor voltage, 3 V; and RF lens, 0.2 V. Nitrogen was used as both the nebulizing and the desolvation gas at 630 L/h. For operation in the MS/MS mode, argon was used as collision gas with a pressure of 2.6×10^{-3} mbar. Instrument control and data acquisition and evaluation were performed with the Analyst 1.4.2 software package purchased from Applied Biosystems.

3. Results and discussion

3.1. On-line SPE optimization

Extraction efficiency of a SPE procedure is controlled mainly by (1) the nature of the adsorbing material, (2) the conditioning and elution solvents used and (3) the sample volume loaded.

3.1.1. Adsorbing material

The first stage of the SPE optimization was to determine the most appropriate adsorbing material. For this purpose, three different disposable trace enrichment cartridges were evaluated for their efficiency in the on-line SPE of the target sulfonamides from water: the polymeric cartridge Oasis HLB (macroporous polymer of divinylbenzene and *N*-vinylpyrrolidone, 30 μm particle size) from Waters (Barcelona, Spain), the polymeric phase PLRP-s (cross-linked styrenedivinylbenzene polymer, 15–25 μm particle size) from Spark Holland, and the silica-based cartridge Hysphere C18 EC (endcapped octadecyl phase, 8 μm particle size) also from Spark Holland. 10 mL of HPLC water spiked with a mixture of all the analytes at 100 ng/L were loaded at 1 mL/min onto the cartridges, previously conditioned with 1 mL of a mixture of methanol and acetone and 1 mL of water (flow rate 1 mL/min). Conditioning and equilibration were performed with the same solvents as in the off-line SPE procedure previously developed by the same authors [13]. Triplicates were run for each kind of cartridge. After sample loading and prior to elution, the cartridges were washed with 1 mL of water at a flow rate of 1 mL/min to improve the complete transfer of the sample and remove interferences. Recoveries were based on the ratio between the peak areas obtained with the on-line analysis and the results from a parallel off-line analysis of a standard mixture of the sulfonamides (same total mass injected in both cases). Recovery values are shown in Fig. 2, where a goodness range between 75% and 125% has been marked. As it can be seen, despite recoveries are higher for the Hysphere C18 cartridges, these values are for most of the analytes far above 100%, being this the main reason why this adsorptive material was discarded. Although PRLPs and Oasis HLB showed similar recoveries, the latter was finally selected as it has been previously proved to be suitable in on-line SPE [19] and off-line SPE procedures for the same family of compounds [2,13,18,25–26].

3.1.2. Solvents

Once the extraction cartridge has been selected, different solvents for the conditioning step of the cartridge were evaluated (acetonitrile, methanol and acetone separately). The same volume

Table 3
Method limits of detection (MLOD) and quantification (MLOQ) corresponding to the first SRM transition for all the sulfonamides.

Compounds	WWTP influent		WWTP effluent		Ground water		Surface water	
	MLOD	MLOQ	MLOD	MLOQ	MLOD	MLOQ	MLOD	MLOQ
Sulfisomidin	0.10	0.33	0.13	0.43	0.03	0.11	0.05	0.16
Sulfanitran	0.56	1.87	0.24	0.79	0.05	0.17	0.03	0.10
Sulfaguanidina	7.84	26.1	1.01	3.37	12.0	40.0	1.01	3.37
Sulfamerazine	0.25	0.83	0.19	0.62	0.12	0.40	0.21	0.70
Sulfaquinoxaline	0.55	1.83	0.04	0.15	0.02	0.08	0.19	0.63
Sulfadoxine	0.12	0.39	0.04	0.14	0.02	0.08	0.19	0.63
Sulfacetamide	–	–	6.90	23.0	5.13	17.1	4.06	13.5
Succinyl-sulfathiazole	7.23	24.1	2.73	9.10	3.29	11.0	2.58	8.60
Sulfabenzamide	0.05	0.17	0.11	0.38	0.22	0.74	0.41	1.38
Sulfadiazine	1.12	3.72	0.90	2.99	0.18	0.59	0.34	1.12
Sulfadimethoxine	0.16	0.54	0.01	0.02	0.02	0.08	0.02	0.07
Sulfamethazine	0.40	1.33	0.06	0.19	0.04	0.13	0.05	0.15
Sulfamethizole	3.01	10.0	1.04	3.47	3.10	10.3	4.52	15.1
Sulfamethoxazole	1.14	3.79	0.77	2.55	0.81	2.69	0.86	2.87
Sulfamethoxypyridazine	0.14	0.47	0.05	0.17	0.03	0.09	0.09	0.31
Sulfapyridine	0.32	1.08	0.13	0.42	0.02	0.05	0.06	0.20
Sulfathiazole	0.28	0.94	0.45	1.48	0.21	0.70	0.22	0.74
Sulfisoxazole	0.50	1.67	2.15	7.16	0.63	2.11	0.31	1.03
N^4-acetylsulfamethazine	2.17	7.25	0.14	0.48	0.02	0.08	0.02	0.06

