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Extraction and determination of sulfonamides, macrolides, and trimethoprim in sewage sludge

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Abstract

Pressurized liquid extraction (PLE) was optimized and validated for the determination of sulfonamide and macrolide antimicrobials and trimethoprim in sewage sludge samples. A mixture of water/methanol (50:50, v/v) was found as the most efficient extraction solvent. A temperature of 100 °C and a pressure of 100 bar were chosen for extraction. Two cycles of 5 min each efficiently extracted at least 97% of the total extractable amount of all studied analytes from activated sludge. The limits of quantification ($S/N = 10$) varied between 3 and 41 μg/kg dry weight (dw) and the relative recoveries ranged between 78 and 142%. Additionally, the influence of pH and different LC/MS/MS systems on the absolute recoveries was assessed. Of the investigated antimicrobials sulfapyridin, sulfamethoxazole, trimethoprim, azithromycin, clarithromycin and roxithromycin were detected in municipal sewage sludge samples. Concentrations in activated sludge ranged up to 197 μg/kg dw. In comparison, results obtained by ultrasonic solvent extraction were significantly lower for sulfonamides and in tendency lower for macrolides.

Keywords: Sewage sludge; Pressurized liquid extraction; Sulfonamide; Macrolide; Antimicrobials

1. Introduction

Antimicrobial agents are widely used in human and veterinary medicine. The overall human consumption of antimicrobials amounts to over 30 t per annum (t/a) in Switzerland and over 400 t/a in Germany – resulting in a similar consumption of approximately 5 g per person and year in both countries [1–3]. Sulfonamides (16–21% of the total human consumption) and macrolides (9–12%) are the most important groups of antimicrobials used by humans, following the beta lactams (50–60%).

Human-use pharmaceuticals, including antimicrobial agents, are excreted unchanged or metabolized from the patients' body. Therefore, they mainly reach wastewater treatment plants (WWTPs) through household wastewater. The occurrence and fate of pharmaceuticals in WWTPs and receiving surface waters has hence been of increasing interest in recent years [4–10]. In the case of antimicrobials, this is also motivated by the possible maintenance and spread of resistance caused by the constant input of low concentrations of antimicrobials. They have been detected in WWTP effluents and receiving surface waters illustrating the importance of WWTPs as point sources and the almost ubiquitous presence of these emerging contaminants [11–17]. The occurrence of macrolides and sulfonamides in WWTP effluents also indicates an incomplete removal during conventional wastewater treatment. No distinction between sorption and degradation can be made since the studies performed so far focus on the fate and occurrence in the aqueous phase, except for fluoroquinolones. Golet et al. [18] showed that specific sorption to sludge is the main removal route of the highly polar fluoroquinolones in wastewater treatment. This clearly illustrates the need for analytical methods for sewage sludge

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when assessing the fate and occurrence of contaminants in wastewater treatment. Methods published so far for the determination of other antimicrobials in environmental biosolids focus on the veterinary use and on the spread of contaminated manure onto soil. Analytical methods and studies performed range from animal food products [19] to manure [20–22] and to soil [23–29]. Additionally river sediments [30] and meat from production animals [31,32] were analyzed for sulfonamide and/or macrolides. A review on part of the literature available can be found in [33]. In most cases the compounds of interest were extracted from the samples by ultrasonic solvent extraction (USE) or blending with a suitable solvent. USE represents a simple and relatively low priced approach. In a few cases, pressurized liquid extraction (PLE), also known as accelerated solvent extraction (Dionex), was applied [18,26]. Using PLE the sample is extracted under high pressure and high temperature to enhance solubility and mass transfer [34]. Further advantages of PLE are the minimal solvent usage and automation, which enables the simultaneous extraction of a high number of samples.

In this study we aimed at developing a sensitive and reliable method for the extraction of macrolides, sulfonamides and trimethoprim (Fig. 1) from activated and digested sewage sludge. By comparing different extraction procedures (PLE and USE) and the application of different analytical methods in two different laboratories, an expanded validation of the method is achieved. Results from the analysis of municipal activated and digested sludge samples from Germany and Switzerland are given to show the applicability of the methods presented.

2. Experimental section

2.1. Chemicals and reagents

HPLC-grade methanol, acetonitrile, and water were purchased from Scharlau (Barcelona, Spain). Analytical grade ethyl acetate, acetone, ammonia solution, 25% sulfuric acid, sodium chloride, sodium hydroxide, ammonium acetate, and formic acid were obtained from Merck (Darmstadt, Germany).

Sulfamethazine (SMZ), sulfamethoxazole (SMX), sulfadiazine (SDZ), oleandomycin (OLE) and roxithromycin (ROX) were purchased from Sigma-Aldrich (Buchs, Switzerland). Sulfathiazole (STZ), sulfapyridine (SPY), trimethoprim (TMP), tylosin (TYL), and erythromycin (ERY) were obtained from Fluka Chemicals (Buchs, Switzerland). Sulfamethazine-phenyl-$^{13}C_6$ ($^{13}C_6$SMZ) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and sulfamethoxazole-d_4 (d_4SMX), sulfadiazine-d_4 (d_4SDZ), sulfathiazole-d_4 (d_4STZ) as well as N^4-acetylsulfamethoxazole-d_5 (d_5N^4AcSMX) were purchased from Toronto Research Chemicals (North York, ON, Canada). Clarithromycin (CLA) was kindly supplied by Abbott (Wiesbaden, Germany) and azithromycin (AZI) by Pfizer (Zurich, Switzerland). Azithromycin is also available from Sigma-Aldrich (Buchs, Switzerland). Standard solutions for dehydro-erythromycin (ERY-H_2O) were prepared from erythromycin as described by McArdell et al. [16]. The acidic solution was readjusted to pH 6 after 4 h using 1M NaOH to ensure stability during storage.

