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Fully automated online solid phase extraction coupled directly to liquid chromatography–tandem mass spectrometry Quantification of sulfonamide antibiotics, neutral and acidic pesticides at low concentrations in surface waters

Krispin Stoob, Heinz P. Singer *, Christian W. Goetz, Matthias Ruff [1], Stephan R. Mueller [2]

Swiss Federal Institute of Aquatic Science and Technology, Eawag Water and Agriculture Department, Ueberlandstrasse 133, Duebendorf CH-8600, and Swiss Federal Institute of Technology, ETH, Zurich, Switzerland

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Abstract

A fully automated online solid phase extraction–liquid chromatography–tandem mass spectrometry (SPE–LC–MS/MS) instrumental setup has been developed for the quantification of sulfonamide antibiotics and pesticides in natural water. The direct coupling of an online solid phase extraction cartridge (Oasis HLB) to LC–MS/MS was accomplished using column switching techniques. High sensitivity in the low ng/L range was achieved by large volume injections of 18 mL with a combination of a tri-directional autosampler and a dispenser system. This setup allowed high sample throughput with a minimum of investment costs. Special emphasis was placed on low cross contamination. The chosen approach is suitable for research as well as for monitoring applications. The flexible instrumental setup was successfully optimised for different important groups of bioactive chemicals resulting in three trace analytical methods for quantification of (i) sulfonamide antibiotics and their acetyl metabolites; (ii) neutral pesticides (triazines, phenylureas, amides, chloracetanilides) and (iii) acidic pesticides (phenoxyacetic acids and triketones). Absolute extraction recoveries from 85 to 112% were obtained for the different analytes. More than 500 samples could be analyzed with one extraction cartridge. The inter-day precision of the method was excellent indicated by relative standard deviations between 1 and 6%. High accuracy was achieved by the developed methods resulting in maximum deviation relative to the spiked amount of 8–15% for the different analytes. Detection limits for various environmental samples were between 0.5 and 5 ng/L. Matrix induced ion suppression was in general smaller than 25%. The performance of the online methods was demonstrated with measurements of concentration dynamics of sulfonamide antibiotics and pesticides concentrations in a little creek during rain fall events.

Keywords: Fully automated online extraction; Acetyl sulfonamide antibiotics; Sulcotrione; Mesotrione; ESA OXA metabolites; Biocides

1. Introduction

Bioactive compounds, such as antibiotics and pesticides represent water contaminants of particular interest because of their potential unwanted side effects to humans and aquatic organisms. Both groups are used in agriculture as well as in private households. Antibiotics enter the environment due to the land spreading of antibiotic-containing manure in agriculture [1], or by input from waste water treatment plants after use as human medicals [2]. Pesticides are introduced into the environment intentionally for crop protection in agricultural or non-agricultural use in urban areas. Many different antibiotics (e.g., [3–5]) and pesticides (e.g., [6–8]) have been found in surface and ground water. Antibiotics including β-lactams, tetracyclines, sulfonamides, macrolides and fluoroquinolons are often administered in veterinary and human medicine. The sulfonamides are of special interest due to their high excretion rate [9] and

* Corresponding author. Tel.: +41 1 823 55 77; fax: +41 1 823 50 28.
E-mail address: heinz.singer@eawag.ch (H.P. Singer).
[1] Present address: University of Berne, Laboratory for Radio and Environmental Chemistry, Freiestrasse 3, 3012 Bern, Switzerland.
[2] Present address: Swiss Agency for the Environment, Forests and Landscape (SAEFL), Worblentalstrasse 32, 3003 Bern-Ittigen, Switzerland.

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their persistence in the environment [10] or during waste water treatment [11]. Their high mobility increases the leaching potential from agricultural fields where manure from medicated live stock was applied. Widely used pesticide groups are the triazines, phenylureas, amides, chloracetanilides, phenoxyacetic acids and the recently introduced triketone pesticides, sulcotrione and mesotrione, which are increasingly used [8]. Even though modern pesticides are fairly degradable high concentration can be found in surface waters due to losses from agricultural land or due to direct input from point sources.

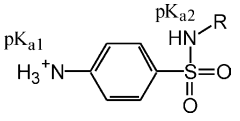
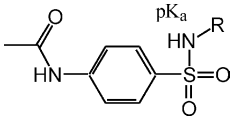
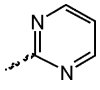
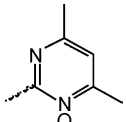
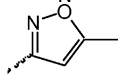
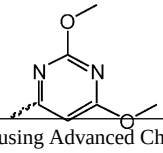
In contrast to apolar contaminants, the compounds mentioned above represent low octanol–water partitioning coefficients ($\log K_{ow} < 3$) and high water solubilities (mg/L to g/L) because of their functional groups with H-donor/-acceptor properties. Furthermore, most of them show pK_a-values in the environmentally relevant range and are typically anionic in natural waters. Physico-chemical properties of the sulfonamides are given in Table 1, those of the pesticides are published elsewhere [12].

The input of theses substances from diffuse and point sources to surface water is highly dynamic [13]. High sample throughput and a dynamic measuring range over several orders of magnitudes are imperative needs for the reliable quantification of the load or the concentration dynamic of these substances in catchment studies for mass balance or risk assessment purposes. To this end, analytical methods exhibiting sensitivity in the low ng/L-range are necessary. In addition, high selectivity is required in order to avoid interference by matrix constituents. Presently, LC–MS/MS has become the method of choice. One of the major advantages of using liquid chromatography instead of gas chromatography is that there is no need for derivatization of polar analytes [14]. However, water samples must usually be pre-concentrated before analysis, which is typically done by time-consuming and costly offline SPE.

