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Determination of six sulfonamide antibiotics, two metabolites and trimethoprim in wastewater by isotope dilution liquid chromatography/tandem mass spectrometry

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ABSTRACT

A highly sensitive method for the analysis of six sulfonamide antibiotics (sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfamethazine and sulfamethoxazole), two sulfonamide metabolites (N_4-acetyl sulfamethazine and N_4-acetyl sulfamethoxazole) and the commonly co-applied antibiotic trimethoprim was developed for the analysis of complex wastewater samples. The method involves solid phase extraction of filtered wastewater samples followed by liquid chromatography–tandem mass spectral detection. Method detection limits were shown to be matrix-dependant but ranged between 0.2 and 0.4 ng/mL for ultrapure water, 0.4 and 0.7 ng/mL for tap water, 1.4 and 5.9 ng/mL for a laboratory-scale membrane bioreactor (MBR) mixed liquor, 0.7 and 1.7 ng/mL for biologically treated effluent and 0.5 and 1.5 ng/g dry weight for MBR activated sludge. An investigation of analytical matrix effects was undertaken, demonstrating the significant and largely unpredictable nature of signal suppression observed for variably complex matrices compared to an ultrapure water matrix. The results demonstrate the importance of accounting for such matrix effects for accurate quantitation, as done in the presented method by isotope dilution. Comprehensive validation of calibration linearity, reproducibility, extraction recovery, limits of detection and quantification are also presented. Finally, wastewater samples from a variety of treatment stages in a full-scale wastewater treatment plant were analysed to illustrate the effectiveness of the method.

1. Introduction

The occurrence and fate of antibiotic pharmaceuticals in municipal wastewater treatment plants (WWTPs) is currently a subject of rapidly increasing interest to process scientists and water quality regulators. Much of this interest is in regard to public health concerns over the presence of residual antibiotics in the treated effluents, the disturbance to microbial ecology in receiving environments, and the potential for proliferation of antibiotic resistant pathogens [1]. The presence of antibiotics in treated effluents, even at very low concentrations, can cause toxic effects to several aquatic species and resistance among natural bacterial populations [2]. A high proportion of some administered antibiotics are excreted into domestic sewage in their original pharmacological form or as simple conjugates that may subsequently be transformed back into their active parent compounds [3,4]. Detailed knowledge of the occurrence and fate of these antibiotics during wastewater treatment is a key requirement for any assessment of public health or environmental risk.

Sulfonamides are an important group of antibiotics widely used in human and veterinary medicine to treat urinary tract infections, ear infections, bronchitis, skin and soft tissue infections. Important sulfonamides administered for these applications include sulfamethoxazole, sulfadiazine, sulfamerazine, sulfamethazine, sulfapyridine, and sulfathiazole. Trimethoprim is another antibiotic agent often co-administered with sulfamethoxazole to enhance treatment against a variety of bacterial infections by synergistically disrupting an additional step in the bacterial synthesis of folic acid, which is required for bacterial growth [5,6]. In Australia, sulfamethoxazole and trimethoprim are among the top 50 most dispensed pharmaceuticals by mass, accounting for around seven and three tonnes per anum, respectively [7]. A number of analytical methods have been recently developed to determine trace concentrations of sulfonamides and trimethoprim in several municipal

Abbreviations: APCI, atmospheric pressure chemical ionisation; CE, collision energy; CXP, cell exit potential; DP, declustering potential; ESI, electrospray ionisation; IDL, instrument detection limit; LC, liquid chromatography; MBR, membrane bioreactor; MDL, method detection limit; MRM, multiple reaction monitoring; MS, mass spectrometry; Q, quadrupole; S/N, signal to noise ratio; SPE, solid phase extraction; UV, ultraviolet; WWTP, wastewater treatment plant.

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and livestock wastewater matrices [8–15]. Some of these studies have provided analytical methods for quantifying these antibiotics in wastewater using liquid chromatography (LC) coupled with ultraviolet (UV) detection [11,15,16]. However, analytical detection limits of these methods are generally limited by significant signal interference associated with UV spectral overlaps with other wastewater constituents. Consequently, more selective mass spectral (MS) techniques have been generally preferred for analysis in wastewater applications [8–10,13,14,17].

Only a few previous studies have incorporated the analysis of the metabolites of sulfonamide in wastewater such as N_4-acetyl sulfamethoxazole [10] and N_4-acetyl sulfamethazine [9]. The occurrence of these metabolites during wastewater treatment should be routinely considered along with their active parent compounds because they are known to be transformed back to the parent compounds in wastewater environments [10]. Significant complications in the quantification of antibiotics by liquid chromatography–mass spectrometry (LC–MS) arise due to matrix-specific factors, which significantly vary depending on the origin and composition of the samples. Wastewater matrix components may reduce the extraction efficiency as well as enhance or suppress mass spectral ionisation [9]. Thus a reliable analytical method should minimise the impact of these matrix effects and must account for any variability in extraction recoveries and ionisation efficiencies derived from different sample matrices. On the other hand, their adsorption into sludge or biomass is an indispensible factor that needs to be taken into account in order to understand the removal mechanism of antibiotics in wastewater treatment processes. However, the number of analytical methods available to determine sulphonamides, their metabolites and trimethoprim in biomass is very limited [17,18].