Both are given in ng/L. Values for sulfacetamide in influent water could not be estimated, as this sulfonamide was not detected in any of the water samples.

of water spiked at 100 ng/L was loaded afterwards (triplicate analysis). As it can be seen in Fig. 3a, acetonitrile showed the lowest recoveries, whereas values for methanol and acetone were quite similar. Two mixtures of both solvents (1:1, v/v) were also studied, one of them at neutral pH and the second slightly acidified with formic acid at 0.1% (Fig. 3b). Differences between these two mixtures were hardly noticeable in the recoveries obtained; in order to make the procedure easier to handle, the mixture without acid was eventually selected.

3.1.3. Sample volume

Natural water samples were spiked with a mixture of the analytes at a concentration of 100 ng/L and volumes from 5 mL to 20 mL were loaded onto the HLB cartridges (5–50 mL for groundwater). Breakthrough curves were made for the different water matrices, where the sample volume extracted was represented against the integrated area obtained under the respective chromatogram peak (Fig. 4). The highest peak areas were generally bigger after extracting 15 mL of surface water and WWTP effluent, 5 mL for WWTP influent and 40 mL for ground water.

3.2. MS/MS detection optimization

The analytical method developed is based on a method previously described by the authors for the off-line SPE extraction and LC–MS/MS analysis of 10 sulfonamides (one of them a metabolite), in environmental water matrices [13]. Nine new sulfonamides were added to the method and correspondingly optimized, first by infusion and afterwards by on-column off-line injection of standard solutions of the individual compounds and a mixture solution of all of them. Identification of the precursor ions and optimum ionization conditions was performed in the full scan mode by recording mass spectra from m/z 50 to 500. Further identification of the most abundant fragment ions and selection of the optimum gas collision energies (CE) for each analyte were carried out in the product ion scan mode.

For the positive confirmation of the target analytes in the samples, strict criteria had to be met in order to avoid false positives. Following the European Commission Decision 2002/657/EC [27], a minimum of three identification points (IPs) is required for this purpose. Besides, the chromatographic retention time of the ana-

Table 4
Sulfonamide concentrations detected in the different WWTPs samples studied (given in ng/L).