Automated SPE is routinely used in the pharma-industry to increase the sample throughput [15]. The simplest approach to automation is generally a "single cartridge approach" [16]. Several working steps, such as evaporation, reconstitution and injection are eliminated by the direct coupling of SPE to LC. This results in a faster and more precise procedure since the total enriched amount of substance is eluted directly to the LC [17]. In addition, procedural errors are reduced. In contrast to applications used in pharmacological studies, where cleanup is often the main issue, achieving quantifiable analyte amount is generally the main challenge in environmental analysis. Sample volumes of a few 10 mL often have to be enriched to quantify analytes in the low ng/L range with conventional LC–MS/MS systems, which typically have absolute sensitivities of some 10 pg.

Online SPE-methods using manual loop injections [18] or a LC pump [19] for sample delivery are not compatible for routine analysis. Applications designed for multiple sample handling – but without autosampler – either use a multi-port valve [20] or a solvent delivery system [21–23]. They are well suited for all applications where only a restricted number of samples have to be analyzed, e.g., to investigate degradation processes of pesticides in water over time in the same solution [24,25]. The main drawback of these systems for routine analysis is the limited number of individual samples which can be processed. Gen-

Table 1
Structure and substance properties of the sulfonamide antibiotics and their acetyl metabolites

	R	Sulfa- (SA)			Acetylsulfa- (Ac SA)		
							
		CAS	pK_{a1} pK_{a2} [38]	$K_{ow\ (exp)}$ [39] $K_{ow\ (calc)}$[a]	CAS	pK_a[a]	$K_{ow\ (calc)}$[a]
-Diazine		[68-35-9]	2.0 [40] 6.4	0.8 1	[127-74-2]	6.3 ± 0.3	3
-Thiazole		[72-14-0]	2.4 [40] 7.1	1 2	[127-76-4]	7.0 ± 0.1	7
-Methazine		[57-68-1]	2.4 [40] 7.4	2 6	[100-90-3]	7.2 ± 0.5	21
-Methoxazole		[723-46-6]	1.8 [41] 6.0	8 8	[21312-10-7]	5.6 ± 0.5	30
-Dimethoxine		[122-11-2]	1.9 [41] 6.1	40 28	[555-25-9]	6.0 ± 0.5	111

[a] Calculated values using Advanced Chemistry Development (ACD/Labs) Software Solaris V4.67.

erally, a maximum of 22 samples could be analyzed without manual interaction by combining several valves [26]. This precludes "unattended" analysis of large sample sets over several days, e.g., during weekends. Therefore, an autosampler is an indispensable prerequisite for high sample throughput in routine analysis.

However, conventional LC-autosamplers are designed to typically inject 10–100 μL from a sample only. Online SPE–LC applications with large volume injection by autosampler using single or repeated injection have only been realized for the analysis of different pesticides with a injection volume up to 4.3 ml [27–30], respectively 10 mL [31]. Overall, the dispensable sample volume which can be handled by the autosampler is the key factor for method sensitivity. The amount of available vial positions is limiting the sample throughput. To date, the maximum capacity for high volume vials is 24 [31].

In this paper, we describe a fully automated online SPE–LC–MS/MS setup for analysing different groups of polar contaminants in natural waters. The cost effective instrumental approach (i) incorporates all the advantages of the different existing online SPE methods: large volume injection, unattended 24 h/7 days operation, low risk for contamination, parallel extraction and separation for high sample throughput and (ii) is applicable for very polar analytes, such as sulfonamide antibiotics and triketone pesticides. Furthermore, the flexible instrumental setup allows the transfer of established offline SPE procedures into online SPE–LC–MS/MS applications. A combination of commercially available components was used, which were added to a standard LC–MS/MS using column switching techniques. By combining a standard tri-directional autosampler with a large-volume dispenser system, it was possible to achieve high sensitivity using standard chromatographic and detection equipment at low investment costs.

The efficiency and applicability of the developed setup is demonstrated with three analytical methods for (i) the sulfonamides: sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethoxazole, sulfathiazole including their acetyl metabolites; (ii) the neutral pesticides: atrazine and its desethyl metabolite, dimethenamide, diuron, isoproturon, metolachlor, simazine, tebutam and terbuthylazine and (iii) the acidic pesticides: 2,4-D, dimethenamide-ethanesulfonic acid (ESA) and oxanilic acid (OXA), MCPA, mecoprop, mesotrione, metolachlor-ESA and -OXA and sulcotrione. To the authors' best knowledge, this is the first online SPE method developed for the quantification of sulfonamide antibiotics including their acetyl metabolites as well as for the triketone pesticides (i.e., mesotrione and sulcotrione) in ambient waters. The three analytical methods were successfully applied for a field study of sulfonamide antibiotics, neutral and acidic pesticides in an agricultural region within the catchment area of Lake *Greifensee* near Zurich, Switzerland.

2. Experimental

2.1. Hardware

A tri-directional autosampler (HTC PAL, CTC Analytics, Zwingen, Switzerland) with 80-μL side-port syringe (80-mm needle, Hamilton, Bonaduz, Switzerland) combined with a large volume dispenser module (10-mL dispenser syringe with 10-mL loop, CTC Analytics, Switzerland) and two sample trays with 64 positions for 20-mL vials (BGB Analytik, Böckten Switzerland) was used for sample injection and buffer addition. Sample enrichment was achieved with an 18-mL sample loop (custom product, BGB Analytik, Switzerland) on an Oasis hydrophilic-lipophilic balance (HLB) extraction cartridge 20 mm × 2.1 mm I.D., 25 μm particle size (Waters, Rupperswil, Switzerland) using two six-port valves (VICI, Schenkon Switzerland). The LC pump system consisted of a binary pump (load pump), a quaternary low pressure mixing gradient pump (elution pump), an isocratic pump (precolumn addition pump) (all Rheos 2000, Flux instruments, Switzerland) and a column oven (Jones, Omnilab, Mettmenstetten, Switzerland). Two different analytical columns equipped with guard columns were used: A Nucleodur C_{18} Gravity 125 mm × 2 mm I.D., 5 μm (Macherey&Nagel, Oensingen, Switzerland) for the sulfonamides and the neutral pesticides and a GromSil ODS 3 CP, 125 mm × 2 mm I.D., 3 μm (Stagroma, Reinach, Switzerland) for the acidic pesticides. The LC was coupled with an electro spray probe (ESI) to a TSQ Quantum triple quadrupole MS (Thermo Electron, San Jose, CA, USA), operated under unit resolution in the selected reaction monitoring (SRM) mode. Details of the substance specific parameters for the ionization and detection of the sulfonamides are given in Table 2; those for the pesticides are published elsewhere [8].