This paper describes the development of a sensitive and reliable analytical method based on solid phase extraction (SPE) followed by analysis by liquid chromatography–tandem mass spectrometry (LC–MS–MS) for the simultaneous determination of six sulfonamide antibiotics, two N_4-acetyl sulfonamide metabolites and trimethoprim in complex matrices. The isotope dilution method, using direct structural analogues as internal standards, is considered to be the optimal approach to account for variable recoveries and matrices, and thus improve quantitative determination [19]. Isotope dilution involves the addition of a known quantity of isotope-labelled antibiotics to all sample and calibration solutions in order to normalise observed peak intensities for variable samples.

2. Materials and methods

2.1. Materials

Sulfadiazine (99% purity), sulfathiazole (98% purity), sulfamethoxazole (98% purity), sulfamethazine (99% purity), sulfamerazine (99% purity), sulfapyridine (99% purity) and trimethoprim (98% purity) were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). N4-acetylsulfamethoxazole (99% purity) was obtained from Frinton Laboratories Inc. (Vineland, NJ, USA). N4-acetyl sulfamethazine (99% purity), N4-acetyl sulfamethoxazole-d5 (98% purity), N4-acetyl sulfamethazine-d4 (98% purity), sulfadiazine-d4 (98% purity), sulfathiazole-d4 (98% purity), sulfamethoxazole-d4 (98% purity), sulfamethazine-d4 (98% purity), sulfamerazine-d4 (98% purity) and trimethoprim-d4 (98% purity) were purchased from Toronto Research Chemicals (North York, Canada). The chemical structures and properties of the above compounds can be found in Table 1. All solvents were supplied from Ajax Finechem (Tarron Point, NSW, Australia). Acetonitrile and methanol were of HPLC grade. Formic acid (99% purity), H_2SO_4 and NH_4OH solutions were of analytical grade. Ultrapure water was produced using a Driec-Q filtering system from Millipore (North Ryde, NSW, Australia). Whatman glass fibre filters and filtering system were obtained from Sigma–Aldrich (Castle Hill, NSW, Australia). Kimble culture tubes (13 mm I.D. × 100 mm height) were purchased from Biolab (Clayton, Vic, Australia). Oasis hydrophilic lipophilic balance (HLB) extraction cartridges (6 cc, 500 mg) were purchased from Waters (Rydalmere, NSW, Australia).

Stock standard solutions of antibiotics were initially prepared at 100 mg/L in methanol in amber vials and then further diluted with methanol in series to obtain working standard solutions of lower concentrations. All standard solutions of unlabelled antibiotics were stored at −18 °C and remade every three months. Stock standard solutions of isotope labelled antibiotics were also prepared in methanol and stored in amber vials at −18 °C. Working solutions of labelled antibiotics at lower concentrations were stored at 4 °C and remade from concentrated stock standards every month.

2.2. Sample collection

The analytical method validation was undertaken using a series of samples collected from a laboratory-scale membrane bioreactors (MBR) at University of New South Wales and a full-scale municipal WWTP. The WWTP treats municipal sewage from a small town (a capacity of 3800 EP) approximately 320 km north of Sydney, Australia and receiving influents of exclusively domestic origin. The main treatment processes of the WWTP were activated sludge, secondary clarification and UV disinfection. A small side-stream of the screened influent from this WWTP was diverted for treatment by a pilot-scale MBR (capacity of 25 EP, treating 4.5 kL/day) followed by electrochlorination. Grab samples (in triplicates) of screened influents, activated sludge effluents after secondary clarifier, UV effluents, pilot-scale MBR, activated sludge and MBR electrochlorination effluents were collected in glass bottles, transported at 4 °C to the University of New South Wales laboratory and then processed within 24 h of collection to minimise microbial degradation.

2.3. Sample treatment

2.3.1. Aqueous samples

Influent and effluent samples were filtered under vacuum pressure using 11 μm particle size retention glass fibre filters (Whatman No. 1, Sigma–Aldrich, Castle Hill, NSW, Australia) and then 0.7 μm pore size glass fibre filters (GF/F Whatman filters Sigma–Aldrich) to minimise cartridge clogging during the SPE step (Fig. 1). Glass fibre filters were rinsed with methanol and MiliQ before use to prevent any cross-contamination. Isotopes labelled standards were added to the filtrate for correction.

2.3.2. Sludge samples

After collection, solid components of the sludge were separated by centrifuging for 30 min at a rotation speed of 3000 rpm (Thermo Fisher Scientific). Solid samples were completely frozen and then freeze-dried (Thermo Fisher Scientific) to remove all moisture content. Since the principal concept of freeze-drying is to sublime ice into vapour without liquid formation, the sample was gradually dried without degrading other contents inside the solid. After drying (approximately 2–3 days) freeze-dried samples were stored in an amber desiccator (a moisture-free container with silica gel adsorbents) until analytical analysis using an ultrasonic solvent extraction technique.