2.2. Sample collection

Grab samples were taken from the end of the nitrification compartment at different municipal WWTPs in Germany and Switzerland (activated sludge). All plants consist of primary clarification and a denitrification – nitrification cascade with an internal recirculation of sludge as secondary treatment. Phosphate removal is performed by the addition of iron salts to different treatment steps. In WWTP-W, located at Wiesbaden, Germany, serving 350,000 population equivalents (PE), Fe(II)Cl_2 is added to the final clarification. Simultaneous precipitation with Fe^{3+} in secondary treatment is performed at WWTP-K, located in Kloten-Opfikon, Switzerland, near the international airport of Zurich (55,000 PE), and at WWTP-A, located in Altenrhein, Switzerland, close to the border with Austria (40,000 PE). Additionally, a grab sample was collected from the outlet of the anaerobic, mesophilic digester at WWTP-K containing a mixture of primary and secondary sludges (digested sludge).

Activated sludge samples were filtered through glass fiber filters (GF8, Whatman) and the solid fraction was frozen. Digested sludge was directly frozen without filtration. Samples were subsequently freeze-dried and finely ground in a mortar. The dry sludge samples were stored in amber glass bottles at −25 °C until analysis. Consequently, the results obtained for activated sludge are given in μg/kg dry weight (dw), while those for digested sludge, including the aqueous phase, are given in μg/L. The concentration of solids in the freeze-dried digested sludge was determined to be 17 ± 6 g/L.

2.3. Sample preparation

For USE an aliquot (500 mg) of freeze-dried sludge was successively extracted with 4 and 2 mL methanol and then two times with 2 mL acetone (Table 1). In each extraction step, the sample slurry was ultrasonicated for 5 min. Surrogate standards (see Section 2.4) were spiked into the slurry of the first methanol extraction before ultrasonication. The slurries were centrifuged at 3000 rpm for 5 min after each extraction step and the supernatants collected. The solvent of the combined supernatants was evaporated to a volume of ∼200 μL, which was then diluted with 150 mL of local groundwater for solid phase extraction as a clean-up step.

For PLE samples of freeze-dried sludge were weighed (200 mg) and transferred into 11-mL extraction cells (Dionex) partly filled with quartz sand (Table 1). During mixing, more sand was added until the cell was completely filled. For extraction an automated Dionex ASE 200 accelerated solvent extractor (Sunnyvale, CA, USA) equipped with