Table 2
Substance specific MS/MS parameters for the sulfonamides: precursor, quantifier-product, qualifier-product, collision energy (in parentheses)

Analyte	Precursor	Quantifier	Qualifier
Sulfadiazine	251.1	156.0 (22)	92.0 (34)
D_4-Sulfadiazine	255.1	160.0 (20)	96.0 (32)
Sulfathiazole	256.0	108.0 (28)	92.0 (34)
D_4-Sulfathiazole	260.1	112.0 (32)	96.0 (32)
Acetylsulfadiazine	293.1	134.1 (28)	198.0 (24)
Acetylsulfathiazole	298.1	134.1 (30)	198.0 (24)
D_5-Acetylsulfathiazole	303.1	139.1 (20)	203.0 (10)
Acetylsulfamethazine	321.1	186.0 (26)	134.1 (34)
Sulfamethazine	279.1	186.0 (24)	108.0 (36)
$^{13}C_6$-Sulfamethazine	285.1	114.0 (34)	186.0 (22)
Sulfamethoxazole	254.1	156.0 (22)	92.0 (32)
D_4-Sulfamethoxazole	258.1	160.0 (20)	96.0 (28)
Acetylsulfamethoxazole	296.1	134.1 (30)	108.0 (30)
D_5-Acetylsulfamethoxazole	301.1	139.1 (32)	203.1 (26)
Acetylsulfadimethoxine	353.1	156.0 (28)	134.1 (32)
Sulfadimethoxine	311.1	156.0 (26)	92.0 (36)
D_4-Sulfadimethoxine	315.1	160.0 (36)	96.0 (24)

ESI conditions (positive mode): spray voltage 3500 V, sheath gas 40 bar, auxiliary gas 5 bar, ion transfer capillary temperature 350 °C.

2.2. Online SPE–LC setup

The setup of the online SPE–LC coupling with the two switching valves is shown in Fig. 1. The dispenser system consisted of a large volume dispenser syringe connected to the autosampler syringe and to a wash solution via dispenser

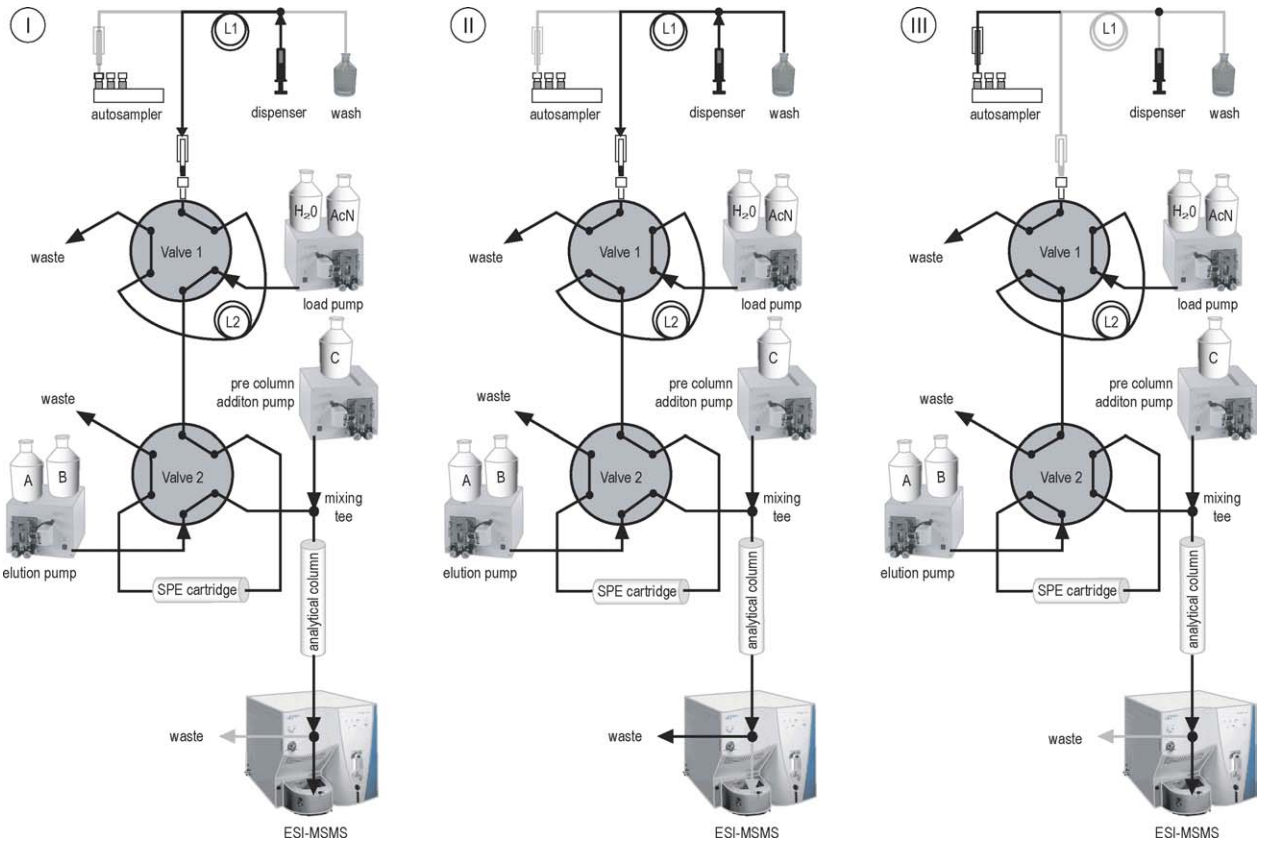


Fig. 1. Schematic view of the online SPE–LC–MS/MS setup during the three SPE steps: (I) "loading"; (II) "enrichment"; (III) "elution", according to Table 3. L1: dispenser loop; L2: sample loop; H_2O: HPLC grade water; AcN: HPLC grade acetonitrile; composition of eluents A, B and C, see Table 4.