For ultrasonic solvent extraction (USE), 500 mg of freeze-dried solid samples (i.e. sludge) was successively extracted with 5 mL MeOH, 5 mL of acetone and 4 mL of MeOH in capped Kimble culture tubes. Before the first extraction, isotope-label standards were spiked into the freeze-dried samples, which were then mixed and

Table 1
Chemical structures of target compounds and their isotope labelled standards in the study.

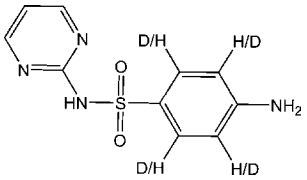
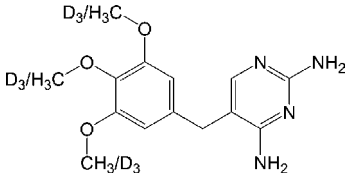
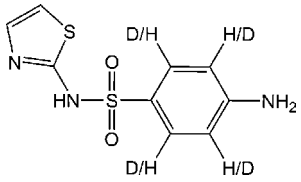
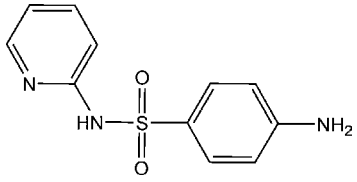
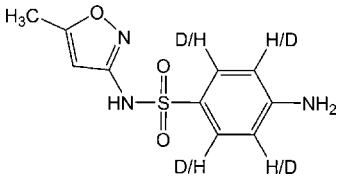
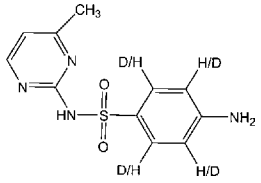
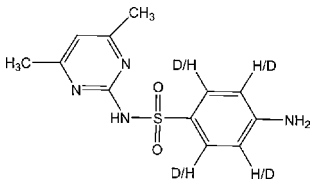
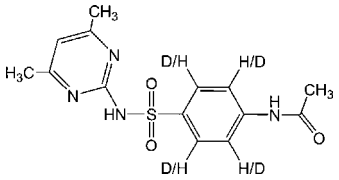
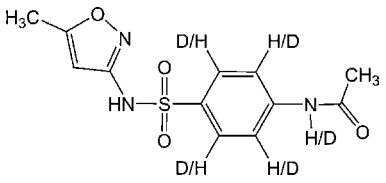
Unlabelled antibiotics (isotope labelled)	MW	Compound structure	pK_a of the antibiotics
Sulfadiazine (Sulfadiazine-d4)	250.05 (254.08)		$pK_{a,1}$ = 2.10 $pK_{a,2}$ = 6.28 [24]
Trimethoprim (Trimethoprim-d9)	290.14 (299.21)		$pK_{a,1}$ = 3.23 $pK_{a,2}$ = 6.76 [25]
Sulfathiazole (Sulfathiazole-d4)	255.01 (259.05)		$pK_{a,1}$ = 2.08 $pK_{a,2}$ = 7.07 [24]
Sulfapyridine[a]	249.06		$pK_{a,2}$ = 8.40 [26]
Sulfamethoxazole (Sulfamethoxazole-d4)	253.05 (257.08)		$pK_{a,1}$ = 1.83 $pK_{a,2}$ = 5.57 [24]
Sulfamerazine (Sulfamerazine-d4)[a]	264.07 (268.10)		$pK_{a,1}$ = 2.17 $pK_{a,2}$ = 6.77 [24]
Sulfamethazine (Sulfamethazine-d4)	278.08 (282.12)		$pK_{a,1}$ = 2.28 $pK_{a,2}$ = 7.42 [24]

Table 1 (*Continued*)

Unlabelled antibiotics (isotope labelled)	MW	Compound structure	pK_a of the antibiotics
N4-acetyl sulfamethazine (N4-acetyl sulfamethazine-d4)	320.09 (324.13)		N/A (not available)
N4-acetyl sulfamethoxazole (N4-acetyl sulfamethoxazole-d5)	295.06 (300.10)		N/A

[a]Isotope labelled standard of sulfapyridine is not commercially available. Sulfamerazine-d4 is used as a surrogate standard in the analysis for the unlabelled sulfapyridine.