Compounds	WWTP1		WWTP2		WWTP3		WWTP4	
	I	E	I	E	I	E	I	E
Sulfisomidin	–	–	–	–	–	<MLOQ	–	–
Sulfanitran	<MLOQ	–	<MLOD	–	<MLOQ	–	–	–
Sulfaguanidine	–	–	<MLOQ	1.88	–	–	–	–
Sulfamerazine	–	–	–	4.94	–	34.6	–	9.85
Sulfaquinoxaline	–	<MLOD	–	<MLOQ	–	<MLOQ	–	–
Sulfadoxine	–	<MLOQ	<MLOQ	<MLOQ	–	–	–	–
Sulfacetamide	–	–	–	–	–	–	–	–
Succinyl-sulfathiazole	–	<MLOQ	–	–	–	–	–	–
Sulfabenzamide	–	–	–	–	–	<MLOQ	–	–
Sulfadiazine	–	<MLOD	–	–	–	–	181	104
Sulfadimethoxine	–	–	–	–	–	–	20.1	10
Sulfamethazine	–	–	–	–	–	18	–	14.7
Sulfamethizole	–	–	247	<MLOD	–	<MLOQ	–	–
Sulfamethoxazole	–	12.4	–	302	–	77.4	89	133
Sulfamethoxypyridazine	–	–	–	–	–	–	–	–
Sulfapyridine	<MLOQ	<MLOQ	2.15	38.3	5.23	8.53	855	113
Sulfathiazole	–	–	–	5.12	–	9.21	37.5	7.46
Sulfisoxazole	–	<MLOQ	–	–	<MLOQ	<MLOQ	–	8.17
N^4-acetylsulfamethazine	<MLOD	<MLOD	<MLOD	<MLOQ	–	–	–	5.29

–: not detected; <MLOD: under the method limit of detection; <MLOQ: under the method limit of quantification.