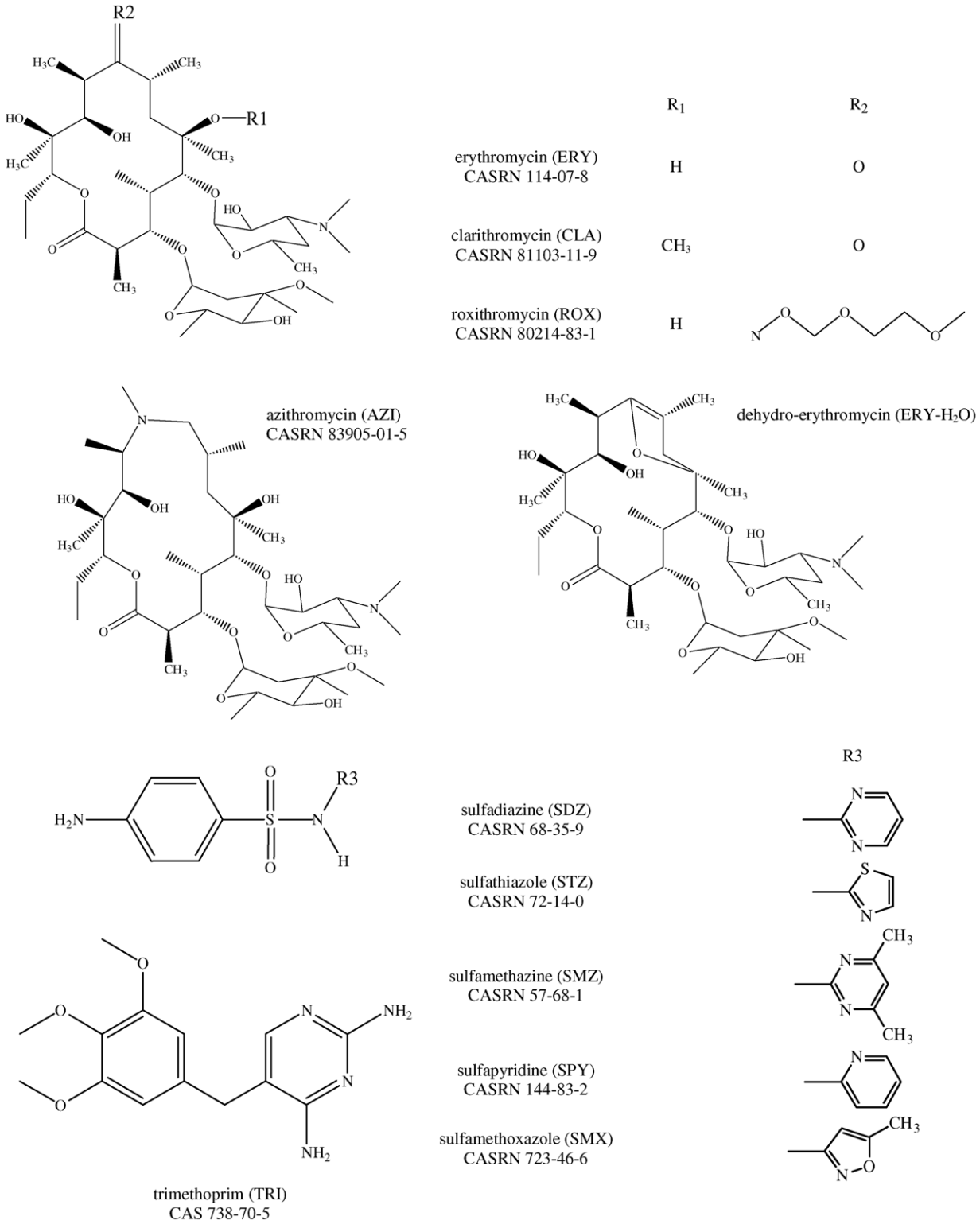

Fig. 1. Chemical structures of the investigated sulfonamides, macrolides and trimethoprim.

a solvent controller was used. A methanol–water mixture (50/50, v/v) proved to be optimal as extraction solvent. An extraction temperature of 100 °C and an extraction pressure of 100 bar were chosen as operating conditions. Preheating time and static time were set to 5 min each. A total flush volume of 120% the cell volume and a purge time of 60 s with nitrogen was used. The final extraction volume was ~22 mL with three extraction cycles for activated sludge and two for digested sludge. The PLE extracts were completely transferred to 500 mL amber glass bottles by rinsing the col-

Table 1
Extraction procedures for sulfonamides, macrolides and trimethoprim from activated sludge

Parameter	Pressurized liquid extraction (PLE)	Ultrasonic solvent extraction (USE)
Sample amount	200 mg	500 mg
Solvent	methanol:water (1:1, v/v)	methanol acetone
Time	three cycles of 5 min (preheating time 5 min)	four times for 5 min (4 mL methanol, 2 mL methanol, 2 mL acetone, 2 mL acetone)
Temperature	100 °C	–
Pressure	100 bar	–
Flush	120% of cell volume for all three cycles	–
Nitrogen purge	60 s	–

lection vial with ~100 mL of de-ionized water in three steps. They were further diluted with ~350 mL de-ionized or local groundwater to reduce the methanol content of the sample for solid-phase extraction to below 5%. Surrogate standard (see Section 2.4) was spiked directly on the sludge in the extraction cell (method 2 and method 1 during method development) or in the PLE extract prior to dilution (method 1).

The respective extracts of both extraction methods (USE and PLE) were adjusted to pH 4 with sulfuric acid or directly enriched without pH adjustment (pH 7). Solid-phase extraction was performed on 6 mL Oasis HLB sorbent cartridges (200 mg) (Waters, Bergen op Zoom, The Netherlands). Detailed information on solid-phase extraction can be found in Göbel et al. [17].

2.4. LC tandem MS analysis

Different methods were used at two different laboratories for the separation and detection of sulfonamide and macrolide antimicrobials in sludge extracts (Fig. 2), both based on methods published for aqueous wastewater samples [17,35]. In method 1, separation was achieved using a 150 mm × 2 mm YMC Pro C18 column (120 Å, 3 μm, Stagroma, Reinach, Switzerland) and a mobile phase of methanol–water containing 1% (v/v) formic acid. Gradient elution was used at a flow rate of 150 μL/min. A triple quadrupole mass spectrometer, TSQ Quantum Discovery (Thermo Finnigan, San Jose, CA, USA), equipped with electrospray ionization was used for detection. A spray voltage of 3500 V and a capillary temperature of 350 °C were applied. Analyses were performed in the positive multiple reaction mode using two transitions per analyte. An external calibration curve in de-ionized water was used for quantification. For accurate amounts the results were corrected with the corresponding relative recovery rates (over SPE and measurement) obtained from spike experiments in the same matrix. Therefore, the following substances (100 ng) were added to the PLE extracts: d_4SDZ, d_4STZ, d_4SMX, $^{13}C_6$SMZ as surrogate standards for sulfonamides and TYL as surrogate standard for macrolides. $^{13}C_6$SMZ was also used as surrogate standard for trimethoprim.

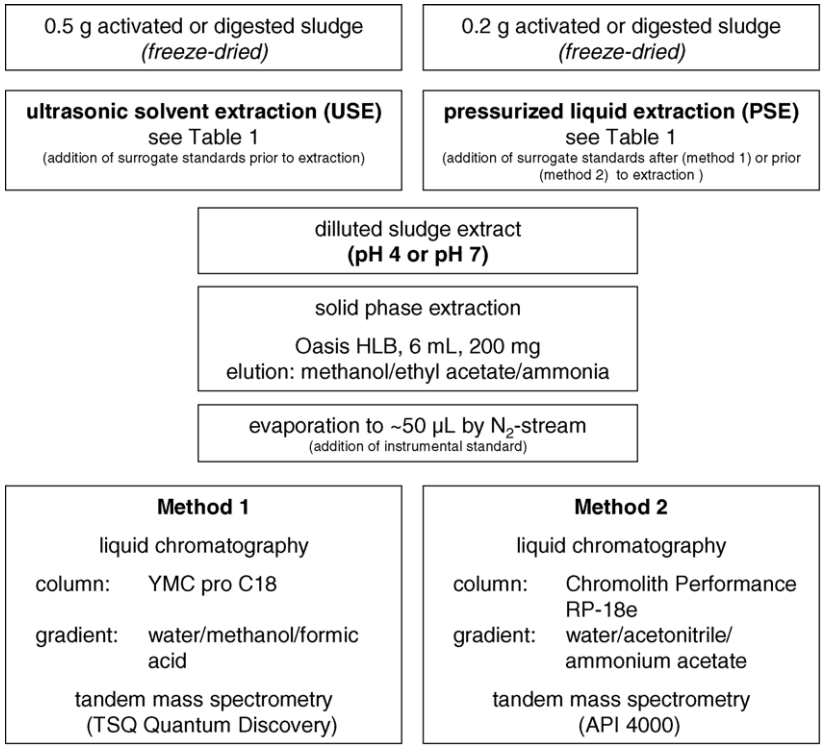

Fig. 2. Scheme for extraction and analysis of sulfonamides, macrolides and trimethoprim in sewage sludge.