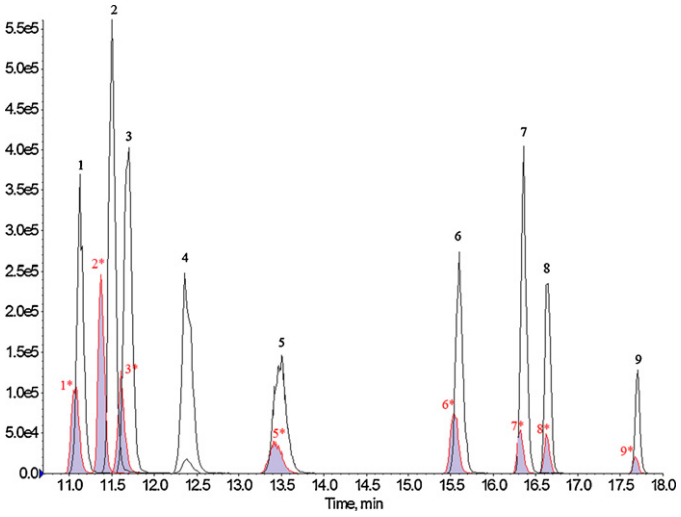


Fig. 1. Liquid chromatogram showing separation of the 9 analytes and corresponding isotopically labelled standards. Key: (1) sulfadiazine, (1*) sulfadiazine-d4, (2) trimethoprim, (2*) trimethoprim-d9, (3) sulfathiazole, (3*) sulfathiazole-d4, (4) sulfapyridine, (5) sulfamerazine, (5*) sulfamerazine-d4, (6) sulfamethazine, (6*) sulfamethazine-d4, (7) sulfamethoxazole, (7*) sulfamethoxazole-d4, (8) N_4-acetyl sulfamethazine, (8*) N_4-acetyl sulfamethazine-d4, (9) N_4-acetyl sulfamethoxazole and (9*) N_4-acetyl sulfamethoxazole-d5.

left undisturbed for more than an hour. In each extraction step, sample slurry was shaken well and then ultrasonicated (Thermo Fisher Scientific Ultrasonic Water Bath) for 10 min at 40 °C. After that, the slurries were centrifuged at 3000 rpm for 5 min. The combined supernatant of the three extractions were collected in another clean Kimble culture tube and evaporated to a volume of approximately 1 mL. The final concentrated extract was diluted with MiliQ water to 500 mL for solid phase extraction as a clean-up step.

2.4. Solid phase extraction

Sample enrichments were undertaken by SPE on Oasis HLB cartridges (Waters, Rydalmere, NSW, Australia). Prior to loading, the cartridges were conditioned with 4 mL of ultrapure water, 4 mL of methanol, 4 mL of methanol (1% NH_4OH, v/v) and finally with 4 mL of ultrapure water adjusted to pH 3.5 (acidified with 1 M H_2SO_4 solution). The pH adjustment was selected to minimise ionisation of the analytes according to the pKa values shown in Table 1, thus enhancing hydrophobic solid phase extraction. Several previous studies have demonstrated improved SPE recoveries of sulfonamides with acidification to pH 3–4 [10,20]. SPE cartridges were loaded by drawing through 1 L of water samples under vacuum, maintaining a loading flow rate of less than 5 mL/min at all times. The SPE cartridges were rinsed with 4 mL of ultrapure water before drying under a gentle flow of nitrogen gas (N_2) (BOC Gas) for 1 h. If required, dried cartridges were stored under N_2 at −18 °C prior to elution and quantitative analysis. Analytes were eluted from the cartridges with 2 × 4 mL of methanol into Kimble culture tubes (Biolab, Clayton, Australia). The extracts were centrifugally evaporated under vacuum at 35 °C using a Thermo Speedvac concentrator (Biolab, Clayton, Australia). The eluents were dried to about 50 μL in the Speedvac concentrator and then reconstituted to 1 mL with a solution of water/methanol (70/30, v/v). The reconstituted samples were sonicated for 10 min at ambient temperature in an ultrasonic water bath (Thermo Fisher Scientific) to ensure full re-dissolution. The reconstituted samples were then transferred to amber LC autosampler vials for quantitative analysis.

2.5. Liquid chromatography separation

An Agilent 1200 LC system (Forest Hill, Australia) comprised of a binary pump, a degasser, an auto-sampler with 10 μL sample loop and a column heater was used for all analysis. Chromatographic separation was undertaken on a Luna C18, 5 μm, 150 mm × 4.6 mm, 100 A analytical LC column equipped with a C18, 5 μm, 4 mm × 2 mm, 100 A column guard (Biolab, Clayton, Australia). The mobile phase was comprised of ultrapure water with 0.1% formic acid (mobile A) and methanol with 0.1% formic acid (mobile B). The column flow rate was maintained at 800 μL/min throughout the analysis. Optimal separation was obtained with an elution gradient consisting of a 2 min equilibrium at 5% mobile B, held for a further 6 min after injection and increased to 20% mobile B in 1 min and held for 3.5 min, before increasing to 100% mobile B over 10 min. To avoid carry-over and to maintain a stable separation, the column was further washed with 100% mobile B for 2 min before re-equilibration at initial mobile phase condition at 5% mobile B in 1 min, and further post-conditioned for 2 min for stabilization. The column heater was maintained at 50 °C for optimal separation and consistency.

Table 2
Optimal compound dependent parameters for tandem MS.