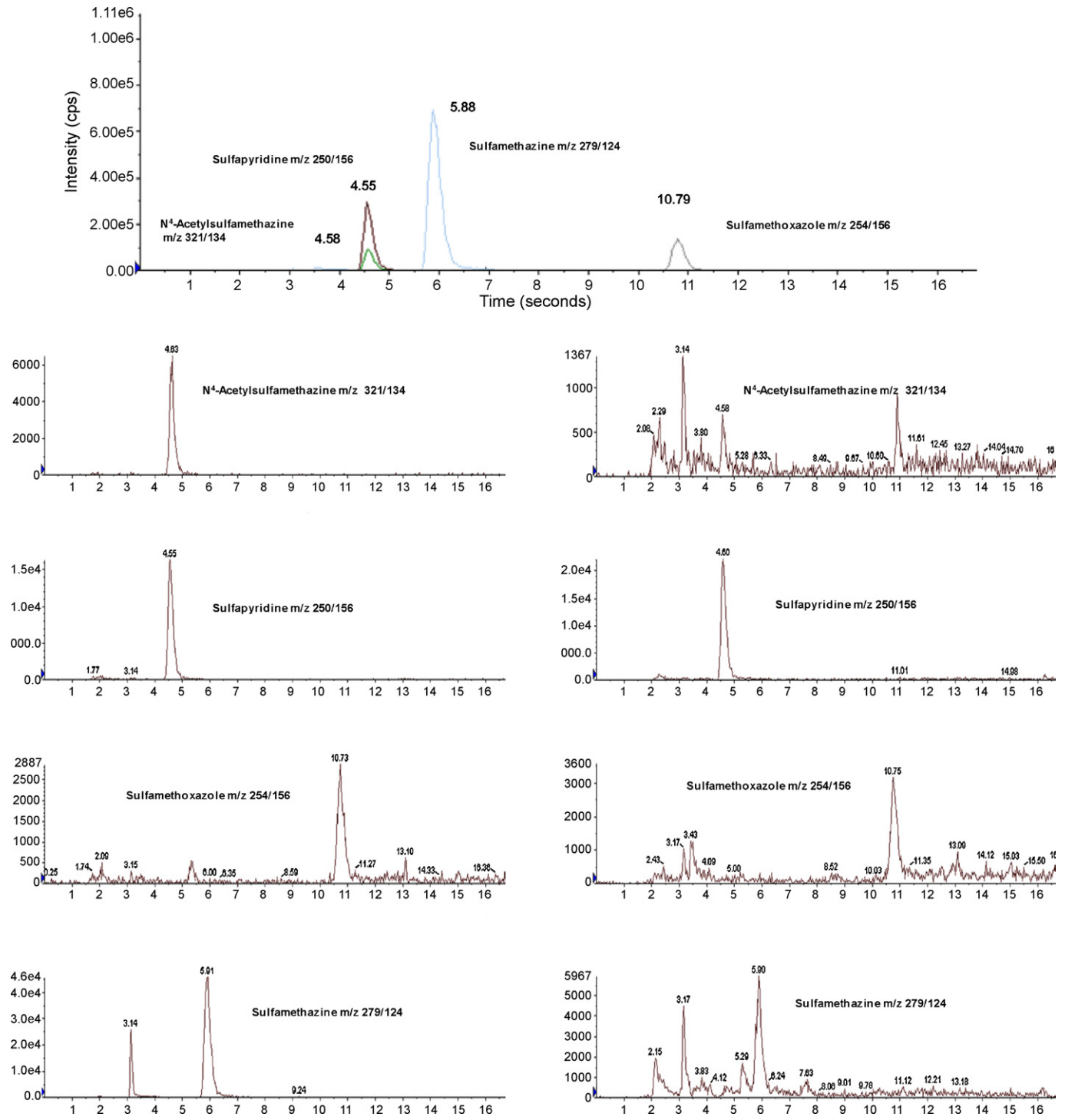

Fig. 5. Reconstructed chromatogram for four of the sulfonamides studied in surface and WWTP effluent water samples, and a reference chromatogram corresponding to an HPLC grade water spiked at 500 ng/L.

lyte in the sample should not vary more than 2% in comparison to the calibration standards', and the relative abundance of the two SRM transitions monitored must also be compared to the standards' corresponding values.

3.3. Method validation

After optimization, the analytical method developed was evaluated in terms of linearity, repeatability, accuracy, selectivity and sensitivity.

Quantification was performed based on peak areas and by the internal standard calibration method, crucial to correct potential matrix effects. Concentrations were estimated for the most abundant SRM transition selected. d_4-sulfathiazole was added to all the samples at a concentration of 500 ng/L right before analysis.

Five to eight point matrix matched calibration curves were constructed for each of the water types, using least-squares linear regression analysis at concentrations ranging from 0.05 ng/L to 1000 ng/L. Correlation coefficients (r^2) were higher than 0.999 for all of the sulfonamides studied.

Sensitivity is one of the method parameters enhanced when peforming on-line SPE analysis. Despite the low sample volumes required, it has been proved that sensitivity is not affected but, on the contrary, improved considerably. Table 2 shows the instrumental limits of detection (iLODs) for each of the four water matrices. Method limits of detection (MLOD) and quantification (MLOQ) were also calculated as the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10, respectively. MLOD values were in the range of 0.03–8.44 ng/L for WWTP influent water, 0.21–7.31 ng/L for WWTP effluent water, 0.02–5.13 ng/L for ground water and 0.02–4.52 ng/L for surface water samples (Table 3).

The precision of the method was evaluated by analyzing five consecutive times the corresponding water matrices spiked with a standard mixture of the analytes at 100 ng/L. The relative standard deviations obtained varied from 0.02% to 26% (Table 2).

3.4. Practical application

The applicability of the method was assessed through the analysis of the target sulfonamides in the four different water matrices considered. Water from four WWTPs (influent and effluent samples) and surface water samples from four different rivers (Ebro River and three of its tributaries) were sampled in spring–summer 2008. Ground water samples from four wells in the same area were also taken that year and in October 2007. Fig. 5 shows the chromatograms of four of the sulfonamides most frequently detected in surface and WWTP effluent water samples. The reconstructed chromatogram corresponding to HPLC grade water spiked at 500 ng/L has been also included as a reference for the retention time of the analytes.