In method 2, separation was achieved on a 100 mm × 4.6 mm Chromolith Performance RP-18e column at a flow rate of 400 μL/min and a total run time of 50 min. Gradient elution was performed with solvent A (water containing 10% acetonitrile and ammoniumacetat (10 mM)) and solvent B being a mixture of 80% acetonitrile and 20% solvent A. Initial conditions were set to 100% A. After 10 min the percentage of B was increased to 26% within 5 min and to 38% in the following 2 min. After 7 min of 38% B, the percentage of B amounts to 100% in a time span of 6 min, where it was kept for 4 min. Within 2 min initial conditions were restored and run for another 14 min. Detection was performed using a triple quadrupole mass spectrometer, API 4000 (Applied Biosystems, Foster City, CA, USA), equipped with electrospray ionization. An ion source voltage of 5.000 V and a temperature of 750 °C were applied, while the declustering potential was compound dependent and ranged between 56 and 106 V. Analyses were performed in the positive multiple reaction mode using two transitions per analyte. Quantification was performed using an internal calibration curve in local groundwater. Surrogate standards (100 ng) were added prior to USE or PLE extraction. d_4SMX was used as surrogate standard for all sulfonamides and trimethoprim and OLE for all macrolides. No surrogate standard was used for azithromycin and sulfapyridine, which were subsequently quantified by comparing peak areas of the samples and the calibration. In the case of sulfapyridine, all results obtained were additionally corrected by the respective absolute recoveries obtained using a matrix spike recovery for each sample.

In both methods, the SPE extracts were mostly diluted up to 10-fold with de-ionized water prior to measurement. Filtration of the final extracts prior to measurement led to significant losses of the analytes, especially in the case of the macrolide antimicrobials.

2.5. Extraction development

For PLE method development, an additional activated sludge sample from WWTP-K was filtered and the solid fraction was spiked with an aqueous solution raising the concentration of analytes by approximately 400 μg/kg dw. The mixture was stirred manually for (1/2) h and subsequently freeze-dried. This was considered to be the best substitute for native sludge where the interaction between compounds and sludge may be different due to aging effects. Spiking was necessary since not all compounds investigated were present in the sludge sample taken. By varying extracting conditions the following parameters were optimized by duplicate analyses in the order given: extraction solvent (nine solvents and mixtures), extraction temperature (60/80/100/150/200 °C), cycle time (1/3/5/10/20 min), extraction pressure (60/80/100/120/150 bar) and sample amount (100/200/400 mg). Multiple sequential extraction (4 × 5 min, $n = 2$) of the same sludge sample (activated and digested) was performed to ensure quantitative extraction. Therefore, the extracts of the individual cycles were collected separately. As with all extraction methods, so called "bound residues" are not assessed with the sequential extraction. The maximum extractable amount was defined as the sum of the amounts measured in the four cycles. The amount recovered in each cycle was expressed as a percentage of this sum (extraction yield). To assess the stability of the compounds investigated during PLE extraction, quartz sand as inert matrix was spiked with analytes (100 ng) and extracted ($n = 2$) as described.

In the case of the USE method, parameters generally suitable for the extraction of sewage sludge were chosen (Table 1) [36]. Exhaustive extraction under the given conditions was tested by prolonged extraction of activated sludge with acetone.

2.6. Method validation

Accuracy was assessed by relative recovery studies using area ratios (analyte/surrogate standard) for quantification. Freeze-dried activated sludge was spiked prior to extraction in the extraction cell with analytes (50–100 ng) in methanol and surrogate standard and subsequently analyzed ($n = 2$–3). Therefore, the surrogate standard is only used to account for experimental losses during extraction and enrichment of the sample as well as for matrix effects (e.g. ion suppression) during measurement. It cannot account for the interactions of the analyte with the sludge itself. For relative recoveries over solid-phase extraction and measurement, activated sludge extracts were spiked with analytes (50–100 ng) and surrogate standards prior to solid-phase extraction ($n = 2$). The calculated amount of antimicrobials minus the amount already present before spiking ($n = 2$–3) was related to the spiked concentration. Absolute recoveries were obtained using absolute areas instead of area ratios. The areas obtained in spiked activated sludge (50–100 ng, prior to or after extraction) minus the areas obtained in the respective non-spiked samples, were compared to the areas obtained from an external standard with the same concentration as the spike.

Breakthrough of the analytes on the SPE cartridges was determined by the enrichment of spiked activated sludge (400 μg/kg dw) in duplicate analyses using two stacked cartridges. A breakthrough on the first cartridge triggered an enrichment on the consecutive cartridge, which was then eluted separately. Complete elution of the cartridges was verified by eluting cartridges for a second time with 1.5 mL acetone as a stronger solvent ($n = 2$). The acetone extract was then treated as a separate sample. The precision of the entire method was determined by extracting replicates ($n = 3$–6) of spiked activated sludge (90–500 μg/kg dw). It was defined as the relative standard deviation of the amount measured. Limits of quantification (LOQ) were defined by two methods. In the case of PLE, the LOQ was defined as concentrations in a sample matrix resulting in signals with signal-to-noise (S/N) ratios of 10. The concentration corresponding to the defined S/N was determined by scaling down, using the measured concentration and the assigned S/N ratio of the peak

– assuming a linear correlation through zero. Results from several samples ($n = 6$) were used to yield an average value. In the case of USE, the second lowest concentration in the linear range of the internal calibration curve in local groundwater with a S/N ratio exceeding 10 was used to estimate the LOQ.