valve. An additional loop was inserted between dispenser valve and autosampler syringe to avoid contamination of the dispenser syringe. Sample enrichment was performed by the load pump, which was also used for washing and conditioning the extraction cartridge. The load pump was connected to the 18-mL loop via valve 1. Valve 1 was linked up with valve 2, where the elution pump and the extraction cartridge where attached. The precolumn addition pump was placed between valve 2 and the analytical column using a mixing tee (Omnilab, Mettmenstetten, Switzerland). The whole procedure was controlled through Xcalibur software version 1.4 (Thermo Electron).

The online SPE procedure consists of three main steps: loading, enrichment and elution (Fig. 1, Table 3). The 18 mL sample loop was loaded with two times 9.5 mL sample. The sample was enriched with a flow rate of 2 mL/min. Elution was done in the back-flush mode. The SPE eluate was mixed with buffered water from the precolumn addition pump prior to the analytical

Table 3
Actions of the different components during the SPE-steps

SPE-step		Time	Valve 1	Valve 2	Dispenser	Load pump	Elution + precolumn addition pump
III	SPE-Elution sample *n*	0		Switch			
		0.5–3.5				Wash sample loop with H_2O	LC-gradient elution sample *n*
		3.5–5.5				Wash sample loop with AcN	
		5.5–10.5			Buffer addition	Wash sample loop with H_2O	
I	Loading sample *n* + 1	10.5	Switch	Switch	Charge dispenser and sample loop with sample *n* + 1		LC-gradient elution sample *n* (continued)
		10.5–15				Wash SPE cartridge with AcN	
		15–22.5				Conditioning SPE with H_2O	
II	Enrichment sample *n* + 1	22.5	Switch				LC-gradient elution sample *n* (continued)
		22.5–33			Wash diluter system	Extract sample *n* + 1	

Note: the three SPE-steps are arranged according to chromatographic time schedule which is different from the order in Fig. 1. Eluents and gradients for elution and LC, see Table 4. During SPE-elution and LC-gradient elution of a given sample *n*, the next sample *n* + 1 is loaded and extracted.

Table 4
Gradients for the three different methods, all flow rates in μL/min

Time	Sulfonamides[a]				Neutral pesticides[b]				Acidic pesticides[c]			
	%A	%B	%C	Total flow	%A	%B	%C	Total flow	%A	%B	%C	Total flow
0	5	5	90	400	0	40	60	200	0	40	60	150
4	5	5	90	400								
4.1	20	20	60	250								
20	40	40	20	250	0	90	10	200	0	70	30	150
22	0	80	20	250	0	90	10	200				
23									0	90	10	150
24	0	80	20	250	0	40	60	200				
25									0	90	10	150
26	20	20	60	250					0	40	60	150
28	5	5	90	400								
33	5	5	90	400	0	40	60	200	0	40	60	150

Instrumental setup of the eluents, see Fig. 1. Different steps of the online SPE, see Table 3.
[a] A: water, 20 mM formic acid, pH 2.7; B: methanol; C: water, 10 mM ammonia acetat, pH 7.
[b] A: not used; B: methanol, 20 mM formic acid; C: water, 20 mM formic acid, pH 2.7.
[c] A: not used; B: methanol, 120 mM formic acid; C: water, 120 mM formic acid, pH 2.3.

column. The high pressure gradient for the analytical separation was achieved by changing the ratio of the elution pump (eluents A and B) and the precolumn addition pump (eluent C). Different solvents were used for the eluents A, B and C in the three different analytical methods. The composition of the eluents and the gradient tables for the three different analyte groups are shown in Table 4.

2.3. Chemicals

N^4-Acetylsulfamethazine, sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethoxazole and sulfathiazole were supplied by Fluka (Buchs, Switzerland); the isotope labelled internal standards D_5-acetylsulfamethoxazole, D_5-acetylsulfathiazole, D_4-sulfadiazine, D_4-sulfadimethoxine, D_4-sulfamethoxazole and D_4-sulfathiazole by Toronto Research Chemicals (North York, Canada) and $^{13}C_6$-sulfamethazine by Cambridge Isotope laboratories (Andover, MA, USA). Suppliers of the pesticides are published elsewhere [8]. N^4-Acetylated analogues of sulfonamides other than sulfamethazine were synthesized by acetylating the sulfonamides with acetic acid anhydride [32].

Individual stock solutions for all compounds and internal standards were prepared in methanol with concentrations of 1 μg/μL. Aqueous mixture solutions for the different groups of analytes were prepared in concentrations of 0.1 and 1 ng/μL. The latter solutions were used as spiking solutions for sample fortification and for the development of calibration curves. Internal standard solutions, prepared in methanol, contained from 0.5 to 2.5 ng/μL of each substance.

HPLC grade acetonitrile, methanol and water were used (Scharlau, Barcelona, Spain). All other chemicals were of p.a. quality and were purchased from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland), respectively. High purity argon (>99.998%) for use as collision gas (1.5 mTorr) in LC–MS/MS analyses was supplied by Carbagas (Rümlang, Switzerland). Nitrogen gas for the ESI was generated online using a high purity nitrogen generator (NM30L, Peak Scientific Instruments, Renfrew, UK).