Compounds	Retention time (min)	MRM transitions	MRM2/MRM1 Ion ratio	DP (V)	CE (eV)	CXP (V)
Sulfadiazine-d4	11.1	255/160	0.63	56	23	10
		255/96			39	16
Sulfadiazine	11.2	251/156	0.67	61	23	8
		251/92			39	10
Trimethoprim-d9	11.4	300/234	0.67	76	35	14
		300/264			37	16
Trimethoprim	11.5	291/230	0.71	66	33	16
		291/261			35	16
Sulfathiazole-d4	11.6	260/160	0.56	56	23	8
		260/96			41	16
Sulfathiazole	11.7	256/156	0.56	56	23	8
		256/92			39	16
Sulfapyridine	12.4	250/156	0.63	61	23	10
		250/92			35	18
Sulfamerazine-d4	13.4	269/160	1.00	46	27	28
		269/96			43	16
Sulfamerazine	13.5	265/156	1.00	66	25	10
		265/92			41	16
Sulfamethazine-d4	15.5	283/186	0.75	51	27	32
		283/96			47	16
Sulfamethazine	15.6	279/186	0.77	66	25	10
		279/92			45	16
Sulfamethoxazole-d4	16.3	258/160	0.83	61	23	10
		258/96			41	16
Sulfamethoxazole	16.4	254/156	0.77	56	23	8
		254/92			39	16
N_4-acetyl sulfamethazine-d4	16.6	325/138	0.90	71	39	22
		325/186			29	10
N_4-acetyl sulfamethazine	16.7	321/134	0.88	71	39	24
		321/186			31	10
N_4-acetyl sulfamethoxazole-d5	17.60	301/139	0.63	66	37	8
		301/203		66	27	12
N_4-acetyl sulfamethoxazole	17.87	296/134	0.77	66	35	8
		296/198		66	25	12

2.6. Tandem MS analysis

Qualitative and quantitative mass spectral analysis was performed with an Applied Biosystems QTrap 4000 mass spectrometer (Mulgrave, Australia) equipped with an electrospray ionisation source (ESI) operated in positive ionisation mode. Compound dependent parameters and source dependent parameters were optimized using infusion and flow injection analysis, respectively.

For the optimization of compound dependent parameters, each analyte was directly infused at a concentration of 100 ng/mL into the mass spectrometer in a 50/50 (v/v) methanol/water solution at a flow rate of 10 μL/min. Initially, the first quadrupole of mass spectrometer (Q1) was set to scan from *m*/*z* 70 to *m*/*z* [M+100] while the declustering potential (DP), temperature, and gas flow were manually adjusted to achieve a stable and intense signal for the precursor ion, the protonated molecular ion $[M+H]^+$. After the signal was stabilized, the mass spectrometer was set to automatically scan and refine the DP. Then, the collision energy (CE) in the second quadrupole (Q2) was ramped while the ion trap (Q3) scanned thoroughly to identify the four most intense product ions. Finally, the cell exit potential (CXP) was ramped to determine the optimal value for each of four precursor/product ion transitions. After the optimization, the two most intense precursor/product ion transitions were selected for monitoring the corresponding analyte.

Once the optimal compound dependent parameters were determined (Table 2), the source dependent parameters were optimized using flow injection analysis in ESI positive mode. These included the flow rate of the curtain gas, nebulizer gas (GS1), turbo spray gas (GS2), temperature, ion spray voltage, temperature and entrance potential. For the flow injection analysis, consecutive injections of 10 μL of a 50 ng/mL solution in 50% methanol 0.1% formic acid were performed for all target compounds without LC separation. Source dependent parameters were varied and optimized for each injection. The mass spectrometer was set in multiple reaction monitoring (MRM) mode and signal intensities of all analyte transitions were recorded and monitored. The values, corresponding to the most intense signals for most of the analytes (without significant losses of sensitivity for any individual analyte) were selected to be the optimal source dependent parameters. These were as follows: curtain gas 10 psig, ion spray voltage 5500 kV, temperature 470 °C, nebulizer gas (GS1) 60 psig, turbo gas (GS2) 50 psig, collision gas 6 psig (High mode) and entrance potential 10 V. The *X*-position and *Y*-position of the ESI needle were both set at 5 mm. Analyst version 1.5.1 software (Applied Biosystems, Mulgrave, Australia) was used to control the instrument, acquire data and evaluate the results. Two MRM transitions of the precursor ion were monitored for each target compound.

2.7. Method validation studies

The instrumental detection limit (IDL) was determined based on an established standard method [21], which requires the injection of a standard solution to produce a signal that is five times the signal-to-noise ratio (S/N). Initially, ten consecutive injections of 5 pg on the LC column were performed and S/N for each analyte in every injection was calculated. Where the average S/N of any analyte was less than 15 and greater than 5, the injected mass was be used to calculate IDL for that analyte by extrapolation to a S/N of 5. For those analytes with the average S/N greater than 15, consecutive injections of half of the previous mass were performed again until all IDLs could be established. Instrumental stability was assessed on an inter-day and intra-day basis by injecting an

analytes standard solution (25 ng/mL) onto the column ten times per day over two separate days and comparing the variation in the signal intensity of each analyte standard from these injections.

Quantitative determination of the target compounds was undertaken using external calibration principles combined with the isotope dilution technique. The isotope dilution calibration curve was comprised of nine calibration points at 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, and 200 ng/mL spiked with a pre-determined mass of isotope standards. Depending on the types of samples in any batch, the mass of isotope standards added to the calibration solutions could be 10, 25 or 50 ng. The conservative lowest calibration point of 0.5 ng/mL was chosen due to the varying IDLs of the target compounds and the expectation of higher method detection limits (MDLs) in wastewater matrices.