3. Results and discussion

3.1. Method development

For PLE the effect of the different extraction parameters on the extraction efficiency was evaluated to obtain optimal relative extraction conditions for sulfonamides, macrolides and trimethoprim from activated sludge (Table 1). Various solvents and mixtures were tested first. Once the optimum solvent mixture was determined, other extraction parameters such as extraction temperature and pressure, cycle time, number of cycles and sample amount, were investigated.

3.1.1. Extraction solvent

Table 2 shows the results obtained from using water, organic solvents and various mixtures as extraction solvents. A total of 10 substances was investigated. However, only the results of the compounds mainly found in activated sludge samples are presented: sulfamethoxazole, sulfapyridine, azithromycin, clarithromycin, roxithromycin and trimethoprim.

Lower extraction efficiencies were observed for all compounds investigated, especially macrolides, when mixtures of methanol and other organic solvents (acetone or acetonitrile, 1:1) were used. Water itself proved to be a good extraction solvent for the sulfonamides but resulted in low extraction efficiencies for macrolides. More trimethoprim seems to be extracted with increasing amounts of methanol, whereas no significant influence on the sulfonamides was observed. For macrolides the highest extraction efficiencies were observed using a mixture of water and organic solvent at a ratio of 1:1. This is in accordance with previous findings of Salvatore and Katz [37] that reported increasing solubility of macrolides to a maximum with increasing solvent polarity. Mixtures of water with organic solvents other than methanol (1:1) showed similar results for most analytes but resulted in lower extraction efficiencies for sulfapyridine and trimethoprim. Methanol–water at a ratio of 1:1 was finally chosen as extraction solvent representing the best compromise for all compounds investigated. With a pK_a of ∼9 macrolides are weak bases that are positively charged at neutral pH. Since the surface of most particles in sewage sludge are negatively charged [38] ionic interactions may play a role in the sorption of macrolides to sewage sludge. Therefore, the effect of the pH of the chosen extraction solvent was investigated. No significant change in extraction efficiency for any of the analytes was observed when the pH of the water used was adjusted to 10 with sodium hydroxide. This may be caused by the buffer capacity of the sludge or indicate that hydrophobic interactions are predominantly responsible for the sorption of macrolides to activated sludge. Similar conclusions for the macrolide tylosin were made by Tolls [29], when investigating the sorption of veterinary pharmaceuticals in soil.

Table 2
Solvent influence on the extraction of sulfonamides, macrolides and trimethoprim from activated sludge[a]

Extraction solvent	Concentration[b] (μg/kg dw)					
	SPY	SMX	TMP	AZI	CLA	ROX
Methanol/acetone (1:1)	116	527	138	113	42	112
Methanol/acetonitrile (1:1)	120	572	139	133	74	121
Methanol	268	594	321	252	180	195
Methanol/water (3:1)	282	635	295	260	219	251
Methanol/water (1:1)	**287**	**667**	**225**	**368**	**337**	**351**
Methanol/water (1:3)	289	663	217	103	339	369
Water	291	667	228	33	211	231
Water/acetone (1:1)	125	652	144	485	341	364
Water/acetonitrile (1:1)	214	698	222	375	314	343

[a] Selected operating condition in bold letters.
[b] Mean of duplicate analyses using pressurized liquid extraction. Extraction parameters: 100 °C, 100 bar, one cycle of 10 min, 150% flush. Extracts adjusted to pH 4 prior to solid phase extraction. Chemical analysis: method 1.

3.1.2. Extraction temperature and pressure

The effect of extraction temperature on the extraction efficiencies of the analytes turned out to be less profound (data not shown). An extraction temperature of 100 °C was selected as operating condition. Slightly lower extraction efficiencies (10–20%) were observed for all analytes at temperatures below 100 °C. However, if the extraction temperature was increased above 100 °C, the extracted amounts decreased drastically. Compared to the chosen extraction temperature, only 60–80% of most sulfonamides and trimethoprim were measured at an extraction temperature of 200 °C. For sulfamethoxazole a reduction by 95% and for the macrolides investigated a reduction by 60–90% was observed. These findings may be ascribed to a thermal degradation of the analytes at temperatures above 100 °C. Additionally, it was observed that increasingly darker extracts were obtained at higher extraction temperatures, indicating a larger extraction of soluble organic matter. This resulted in problems during solid-phase extraction due to the clogging of the cartridges. An identical effect was observed when increasing the extraction pressure from 60 to 150 bar. However, no significant impact of increasing extraction pressure was observed on the extraction efficiencies of the compounds investigated (data not shown).

3.1.3. Cycle time and sample amount

A cycle time of 5 min resulted in maximum extraction efficiencies for almost all compounds. However, the effect of the extraction time observed was low (variations below 20%) for the investigated sulfonamides, macrolides and trimetho-

prim (data not shown). An influence of the cycle time on the extraction efficiencies may be expected due to the higher extraction temperature used in PLE resulting in a reduction of the viscosity of the solvent. It may therefore, penetrate further into the sample matrix, a process also facilitated by the increased pressure. The extraction efficiencies may furthermore be enhanced by the swelling of the matrix while in contact with the solvent. These processes can also be influenced by the ratio of sample matrix to extraction solvent. However, no significant influence on the extraction efficiency of the analytes from varying sample amounts was observed (data not shown).