WWTP1, WWTP2 and WWTP3 were located in mountain and rural areas and served populations between 500 and 3200 inhabitants. As shown in Table 4, very few sulfonamides could be detected in these three plants, and the estimated concentrations usually remained below the MLOD or MLOQ. Their presence in influent water was usually less frequent and more arduous to determine than in effluents, probably due to matrix effects and the suppression of the signal intensity. For instance, sulfamethoxazole, sulfonamide typically applied in human medicine, was detected in effluent water of the three plants, but not in any of the influent samples. Sulfapyridine, also very common in human therapies to treat intestinal infections, was detected in both influent and effluent samples of WWTP2 and WWTP3, but concentrations in the effluent water were usually higher than that of the influent, which can be attributed also to intense strong matrix effects in the latter, which hampered the identification of the compounds. It could also be attributed to the fact that metabolites and conjugated forms, which are also present in the influent samples, may degrade and retransform into the parent compounds, being these sulfonamides released in the effluent [2]. For these three WWTPs, the highest concentration corresponded to sulfamethoxazole in effluent water of WWTP2 (302 ng/L) and the smaller to sulfaguanidine also in the effluent water sample of WWTP2, with a concentration of 1.88 ng/L.

WWTP4 served around 40 000 inhabitants in an urban area. As expected, the concentrations detected in this location were generally higher than those of the rural WWTPs, with values in influent water up to 855 ng/L for sulfapyridine. Sulfamethoxazole was detected in both influent and effluent waters at concentrations of 89 ng/L and 133 ng/L, respectively.

Ground water samples showed differences in the number of sulfonamides detected as well as in the concentrations estimated, depending on the location (see Table 5). Sulfisomidin, sulfamethazine and sulfamethoxazole were the sulfonamides detected with the highest frequency in both campaigns (Fig. 6) and with the highest concentrations. An occurrence study of these antibiotics in two ground water bodies of Catalonia (Spain) showed that these same sulfonamides were also repeatedly present in this water matrix (66.67%, 89.74% and 58.97% of the ground water samples, respectively) [28]. On the contrary, sulfaguanidine, sulfacetamide, succinyl-sulfathiazole and sulfathiazole were not detected in any of the samples. In those cases where sulfonamides were detected in the two campaigns in the same sampling locations, it could be observed that concentrations in 2007 were usually higher. A feasible explanation for this is that samples in 2008 were taken during the summer campaign which implies smaller infiltration rates to the ground water bodies due to the lack of rain events, meaning less sulfonamides being potentially leached down to the aquifers and, consequently, smaller concentration levels to be detected. However, with the exception of the sampling point GW4, generally a higher number of sulfonamides were detected during the 2008 summer campaign in all the ground water wells which had not been detected the previous year. In all cases, the highest concentrations corresponded to sulfamethoxazole, in a range from 14.8 ng/L to 53.9 ng/L. This data is relevant, as sulfamethoxazole is used mainly in humans and, therefore was not expected to be found so often in ground water from rural areas,

Table 5
Sulfonamide concentrations in the various ground water and surface waters collected (ng/L).