2.4. Environmental samples

Mass flux studies of veterinary sulfonamide antibiotics on grasslands and pesticides in crop protection were carried out during spring/summer 2003 in the basin of Lake *Greifensee*, near Zurich, Switzerland. An automatic water sampling station was installed in the creek at the outlet of a sub-catchment of 0.7 km^2; which is characterised by intensive agricultural production, mainly grasslands and crop production. Catchment discharge volumes were measured and flow-proportional water samples were taken at very high frequency during the whole investigation period. Surface water samples for extraction recovery determinations were collected from the outflow of Lake *Greifensee* on 22nd January and from the creek at the outlet of the investigated sub-catchment on 26th February 2003. All samples were transferred to 1 L glass bottles and stored in the dark at 4 °C for maximal 6 months until analysis; storage stability was proven by repetitive analysis of one fortified sample.

2.5. Sample preparation

Samples were filtered at room temperature in the laboratory with a 250 mL bottle-top filtration unit, using 50 mm diameter 0.45 μm pore size cellulose nitrate membrane filters (Milian, Geneva, Switzerland). Filtration recoveries – validated using fortified lake and creek water samples – were higher than 95% for all substances.

For reproducible trapping on the extraction cartridge the sample pH (ranging from 6.5 to 8.5) was adjusted to 4 by adding 80 μL of 5 M acetate buffer (composition: 5 M acetic acid/5 M sodium acetate 4:1 (v/v)) via the autosampler. This yielded a concentration of 20 mM acetate in the sample (nanopure, creek lake and groundwater), which was sufficient for adequate buffering of the different environmental water samples.

2.6. Quantification quality assurance validation and extraction recovery

For analysis of water samples, 10 μL aliquots of internal standard solution were added to 100 mL sample volume, mixed thoroughly for 10 min and an aliquot of 20 mL was used for analysis. Double blank (nanopure water without analytes and internal standard solution) and blank samples (without analytes but with internal standard solution) were extracted in every sequence to control for carry-over or background concentrations. We used corresponding isotope labelled internal standards for quantification of all substances except for acetylsulfadiazine, acetylsulfadimethoxine, acetylsulfamethazine, ethanesulfonic acid ESA- and OXA- metabolites of dimethenamide and metolachlor. In this case structurally related compounds with respect to the elution in liquid chromatography were used instead: D_4-sulfadimethoxine for acetylsulfadimethoxine, D_5-acetylsulfathiazole for acetylsulfadiazine and acetylsulfamethazine, acetochlor ESA and OXA for the corresponding metabolites of dimethenamide and metolachlor. Calibration curves from extracted nanopure water standards spiked at 2.5, 5, 10, 25, 50, 100, 250, 500, 1000, and 2500 ng/L were used for sample quantification (i.e., extracted calibration). Quality assurance was performed by measuring an extracted calibration curve at the beginning as well as at the end and quantifying the same fortified sample in every sequence.

The following parameters were determined during validation of the three analytical methods: absolute extraction recovery, matrix effects, limits of quantification (LOQ) and detection (LOD), linearity, precision and accuracy. Absolute extraction recovery was determined in nanopure water and natural surface water at six concentration levels (100, 250, 500, 1000, 2500, and 5000 ng/L). Therefore, the SPE elution step (ten minutes) was collected, spiked with internal standard solution and measured by 20 μL loop injection without further pre-concentration to avoid another pathway of potential losses and quantified with standards in nanopure water (i.e., external calibration, levels 2, 5, 10, 20, 50, and 100 ng/mL). The absolute extraction recovery of each analyte resulted from calculating the ratio between the slope of the extracted calibration curve (nanopure or matrix) and the slope of the external calibration. Additionally, breakthrough samples were collected by sampling the waste line of valve 2 during enrichment.

3. Results and discussion

3.1. Instrumental setup development

The instrumental setup for the online SPE–LC coupling was accomplished by several upgrades to a conventional LC–MS/MS system: a dispenser syringe, two loops, two LC pumps, two six-port valves and an online extraction cartridge. The financial investment for the upgrade was 25 k$.

The required sensitivity of a few ng/L was achieved with enrichment of 18 mL realized by dual injection with the dispenser syringe. The combination with the free programmable autosampler allowed automatic sample buffering and could also be used for reagent addition in future applications. The addition of two large capacity sample trays for 20-mL vials enabled to execute sequences with large numbers of samples several days and over night without surveillance on-site.

A sorbent for enrichment should combine a large specific surface area and hydrophilicity in order to have maximum interactions with (bi)-polar analytes [33]. Different copolymer phases designed for that purpose are commercially available. We used a macroporous poly(*N*-vinylpyrrolidone-divinylbenzene) copolymer phase with a surface area of about 800 m^2/g. This material already served as sorbent in many offline SPE applications for the same palette of substances (e.g., [8,34,35]). Accordingly, it proved to be very successful also for online enrichment. This fact enabled the direct transfer of existing offline SPE to the developed setup. All the laborious SPE steps from offline protocol (i.e., washing, conditioning, enrichment) were automated and executed by the load pump (details see Table 3). A satisfactory elution solvent must be able to overcome the interactions of the enriched analytes with the sorbent material. Sharp elution profiles were achieved using (i) back-flush mode and (ii) high organic solvent content for SPE elution with the elution pump.

However, the high organic solvent content of SPE eluate is not suitable for reverse phase chromatography. LC is still beneficial to separate the analytes from remaining sample matrix reducing ion-suppression in the ESI and to achieve sufficient separation of the individual substances. Therefore, dilution of the SPE eluate (eluents A and B) with buffered water (eluent C) was performed by the insertion of the pre column addition pump with a tee piece followed by a low volume (4 μL) mixing chamber. This enabled the trapping and refocusing of the eluted analytes on the analytical column and allowed the readjustment of pH for LC separation.

The applied column switching approach has two major advantages: (i) the gradient formation with two pumps (i.e., elution and precolumn addition pump) is very flexible for the online SPE of very different compounds enabling to adjust organic solvent content and pH for SPE elution and LC separation and (ii) analysis time was cut in half because sample enrichment of sample ($n + 1$) took place at the same time as the previously enriched sample (n) was separated and detected by LC–MS/MS (analysis time of 2 samples/h).