Isotope labelled compounds were used as surrogate standards to correct for matrix effects, SPE recovery variability and instrumental variations for the unlabelled compounds. Direct analogue isotopic standards were used for all target analytes except sulfapyridine, for which no direct analogue could be commercially sourced. Accordingly, sulfamerazine-d4 was used as a surrogate standard for both sulfamerazine and sulfapyridine. Sulfamerazine-d4 was selected as the isotopic standard for sulfapyridine since these chemicals have similar chemical structures (Table 1) and close chromatographic retention times (Fig. 1). The use of isotope labelled compounds in correcting matrix effects was validated in a variety of matrices including ultrapure water, tap water, synthetic wastewater mixed liquor and wastewater effluents. Since it is not possible to find real sewage samples for which there can be confidence that they do not contain any background levels of the target compounds (even if it be below the MDL), the wastewater mixed liquor, effluent and activated sludge were collected from a laboratory-scale MBR treating a synthetic wastewater feed solution (known to be free of the analytes involved in this study). Matrix assessment was undertaken by spiking all of the target analytes (25 ng in mass) and isotopic standards (50 ng in mass) into each matrix prior to the sample extraction step. The sludge samples were spiked with analytes and surrogate standards, mixed and left undisturbed for more than hour to stimulate the process of hydrophilic–hydrophobic partitioning before ultrasonic solvent extraction. Currently, this approach is considered the ideal way to obtain the sludge containing the known concentrations of analytes. These assessments were undertaken with triplicate analysis. Absolute recoveries were determined by directly comparing signal intensity area (counts per second, cps) of the spiked samples with that of the 25 ng/mL calibration solution, while method recoveries were calculated from the isotope dilution calibration curves.

SPE recovery was assessed using spiked ultrapure water samples (six replicates) at low and high concentrations of 10 ng/L and 200 ng/L, respectively. Since the aim was to assess the loss of the target analytes during SPE extraction, the isotope standards (10 ng) were added to the SPE extracts only after the elution step for direct relative comparison to the analytes. To assess potential analyte losses during the drying and reconstitution steps, six centrifuge tubes containing 8 mL HPLC grade methanol were spiked with 10 ng of the target analytes before being vacuum dried and reconstituted. Optimal sample volumes for SPE were also investigated by comparing the SPE recoveries of ultrapure water samples of different volumes (250 mL, 500 mL, 1000 mL and 2000 mL), each spiked with same analyte mass of 25 ng. To assess the effectiveness of solvent elution of the analytes from the SPE tubes, each SPE cartridge was loaded with a 1000 mL ultrapure water sample (spiked with 25 ng of each analyte) and consecutively extracted into three eluent fractions (2×4 mL methanol followed by 1×4 mL acetone). Isotopic standards were added to final eluents prior to analysis.

Efficiencies of ultrasonic solvent extraction (USE) method for activated sludge samples were also evaluated at low and high concentration of 50 ng/g and 200 ng/g, respectively. For each concentration, triplicate samples of 0.5 g freeze-dried laboratory-scale MBR activated sludge were spiked with 25 ng and 100 ng of analytes, mixed well and left undisturbed for more than an hour. Unlike the normal procedure, the isotopic standards (50 ng) were added to the final volume of combined supernatant after three extractions rather than before the first extraction so that the actual recoveries of the analytes through USE can be determined.

Method detection limits (MDL) were determined in each of the matrices described above according to Method 1030C from Standard Methods for the Analysis of Water & Wastewater [21]. For each aqueous matrix, seven samples of 1000 mL volume were spiked with target analytes at concentrations close to the expected MDL. To determine MDL of sludge matrix, seven samples of 0.5 g freeze-dried activated sludge were spiked with target analytes instead. The samples were then spiked with isotopic standards, extracted and analysed through all above sample processing and data quantifying steps. The run of seven samples were not performed sequentially, but were divided into two batches and processed independently on different days to be more representative of day-to-day variability. MDLs were calculated by multiplying the standard deviation of seven replicates by Student's T value of 3.14 (one-side T distribution for six degrees of freedom at the 99% level of confidence). Where the calculated MDLs were greater than the actually spiked concentration of any target analytes, a further seven replicates spiked with higher concentrations were analysed to calculate revised MDLs for those analytes. Alternatively, where the calculated MDLs were 5-times smaller than the actual spiked concentrations, a further seven replicates spiked with lower concentrations were analysed to calculate revised MDLs. This procedure was repeated until MDLs of all target analytes were determined with a signal-to-variability ratio within the bounds of the above criteria.

3. Results and discussion

3.1. Chromatography and mass spectrometry

The mobile phase composition was optimized to achieve the chromatographic resolution of the analytes with the shorter analysis time. A liquid chromatogram showing optimal separation of the 9 analytes and their corresponding isotopically labelled standards is presented in Fig. 1. Isocratic and gradient methods were investigated using different solvents and buffer reagents, which were added to the mobile phases. The addition of 0.1% formic acid to aqueous and organic (methanol) mobile phases was found to give best separation and enhance mass spectrometry intensity. Relatively high column temperature of 50 °C was chosen to increase the separation efficiency and resolution at shorter analysis time. The increase in temperature reduces the dielectric constant of water resulting in a similar effect on the retention time as an increase in proportion of organic solvent in the mobile phase [16]. Due to the excellent LC separation of antibiotics, this LC method could be adapted for other LC instrument using UV or fluorescence detectors.