Compounds	GW1		GW2		GW3		GW4		R1	R2	R3	R4
	2007	2008	2007	2008	2007	2008	2007	2008				
Sulfisomidin	1.14	–	1.83		–	0.62	1.89	0.32	13.70	1.79	6.19	–
Sulfanitran	–	0.80	–	<MLOQ	–	0.82	–	–	–	–	–	–
Sulfaguanidina	–	–	–		–	–	–	–	–	–	–	–
Sulfamerazine	–	3.22	–	<MLOQ	–	–	0.77	–	15.50	–	10.10	3.18
Sulfaquinoxaline	<MLOQ	1.17	–	-	<MLOQ	–	0.32	–	20.80	–	5.89	–
Sulfadoxine	–	4.48	–	–	–	–	–	–	20.00	–	6.77	–
Sulfacetamide	–	–	–	–	–	–	<MLOD	–	–	–	–	–
Succinyl-sulfathiazole	–	–	<MLOD	–	<MLOD	–	–	–	–	–	–	–
Sulfabenzamide	–	–	–	3.41	–	–	–	–	–	1.78	–	–
Sulfadiazine	–	–	–	–	0.81	–	–	–	–	–	–	–
Sulfadimethoxine	0.11	1.65	–	–	–	–	0.51		18.10	–	5.02	0.52
Sulfamethazine	–	3.71	<MLOQ	–	0.16	0.43	0.88	0.16	20.10	–	8.04	2.52
Sulfamethizole	<MLOD	<MLOD	–	–	–	<MLOD	<MLOQ	–	2.65	–	–	–
Sulfamethoxazole	17.60	8.55	–	19.70	13.40	53.90	14.80	–	–	–	7.50	32.2
Sulfamethoxypyridazine	–	0.24	–	–	–	0.24	0.77	–	15.50	0.62	7.30	–
Sulfapyridine	–	0.70	–	0.05	–	0.75	1.11	–	11.20	0.16	4.98	0.79
Sulfathiazole	–		–	–	–	–	–	–	13.90	–	10.10	–
Sulfisoxazole	–	<MLOQ	–	<MLOQ	–	<MLOD	<MLOD	–	–	–	12.50	–
N^4-acetylsulfamethazine	–	2.18	–	0.14	–	–	0.38	–	–	0.25	5.32	–

–: not detected; <MLOD: under the method limit of detection; <MLOQ: under the method limit of quantification.

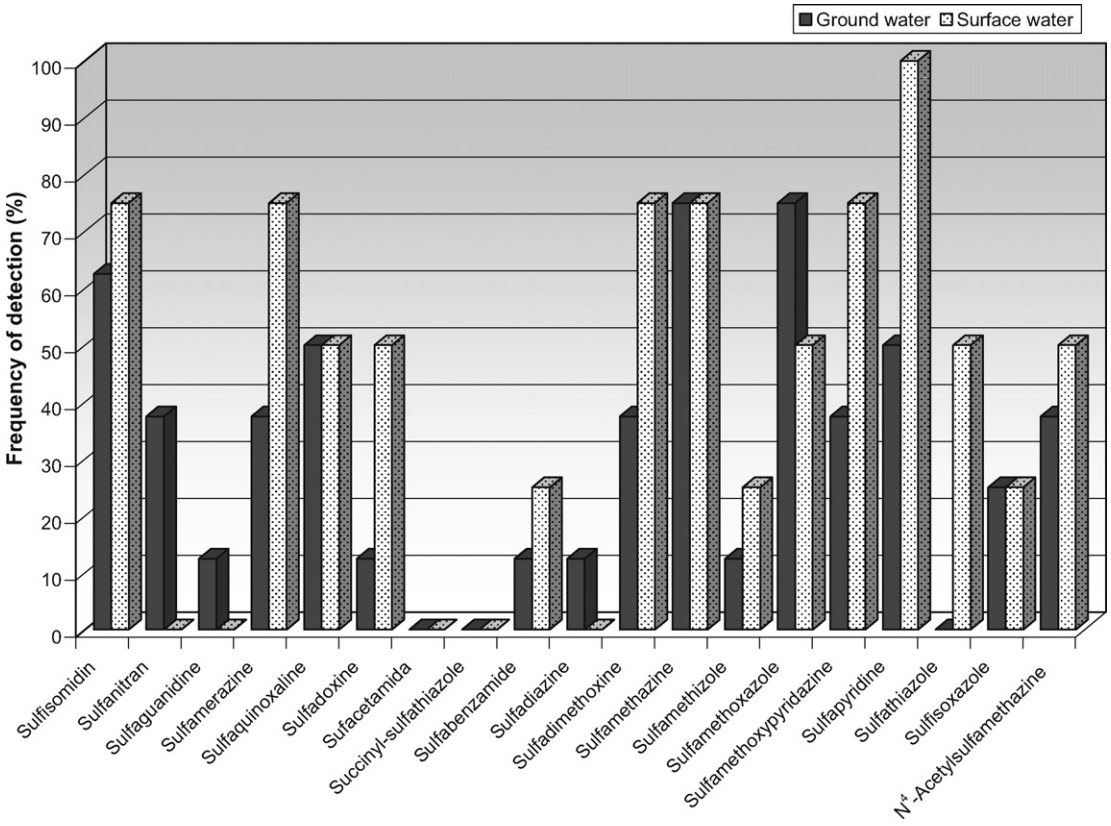

Fig. 6. Frequency of detection for the target sulfonamides studied in both ground water and surface water samples.