3.2. Realization of the elution-trapping for the different analytical methods

The *sulfonamide antibiotics* represent the most polar of the investigated compound classes. In order to trap these analytes on the analytical column, the initial conditions for LC are restricted to very low organic solvent content. Minimum acceptable organic solvent content of the elution mixture and the influence of pH were evaluated to determine optimal elution conditions. The addition of formic acid resulted in sharper elution profiles compared to neutral or basic elution, likely due to the higher water solubility of the cation of the sulfonamides. A 1:1 mixture of formic acid (pH 2.7, 20 mM in water) and methanol proved to be the best solvent for complete elution from the SPE cartridge in less than 4 min. The pH of the SPE eluate

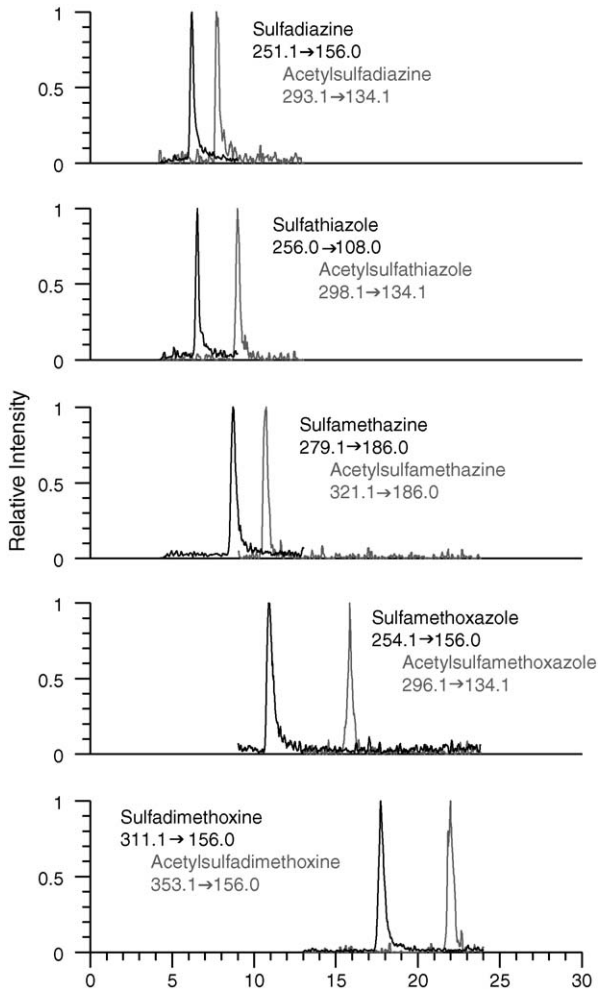


Fig. 2. Illustrative online SPE–LC–MS/MS chromatogram of a 10 ng/L standard for the sulfonamides and their acetylmetabolites.

was re-adjusted to neutral pH by adding ammonium acetate (pH 7, 10 mM in water) to achieve maximal trapping on the analytical column. In addition, a higher flow rate of the precolumn addition pump was applied at the beginning of the chromatographic run to reduce the methanol content to 5% in order to trap the most polar sulfonamides, i.e., sulfadiazine and sulfathiazole. An illustrative chromatogram of the sulfonamides and their acetyl metabolites is shown in Fig. 2.

The development of the analytical procedure for the *neutral pesticides* was much simpler. Elution with acidic methanol (20 mM formic acid) in combination with precolumn addition of acidic water (pH, 2.7 20 mM formic acid) was successful. These acidic eluents even enhanced the ionization efficiency and did not strongly affect analyte trapping and separation on the analytical column.

The elution of the *acidic pesticides* from the extraction cartridge was achieved with acidic methanol (120 mM formic acid). Acidic conditions were required to protonate the acidic pesticides (pK_a between 2.6 and 3.1) for optimal trapping and separation on the analytical column. This was realized by precolumn addition of acidic water (pH 2.3, 120 mM formic acid). Ionization in the positive ESI-mode (ESI^+) is unfavourable for the acidic pesticides because of their electron rich groups (e.g., carboxylic acids, triketones) whereas the negative ESI-mode (ESI^-) is problematic due to interferences with humic acids. However, ESI^- could be highly enhanced with the post column addition of a basic solution (pH 10, 10 mM Tris:MeOH 50:50) [8], which was also used here.

3.3. Cross-contamination

Several cleaning routines were implemented in different method steps to avoid cross-contamination in routine analysis of samples within a broad concentration range using the same equipment: (1) washing of the dispenser syringe and loop with a mixture of water and methanol (90/10, v/v), (2) washing of the cartridge with organic solvent and (3) washing of the analytical column with high organic solvent content after each sample.

The most important potential source for carry-over is the extraction cartridge, where the analyte concentrations are highest. Therefore, the extraction cartridge, as well as the sample loop, were flushed with organic solvent after every extraction to remove any residues of the sample and conditioned with distilled water prior to enrichment of the next sample to make sure no organic solvent was left in the online SPE system. Initial experiments with methanol as washing solution resulted in carry-over of several percent for the less polar substances metolachlor and tebutam. By replacing the washing solution with acetonitrile, carry-over was reduced for the critical substances. Maximum carry-over rates of less than 0.1% were determined when we measured blanks, arranged directly after highly concentrated samples in a sequence. For the more polar pesticides, sulfonamides and acidic pesticides, no carry-over problems were detected even at concentrations of several 1000 ng/L.

The additional loop inserted between the dispenser syringe and the autosampler syringe prevented contamination of the dispenser syringe with sample. Hence, the dispenser syringe was never in contact with any sample. A small air bubble was placed between sample and wash solution in the dispenser loop to prevent contamination of the washing solution with any sample.