3.1.4. Number of cycles

Multiple sequential extractions of the same sample (activated and digested sludge) were performed to evaluate the ability of the method to quantitatively extract sulfonamides, macrolides and trimethoprim from the matrices investigated. For all analytes, except azithromycin, no significant amounts (<2%) were recovered from activated or digested sludge after the first cycle. As shown in Fig. 3 approximately 90% of azithromycin was recovered from activated sludge in the first cycle. Another 7% were recovered in the second cycle, whereas the amounts present in the last two cycles were not quantifiable. Therefore, three cycles were performed in the analyses of activated sludge to assure quantitative extraction. In the case of digested sludge 82% of azithromycin was recovered in the first cycle and another 12% in the second cycle. Even though small amounts could still be detected in the third (4%) and forth (2%) cycle, two extraction cycles were chosen for the extraction of digested sludge. The slightly incomplete extraction of azithromycin was neglected since severe problems were encountered in solid-phase extraction (clogging of the cartridges) and measurement (bad peak shape) when more than two cycles were performed. These findings indicate that the extraction efficiency of azithromycin varies with the sample matrix. It has to be noted however, that complete method development for PLE was performed only for activated sludge.

Also in the case of ultrasonic solvent extraction, exhaustive extraction of activated sludge was achieved with the chosen parameters (Table 1), since no significant amounts of analyte could be detected in the acetone extract of an already extracted sample.

3.1.5. Thermal degradation

Since thermal degradation seems to occur at elevated temperatures, the stability of the analytes under the chosen extraction conditions for PLE was of potential concern. However, recoveries from spiked quartz sand ($n = 2$) varied around 100% for all substances giving no evidence of thermal instability. Deviating results were obtained for trimethoprim (150%) and azithromycin (81%) and are probably due to a different behavior of these analytes and the respective surrogate standards ($^{13}C_6$SMZ and TYL) during solid phase extraction.

3.2. Method validation

3.2.1. Accuracy

The accuracy of the method, expressed by relative recoveries, is influenced by different parameters, e.g. the suitability of the surrogate standard used or the method applied for chemical analysis. For pressurized liquid extraction, solid phase extraction at pH 4 and method 1 for separation and detection the relative recovery ranged between 78 and 106% for the sulfonamides and trimethoprim and between 91 and 142% for the macrolides (Table 4). In that case no major differences were observed between relative recoveries over the entire method (including extraction) and over solid-phase extraction and measurement (excluding extraction). The results from both studies were therefore combined. The small variations obtained when combining both, illustrate the thermal stability of the compounds during extraction.

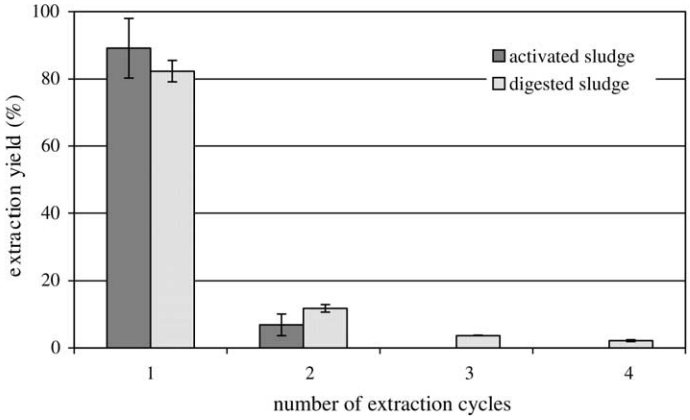

Fig. 3. Results for azithromycin from the multiple sequential extractions of activated and digested sludge. Error bars represent the range of duplicate analyses. Pressurized liquid extraction: parameters of Table 1. Extracts were adjusted to pH 4 prior to solid phase extraction. Chemical analysis: method 1. The extraction yields are displayed as percentage of the total amount extracted in the four cycles.

Additionally, they indicate that the analytes spiked on the freeze-dried activated sludge are extracted quantitatively with the selected extraction conditions. Since spiked analytes are not exposed to the same active sites as native pollutants this result cannot be extrapolated to native activated sludge samples. However, quantitative extraction of native sulfonamides, macrolides and trimethoprim was shown for activated sludge with the developed method by performing multiple sequential extraction experiments.

In the case of absolute recoveries no correction by using surrogate standards is performed. Therefore, they mirror possible losses during extraction, sample preparation and variations in measurement due to matrix effects. From the results obtained during method development and validation it seems that matrix effects, e.g. ion suppression, are the most important factor. Absolute recoveries were determined using two different methods for chemical analysis (see Section 2.4), but the same method for sample preparation. In both cases PLE with identical parameters was used and the extracts were adjusted to pH 4 prior to SPE. Results obtained for method 1 are given in Table 4, while those for method 2 are included in Table 5 (PLE, pH 4). Similar absolute recoveries were obtained with both methods for sulfonamides and trimethoprim. In the case of the macrolides, significantly lower values, and therefore, higher ion suppression, were obtained for method 1 compared to method 2. This could be caused by a different separation of matrix and analytes during liquid chromatography, i.e. by the choice of column and gradient. Differences in separation are also mirrored by the varying retention times of the compounds in the two methods. Additionally, two different mass spectrometers were used, which may also influence the ionization efficiency of macrolides in the samples. Especially, the differences in temperature applied and the amount of in-source fragmentation may lead to different ionization efficiencies for the two methods. Further on, the absolute recoveries were obtained from the analysis of different activated sludge samples, which also has an effect on the matrix present.