With direct infusion, the comparison between atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) was carried out to choose the optimal ionisation source. Although ESI were observed to provide a noisier baseline than APCI, ESI was still chosen for its greater S/N ratio. Some analytes such as trimethoprim have a prominent peak in their mass spectra at m/z $[M+23]^+$ which can be identified as sodium adduct ions. The protonated molecular ions $[M+H]^+$ were selected for all compounds to generate MS/MS spectra because the protonated molecular ions are the most abundant in the MS spectra. The detection of a target compound was confirmed using a 5 point identification criteria. This included the observed presence of the two expected MRM

Table 3
Percent absolute recoveries and method recoveries with isotope dilution of target compounds in different matrices (±RSD).

Compounds	Absolute recovery (±RSD) %					Method recovery (±RSD) %				
	Ultrapure water $n=6$	Tap water $n=3$	MBR effluent $n=3$	MBR mixed liquor $n=3$	MBR sludge $n=6$	Ultrapure water $n=6$	Tap water $n=3$	MBR effluent $n=3$	MBR mixed liquor $n=3$	MBR sludge $n=6$
Sulfadiazine	88 (±5)	32 (±6)	55 (±1)	52 (±1)	44 (±4)	102 (±9)	101 (±1)	101 (±1)	101 (±1)	101(±5)
Trimethoprim	88 (±6)	85 (±3)	103 (±2)	94 (±2)	42 (±3)	101 (±4)	112 (±2)	104 (±2)	104 (±2)	98 (±5)
Sulfathiazole	83 (±4)	31 (±8)	50 (±1)	43 (±3)	35 (±2)	103 (±5)	105 (±2)	105 (±3)	104 (±1)	94 (±8)
Sulfapyridine	86 (±3)	32 (±9)	66 (±1)	62 (±2)	42 (±4)	101 (±4)	104 (±1)	105 (±2)	102 (±4)	95 (±6)
Sulfamerazine	86 (±4)	29 (±7)	75 (±2)	65 (±2)	43 (±4)	101 (±8)	96 (±2)	107 (±4)	106 (±2)	95 (±3)
Sulfamethazine	88 (±5)	25 (±9)	80 (±1)	73 (±2)	44 (±4)	103 (±3)	104 (±1)	111 (±4)	109 (±2)	103 (±4)
Sulfamethoxazole	87 (±3)	40 (±8)	56 (±4)	42 (±1)	48 (±6)	107 (±2)	109 (±0)	102 (±2)	99 (±3)	105 (±6)
N_4-acetyl sulfamethoxazole	93 (±4)	89 (±5)	70 (±6)	52 (±2)	71 (±2)	101 (±3)	98 (±1)	100 (±2)	100 (±2)	98 (±5)
N_4-acetyl sulfamethazine	94 (±3)	91 (±7)	75 (±5)	61 (±4)	80 (±2)	99 (±5)	102 (±3)	104 (±4)	105 (±2)	106 (±2)

transitions at the same retention time, the area ratio of two transitions within a range of 20% variability with respect to the mean area ratio of all calibration solutions, and a consistent analyte-surrogate relative retention time as that of calibration solutions with relative standard deviation of less than 3%.

3.2. Method validation

3.2.1. Quality assurance

The stability of working standard solutions were evaluated by injecting the same amount of stock directly into the LC–MS/MS instrument and comparing the obtained signal intensities over three months. The concentration of stock solution appeared to be consistent over the period of more than three months with the relative standard deviations (RSD) of less than 7% for all antibiotics, indicating the acceptable stability of the working stock under the storage conditions. Degradations of analytes during storage in dark condition at 4 °C were investigated by successively analysing spiked influent and effluent samples over 3 days. Negligible changes in concentrations of all analytes in effluent samples (RSD < 5%) were observed during storage after 3 days. For influent samples, concentrations of sulfonamide antibiotics and their metabolites varied by less than 6.4% during the first 24 h of storage and reduced by 21% after 3 days. Trimethoprim concentrations in influent samples over 3 days were analysed between 92% and 106% compared to the initial concentrations, indicating a stability of this compound during storage (data not shown here).

The instrumental stability was determined to be acceptable with the relative standard deviations less than 5% and 6% for inter-day ($n=10$) and intra-day ($n=20$) runs, respectively. The linear calibration range for the target compounds was determined to be from 0.5 to 200 ng/mL except trimethoprim, for which the calibration curve was only linear from 0.5 to 50 ng/mL. The calibration points for each of the sulfonamides were fitted to linear regressions, while trimethoprim was found to better fit a quadratic regression with a weighting of $1/x^2$. In both cases, calibration curve regression correlation coefficients were always above 0.990 for all sample batches.