where veterinary sulfonamides such as sulfadiazine or sulfamethazine were more likely to be present (via direct deposition of excreta or discharges from cattle farms or via manure amendments in agricultural soil). The smallest concentration detected in the ground water samples corresponded to sulfamethazine in GW1, where only 0.11 ng/L of this compound was detected. The ground water sample which seemed to be less polluted by the sulfonamides studied was GW2, taken from a well located within an urban area. Only sulfisomidin was detected in 2007 in that location, and four other sulfonamides in 2008: sulfabenzamide, sulfamethoxazole, sulfapyridine (typically used in human medicine) and N^4-acetylsulfamethazine. The sample GW1, located in the same village surroundings, close to an agricultural field, showed a higher number of detected sulfonamides (4 in 2007 and 11 in 2008). These results would not allow us to establish a clear distinction between the origin of the sulfonamides present in these ground water samples, as sulfamethoxazole and sulfapyridine, widely use in human medicine, have been detected in frequencies similar to sulfamethazine, typically used in veterinary practices (Fig. 6).

Regarding surface water samples, concentrations ranged from 0.16 ng/L (sulfapyridine in R2) to 32.2 ng/L (sulfamethoxazole in R4). R1 and R3 were the sampling sites where a higher number of sulfonamides were detected (10 and 12, respectively). R1 was situated upstream the WWTP4, close to the same urban area, whereas R3 was close to a village of no more than 1204 inhabitants, where agriculture and cattle were the main economical activities. However, concentrations were slightly higher in R1, with values generally between 10 ng/L and 20 ng/L (see Table 5). Despite being located close to the biggest city of the sampling campaign, with approximately 660 000 inhabitants and a relevant industrial activity, only five sulfonamides could be detected in sampling site R2 and at quite low concentration. Sulfadiazine was not detected in any of the surface water samples analyzed. Except for sulfadiazine, MLODs obtained were slightly better than those previously calculated by Stoob et al. [19], especially for the acetylated metabolite.

4. Conclusion

For the fast and sensitive simultaneous determination of 19 selected sulfonamides and metabolites in natural waters, a new multi-residue analytical method based on on-line SPE-LC–MS/MS was developed. Compared to the existing methods, this analytical approach affords full automation, minimum sample handling by the analyst, low sample volume required (depending on the matrix, from 5 mL to 40 mL), high-throughput (23 min/sample), good reproducibility (with RSD values usually below 10%), improved accuracy (since aqueous calibration standards are processed in the same way as samples), high sensitivity (MLOD values usually <10 ng/L) and high selectivity. Differences are less obvious when comparing to a previous off-line SPE study [10], in which similar MLOD values and sometimes lower were achieved. However, it should be taken into account that the number of compounds to be analyzed by this new on-line SPE methodology is nearly two times higher, and therefore compromise in terms of optimization of the SPE procedure for all the target analytes had to be reached, which may somehow have slightly affected the performance of the methodology. Nevertheless, the application of the developed method to assess the contamination with sulfonamides of different water matrices evidenced the occurrence of most of the studied sulfonamides, even at pg/L level. Sulfonamides typically used in human medicine, such as sulfamethoxazole and sulfapyridine, were the most frequently found in all the water matrices studied, together with sulfamethazine and sulfisomidin. The recurrent presence of sulfamethoxazole in ground water samples of this and other studies by the same author make evident the

environmental health concern and the need of further investigation.

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