Additionally, the influence of the sample pH during solid phase extraction (SPE) on the absolute recoveries was investigated. No distinct influence was observed on the absolute recoveries for the investigated antimicrobials (Table 5). The strong pH dependence of the sulfonamide interaction with the SPE cartridge, as described for aqueous wastewater samples [17], seems not to occur in sewage sludge extracts. More or less comparable absolute recoveries were also observed for the investigated compounds at both pH values independently of the extraction method used. However, a significantly higher relative standard deviation, of up to 33%, was observed if the pH of the sample was adjusted to 4 prior to SPE. This is caused by an increased clogging of the SPE cartridges at the lower pH, which made the enrichment of the total sample volume in some cases impossible.

A dilution of the samples prior to analysis lead to a decrease of matrix effects, since the areas obtained in diluted samples were reduced to a lesser extent than expected by the respective dilution factor. In method 1, for example, absolute recoveries in undiluted samples were 26–50% lower than in six-fold diluted samples for sulfonamides. For macrolides and trimethoprim the reduction ranged between 40 and 80% compared to diluted samples.

3.2.2. Breakthrough and complete elution

Due to the simultaneous extraction of significant amounts of soluble organic matter during extraction of sewage sludge, breakthrough of the analytes from the cartridges and complete elution from the cartridges were investigated. No quantifiable amounts of the analytes could be detected on the second cartridge, which was eluted separately. When testing for complete elution, also no quantifiable amounts of analytes could be measured in the acetone eluates of already eluted cartridges. Thus, the analytes are quantitatively enriched by one cartridge and exhaustively eluted in the case of activated sludge extracts by the procedure applied.

3.2.3. Precision

Precision was characterized as the relative standard deviation determined from extracting replicates of spiked activated sludge. It ranged between 2 and 8% for pressurized liquid extraction and between 7 and 20% for ultrasonic solvent extraction (data not shown). The higher values for USE are probably caused by a higher amount of matrix extracted with the solvents used for ultrasonic solvent extraction. Another reason may lay in the series of manual extraction steps necessary compared to the fully automated extraction during PLE.

3.2.4. Limits of quantification

The limits of quantification for the analytes in activated sludge were defined using two different approaches for pressurized liquid and ultrasonic solvent extraction, respectively (Table 3). Overall, it ranges between 3 and

Table 3
Limits of quantification for sulfonamides, macrolides and trimethoprim in activated sludge

Compound	Limits of quantification (μg/kg dw)		
	Pressurized liquid extraction[a]		Ultrasonic solvent extraction[b]
	Average	Range	
SDZ	4	3–7	4
STZ	41	31–51	–
SMZ	16	12–20	4
SPY	29	21–36	4
SMX	15	10–23	4
TMP	14	9–17	10
AZI	3	2–4	40
ERY-H_2O	6	5–8	–
CLA	4	3–6	10
ROX	3	2–4	10

[a] Concentration estimated from measured samples (method 1) for a signal-to-noise of 10 ($n = 6$).

[b] Defined as the second lowest linear concentration ($S/N > 10$) of the internal calibration curve in local groundwater (method 2).

Table 4
Relative and absolute recoveries for sulfonamides, macrolides and trimethoprim in activated sludge using method 1 for chemical analysis[a]

Compound	Retention time (min)	Relative recovery[b] (%)		Absolute recovery[c] (%)	
		Average	% SD	Average	% SD
SDZ	10.3	106	7	63	6
STZ	12.7	99	5	55	7
SMZ	17.6	97	5	64	17
SPY	12.6	79	5	64	8
SMX	20.4	100	3	64	3
TMP	17.1	78	3	51	4
AZI	21.1	91	10	29	7
ERY-H_2O	30.1	112	9	37	14
CLA	31.5	110	13	33	24
ROX	31.6	142	16	45	27

[a] Pressurized liquid extraction: parameters see Table 1. Extracts adjusted to pH 4 prior to solid phase extraction. Chemical analysis: method 1.
[b] Relative recoveries were determined using area ratios of analyte to surrogate standard. Average and standard deviation (% SD) combing results from experiments with surrogate standard added prior to and after sludge extraction ($n = 4$).
[c] Absolute recoveries were determined using areas. Average and relative standard deviation (% SD) combing results from experiments with surrogate standard added prior to and after sludge extraction ($n = 4$).

41 μg/kg dw for the investigated antimicrobials. The differences observed result from a combination of various factors. Next to the different approaches applied for the estimation of the LOQ, the higher sample amount used in USE compared to PLE plays a role. Additionally, differences in the methods used for separation and detection have an influence, e.g. via peak shape and matrix effects. The results clearly indicate that the limits of quantification given can only be considered as rough estimates. In routine analysis all peaks with a *S*/*N* above 10 were therefore considered valid results.

3.3. Application to sewage sludge samples

The developed methods were applied to selected activated and digested sludge samples from different wastewater treatment plants in Germany and Switzerland (Table 6). The results for the most commonly detected sulfonamides, sulfapyridine and sulfamethoxazole, and macrolides, azithromycin, clarithromycin and roxithromycin, are given. Additionally results for trimethoprim, used almost exclusively in combination with sulfonamides, are included. The occurrence of antimicrobials in activated sludge generally correlates well with the respective aqueous phase [11,17]. Higher concentrations were generally determined in German activated sludge samples (WWTP-W), ranging up to 197 μg/kg dw for sulfapyridine, indicating a lower wastewater dilution compared to Switzerland. A maximum concentration of 73 μg/kg dw was found for sulfamethoxazole in Swiss samples (WWTP-K and WWTP-A). A more detailed discussion on the occurrence of sulfonamides, macrolides and trimethoprim in Swiss municipal wastewater treatment is given elsewhere [39].