3.4. Performance and validation of the online SPE–LC–MS/MS method

3.4.1. Extraction recovery

High extraction recoveries were achieved for all of the compounds: sulfonamides 85–104% (average: 91 ± 5)%, neutral pesticides 95–111% (102 ± 4)%, acidic pesticides 99–112% (105 ± 3)% (analyte specific details see Table 5). There were no significant differences in extraction recoveries between nanopure and surface water for any of the three analyte groups. The recoveries for the substances without corresponding internal standard were not significantly different (two-sided, heteroscedastic *t*-test, $p > 5\%$) from the ones with isotope-labelled internal standard within the substance group. No breakthrough was observed for most of the substances, except for the sulfonamides and dimethenamide OXA, where breakthrough <5% of the enriched amount was detected. This is much lower than with the published offline procedure [8]. We ascribe this improve-

Table 5
Validation parameters for the three different methods: absolute extraction recovery (%) in nanopure and surface water (in parentheses: combined relative standard uncertainty (%)) and LODs in environmental sample matrix

Substance	Absolute extraction recovery (%)		LOD (ng/L)
	Nanopure (n = 6)	Surface (n = 6)	
Acetylsulfadiazine	94 (2)	104 (3)	5
Acetylsulfadimethoxine	85 (1)	92 (2)	5
Acetylsulfamethazine	96 (1)	95 (2)	5
Acetylsulfamethoxazole[a]	87 (2)	91 (2)	5
Acetylsulfathiazole[a]	95 (3)	97 (3)	5
Sulfadiazine[a]	87 (2)	92 (2)	1
Sulfadimethoxine[a]	85 (1)	87 (1)	1
Sulfamethazine[a]	86 (1)	93 (1)	1
Sulfamethoxazole[a]	91 (1)	87 (1)	3
Sulfathiazole[a]	89 (1)	91 (2)	1
Atrazine[a]	103 (1)	111 (2)	0.5
Desethylatrazine[a]	101 (2)	105 (1)	0.5
Dimethenamide[a]	101 (3)	107 (1)	0.5
Diuron[a]	97 (2)	101 (1)	0.5
Isoproturon[a]	100 (2)	104 (1)	0.5
Metolachlor[a]	95 (1)	106 (1)	0.5
Simazine[a]	99 (3)	104 (1)	0.5
Tebutam[a]	102 (2)	106 (1)	0.5
Terbuthylazine[a]	96 (2)	104 (1)	0.5
2,4-D[a]	106 (2)	108 (2)	1
Dimethenamide ESA	110 (5)	102 (3)	3
Dimethenamide OXA	103 (5)	103 (6)	3
MCPA[a]	102 (3)	103 (3)	1
Mecoprop[a]	105 (3)	106 (4)	1
Mesotrione[a]	99 (3)	105 (5)	2
Metolachlor ESA	112 (6)	100 (3)	3
Metolachlor OXA	107 (5)	107 (4%)	3
Sulcotrione[a]	102 (3)	104 (4)	2

Note: matrices for extraction recoveries in surface water are creek water for the sulfonamides and lake water for the pesticides.
[a] Isotope-labelled internal standards were used.

ment to the 15 times higher sorbent to sample volume ratio of the online method compared to offline SPE.

3.4.2. Matrix effects

Matrix effects were evaluated during analysis of several hundred environmental samples from a broad range of different matrices, i.e., creek water from agricultural area, lake water and groundwater, by monitoring the change of the area of the isotope labelled standard, which was spiked at the same concentration in every sample.

In general, matrix effects led to reductions in peak area of less than 25%. This reduction is attributed to ion suppression in the ESI since no differences in absolute extraction recoveries were observed between nanopure and matrix water. However, for selected samples and substances matrix effects were much larger. Maximal ion suppression of up to 70% was observed for the sulfonamides in creek water samples during discharge events. For the neutral pesticides maximum ion suppression up to 50% was found. This result is comparable with findings from offline SPE [8]. Conversely, ion suppression for the acidic pesticides was much smaller, ranging from 0 to 25%. In addition, signal enhancements up to 30–60% for sulfonamides were noticed in groundwater samples. This effect has been reported previously in the analysis of various environmental samples [36,37].

These observations reveal that matrix can affect sensitivity in both directions: signal reduction due to ion suppression as well as signal enhancement. Nevertheless, these matrix effects had no effect on the quantification of analytes – except a slight decrease in sensitivity – when using corresponding isotope labelled internal standard. Analytes without corresponding isotope labelled internal standards were quantified with structural analogues, closely eluting internal standards.

3.4.3. Limit of quantification and detection/linearity

Limits of quantification and detection were influenced by sensitivity of the equipment and were strongly dependent on the sample matrix. Limits of quantification, i.e., 10:1 signal-to-noise, were between 1 and 10 ng/L for the parent substances in environmental waters. The detection for the metabolites of the sulfonamides and acidic pesticides was slightly less sensitive. Nevertheless, quantification was possible for the metabolites above 15 ng/L in surface water samples. The limit of detection, i.e., 3:1 signal-to-noise, for the neutral pesticides (LOD 0.5 ng/L) were slightly lower than for the sulfonamide antibiotics (LOD 1 ng/L, except sulfamethoxazole 3 ng/L, acetyl metabolites 5 ng/L) and the acidic pesticides (phenoxyacids LOD 1 ng/L, triketones LOD 2 ng/L, OXA/ESA metabolites 3 ng/L), respectively (Table 5). This is probably due to lower ionization yield in the spray compared to the neutral pesticides. Even though sensitivity was satisfactory for our purpose, it could be increased by introducing a larger loop charged by repeated injection. Calibration curves were linear over three orders of magnitude – up to 2500 ng/L – in more than 10 extracted calibration curves, indicating the high reliability of the whole procedure for various conditions.

3.4.4. Precision and accuracy

Precision: Run-to-run variation (i.e., intra day precision) within one sequence and day-to-day variation (i.e., inter day precision) were investigated with environmental samples. The latter includes additional effects, such as different operators, calibrations and cartridges. Replicate extraction (n = 10) of aliquots of one environmental sample was used to check the intra-day precision. The relative standard deviation of the average concentration was less than 1.5% (level 25–100 ng/L). Inter-day precision was determined by repeated analysis of aliquots from the same sample over 6 months. Relative standard deviation of the average was 3 to 6% for the sulfonamides (level 250 ng/L, n = 5), 1–3% for the neutral (level 50 ng/L n = 4) and 2–5% for the acidic pesticides (level 50 ng/L, n = 4).