Overall, similar results were obtained in activated and digested sludge using PLE, independently of the sample pH and the method used for chemical analysis (Table 6). However, using ultrasonic solvent extraction, the concentrations determined are generally lower for the investigated sulfonamides and in tendency lower for the investigated macrolides. This may be caused by the less radical extraction conditions, e.g. temperature and pressure, compared to pressurized liquid extraction. Additionally, the extraction conditions used

Table 5
Absolute recoveries for sulfonamides, macrolides and trimethoprim in activated sludge using method 2 for chemical analysis

Compound	Retention time (min)	Absolute recovery[a] (%)							
		PLE[b]				USE[c]			
		pH 4		pH 7		pH 4		pH 7	
		Average	%SD	Average	%SD	Average	%SD	Average	%SD
SDZ	8.6	83	12	54	6	41	13	53	11
SMX	20.4	37	19	41	4	16	16	62	7
TMP	20.0	47	7	44	3	25	10	31	8
d4SMX[d]	20.4	37	15	44	4	16	11	62	8
CLA	33.4	74	21	90	5	55	8	59	15
ROX	33.9	91	33	88	3	73	10	76	8
OLE[d]	25.3	93	9	95	3	67	5	57	14

[a] Absolute recoveries were determined using areas. Average and relative standard deviation (%SD) is given ($n = 3$) Respective relative recoveries can be calculated from the absolute recovery ratio of the analyte and its surrogate standard.
[b] Pressurized liquid extraction (PLE): parameters see Table 1. Extracts adjusted to pH 4 prior to solid-phase extraction (pH 4) or directly enriched (pH 7). Chemical analysis: method 2.
[c] Ultrasonic solvent extraction (USE): parameters see Table 1. Extracts adjusted to pH 4 prior to solid-phase extraction (pH 4) or directly enriched (pH 7). Chemical analysis: method 2.
[d] Used as surrogate standard.

Table 6
Concentrations of sulfonamides, macrolides and trimethoprim in activated and digested sewage sludge from different wastewater treatment plants in Germany (WWTP Wiesbaden) and Switzerland (WWTP Kloten-Opfikon and WWTP Altenrhein)

		Concentration[a] (μg/kg dw)[b]					
		SPY	SMX	TMP	AZI	CLA	ROX
Activated sludges							
WWTP-W	PLE + pH 4[c]	57	113	91	127	34	46
Sample 1	PLE + pH 7[d]	51	100	87	158	41	61
	USE + pH 7[e]	26	41	79	127	34	45
WWTP-W	PLE + pH 4	197	41	107	151	27	131
Sample 2	PLE + pH 7	160	37	133	115	16	83
	USE + pH 7	85	18	96	47	(9)[f]	50
WWTP-K	PLE + pH 4	29	73	30	52	30	nd[g]
	PLE + pH 7	24	51	(18)[f]	(7)[f]	25	nd
	USE + pH 7	na[g]	20	14	(21)[f]	12	nd
WWTP-A	PLE + pH 4	(11)[f]	60	21	56	63	nd
	PLE + pH 7	nd	34	13	(5)[f]	32	nd
	USE + pH 7	nd	27	nd	48	41	nd
		Concentration (μg/L)[h]					
Digested sludges							
WWTP-K	PLE + pH 4	1.0	nd	(0.1)[f]	2.3	0.8	nd
	PLE + pH 7	0.8	nd	nd	1.6	0.3	nd
	USE + pH 7	1.2	nd	nd	1.3	0.3	nd

[a] Mean of duplicate analyses for pressurized liquid extraction (PLE) and single analysis for ultrasonic solvent extraction (USE).
[b] Separation of solid and aqueous phase through filtration before freeze-drying.
[c] Pressurized liquid extraction: parameters see Table 1. Extracts adjusted to pH 4 prior to solid-phase extraction. Chemical analysis: method 1.
[d] PLE (Table 1). Extracts not pH-adjusted prior to solid-phase extraction. Chemical analysis: method 2.
[e] Ultrasonic solvent extraction: parameters Table 1. Extracts not pH-adjusted prior to solid-phase extraction. Chemical analysis: method 2.
[f] Estimated concentrations below the limit of quantification ($S/N < 10$).
[g] nd: Not detected ($S/N < 3$), na: not analysed.
[h] No separation of solid (15–18 g/L) and aqueous phase through filtration before freeze-drying.

for USE, especially the choice of solvent, were not optimized particularly for the extraction of sulfonamide and macrolide antimicrobials.

4. Conclusions

A robust and selective method for the pressurized liquid extraction of sulfonamides, macrolides and trimethoprim from sewage sludge was developed and validated. Several extraction parameters were investigated and the optimized procedure is summarized in Table 1. The method was successfully applied to activated and digested sewage sludge. Even though comparable results were obtained for different sample pHs, it is suggested to not adjust the pH of the extracts prior to solid-phase extraction, to minimize the clogging of the cartridges. The method presented can be used to investigate the occurrence and fate of sulfonamides, macrolides and trimethoprim in wastewater treatment, including the sorption to sewage sludge. Additionally, it may serve as the basis for the determination of pharmaceuticals in general in sewage sludge and other biosolids. Ultrasonic solvent extraction seems to be equally or slightly less efficient for the extraction of macrolides and trimethoprim, while significantly lower extraction efficiencies seem to result for sulfonamides compared to pressurized liquid extraction.

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