Accuracy: Due to lack of reference material the accuracy of the method was determined as recovery of spiked analytes relative to their internal standard. The ratio between the quantified amount (background subtraction, if necessary) and the spiked amount is defined as relative recovery. Environmental samples were spiked at concentration 40–50 ng/L and treated like samples (see Section 2.6) in different sequences. Relative recoveries were in the range of 91–109% for the sulfonamides

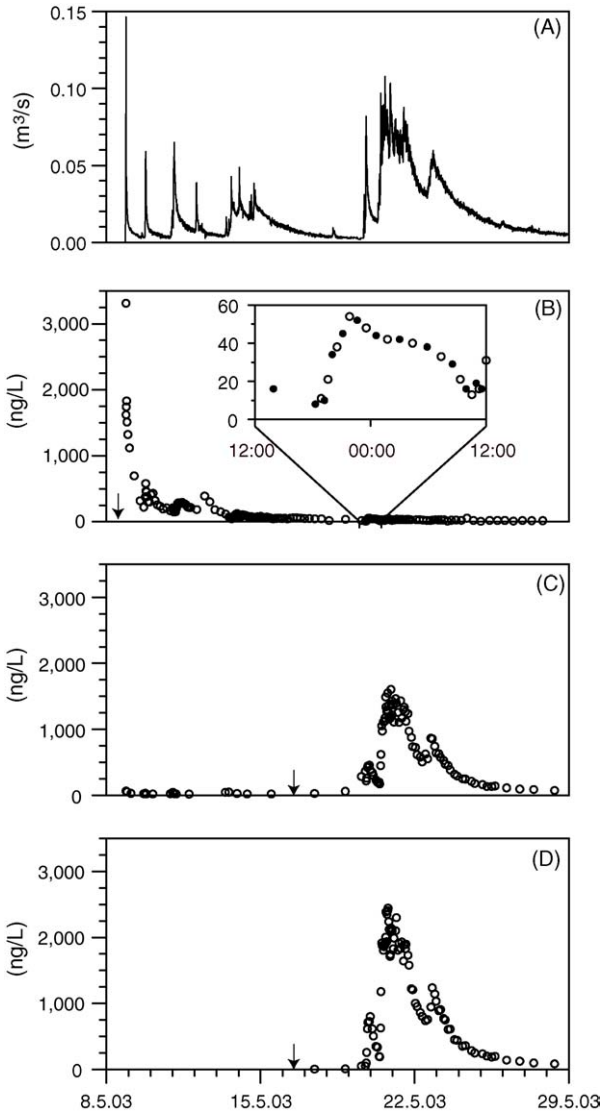


Fig. 3. Overview field study 2003: temporal development of water discharge (A) and concentrations of sulfamethazine (B); atrazine (C) [42]; and sulcotrione (D) [42] in the brook at the outlet of the 0.7 km^2 catchment after manure and pesticide application (marked with arrows). Enlarged section of sulfamethazine: samples measured in June 2003 (open circle) and July 2003 (closed circle).

($n = 2$), 92–115% for the neutral ($n = 4$) and 88–114% for acidic pesticides ($n = 4$) indicating fairly high accuracy.

3.5. Application to environmental samples in catchment studies

The method was used for mass flux studies of sulfonamides antibiotics and pesticides in a small agricultural catchment (0.7 km^2). Sulfamethazine containing manure and pesticides were each applied on several fields with areas of 0.5–1.5 ha in spring 2003. An overview of the temporal variation of analyte concentrations measured in the creek is given in Fig. 3. Generally, the concentrations for each analyte follow discharge dynamics of the creek, which was already shown for neutral pesticides in an earlier study in the same catchment [13]. Measured concentrations were highly variable indicating the need for high frequent sampling and sample analysis. Concentration ranged from a few 10 ng/L up to several 1000 ng/L after the respective applications. This demonstrates the requirement for a large dynamic measuring range of the analytical method as well as a high sensitivity in order to quantify also "base flow concentrations" as well as "pre application samples". Furthermore, prevention of cross contamination is of particular importance in measuring samples with differing concentration by orders of magnitude. The inter-day precision of the developed analytical methods was proven by alternating measurement of the samples in a period of more than 2 months including different extraction cartridges and different calibration standards. The perfect congruency of the time course of the measured concentrations illustrates the reliability of the employed analytical procedure (see Fig. 3B). In total 600 surface water samples, 400 standards and 200 quality control samples were measured during the 3-month lasting field study. The high sample throughput of the developed instrumental setup enabled the fast and precise quantification of sulfonamides and pesticides in the highly dynamic creek water system—an absolute must for in mass balance studies.

4. Conclusions

The fully automated online SPE–LC–MS/MS approach introduced in this paper, based on the combination of commercially available standard components, made it possible to achieve high sensitivity and high sample throughput in the low ng/L range with low financial investment (<25 k$). Manual sample preparation was reduced to sample filtration and spiking of the internal standard solution, which decreases expensive labour time by more than a factor of five. More than 500 samples could be analyzed with one extraction cartridge. The costs for extraction material are reduced by more than 75% compared to offline SPE, where SPE cartridges are for single use only.

Tailor-made analytical methods for the quantification of sulfonamide antibiotics, neutral pesticides and acidic pesticides were validated and successfully applied in different projects, e.g., catchment studies of agricultural chemicals or lake and ground water monitoring. The flexibility of the instrumental setup is supporting the tailor-made optimization according the substance-specific properties at every step of the procedure: pH and solvent composition can be adjusted for enrichment, elution, separation and ionization. This allows applying the instrumental setup to many other polar substances within a broad range of physico-chemical properties, such as other antibiotic classes (e.g., macrolides), pesticides (e.g., glyphosate) or biocides (e.g., benzotriazoles).

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