All isotope labelled standards used in this study included at least four deuteriums (^{2}H or D) atoms in their molecular compositions. The mass spectral signals of the isotopic standards were not affected by the unlabelled analytes since the natural abundances of higher isotope (e.g. carbon-13) compounds is, by comparison, insignificant. The calculated absolute recoveries and method recoveries of the target compounds in ultrapure water, tap water, synthetic wastewater mixed liquor, biologically treated effluent and sludge matrices are shown in Table 3. The absolute recoveries reflected the possible losses during sample preparation, extraction and variation in measurement due to matrix effects with no correction by using surrogate standards (i.e. isotope labelled standards). It was observed that although sample matrices significantly affected the signal intensities of many target analytes, the use of isotope dilution satisfactorily corrected these matrix effects and losses, leading to accurate quantification in all tested matrices (method recoveries of between 94 and 109%). Sulfamerazine-d4 was confirmed to be a reliable isotopic standard for quantitation of sulfapyridine with method recoveries in all tested matrices consistently between 95 and 105% (max RSD = 4%).

3.2.2. Solid phase extraction and ultrasonic solvent extraction recoveries

The results of SPE recoveries of the target compounds from low and high concentration spiking tests showed that the SPE method provided a satisfactory enrichment for all target compounds with recoveries of 88% or greater (Table 4). Although the RSDs from the high concentration spiking test were lower than those from the low concentration spiking test (possibly due to less variation in peak area integration at higher concentration), the mean SPE recoveries were quite consistent regardless of sample concentration. Table 5 shows the mean recoveries during the drying/reconstitution steps and the mean SPE recoveries of target analytes for different sample volumes (250 mL and 2000 mL). Negligible losses of analytes during vacuum centrifuge drying and sonication reconstitution steps were observed. Furthermore, excellent recoveries for all analytes with up to 2000 mL extraction volumes indicate that the SPE breakthrough volume has not been exceeded. In the complete elution tests, the combination of first and second methanol eluents accounted for 93–105% recoveries while no quantifiable amounts of target compounds were detected in the acetone eluent (data not shown here). The USE efficiencies were consistent between the sludge samples containing low and high concentrations of analytes (Table 4). All sulfonamides and their metabolites were effectively extracted using the sonication technique and solvents described above, with efficiencies of greater than 83%. However, efficiencies of 60% and 59% were achieved for low and high spiked concentrations of trimethoprim in sludge. The stronger partitioning to sludge of trimethoprim is possible due to its basicity property. Overall, the result indicates the extraction techniques are optimal for target compounds.

3.2.3. Instrument and method detection limits

The IDLs and MDLs in different matrices are presented in Table 6. IDLs for target sulfonamides and trimethoprim in this study were comparable with those reported in previous studies using the same tandem MS instrument API 4000 QTrap [9] and the similar generation instrument API 4000 [19]. As expected, MDLs increased with the increase in the complexity of the sample matrices. MDLs of all target compounds were between 0.2 and 0.4 ng/L for ultrapure water, 0.4 and 0.7 ng/L for tap water, 1.4 and 5.9 ng/L for laboratory-scale MBR mixed liquor and 0.7 and 1.7 ng/L for biologically treated wastewater effluent. Meanwhile, MDLs of these antibiotics in bench-scale MBR activated sludge ranged from 0.5 to 1.5 ng/g dry weight. In this study, the MDL for all sulfonamide

Table 4
Mean percent solid phase extraction (SPE) recoveries and ultrasonic solvent extraction (USE) efficiencies of target compounds from low and high concentration spiked samples (±RSD).

Compounds	SPE recovery (%) 10 ng/L spiked $n=6$	SPE recovery (%) 200 ng/L spiked $n=6$	USE efficiency (%) 25 ng spiked into 0.5 g sludge $n=3$	USE efficiency (%) 100 ng spiked into 0.5 g sludge $n=3$
Sulfadiazine	93 (±13)	88 (±6)	98 (±3)	103 (±8)
Trimethoprim	92 (±11)	100 (±6)	60 (±3)	59 (±1)
Sulfathiazole	100 (±9)	94 (±4)	84 (±2)	83 (±2)
Sulfapyridine	98 (±8)	94 (±4)	90 (±6)	99 (±4)
Sulfamerazine	95 (±13)	91 (±5)	93 (±3)	98 (±3)
Sulfamethazine	92 (±9)	93 (±7)	101 (±5)	105 (±4)
Sulfamethoxazole	103 (±11)	102 (±7)	103 (±3)	108 (±8)
N_4-acetyl sulfamethazine	102 (±8)	102 (±7)	94 (±4)	102 (±2)
N_4-acetyl sulfamethoxazole	107 (±10)	107 (±5)	108 (±3)	104 (±1)

Table 5
Mean percent recoveries during drying/reconstituting step and SPE recoveries of target compounds for different sample volume spiked with same mass of 25 ng (±RSD).

Compounds	Drying and reconstituting recovery (%) $n=6$	SPE recovery (%) 250 mL volume $n=3$	SPE recovery (%) 500 mL volume $n=3$	SPE recovery (%) 1000 mL volume $n=3$	SPE recovery (%) 2000 mL volume $n=3$
Sulfadiazine	103 (±4)	106 (±4)	98 (±4)	106 (±3)	104 (±4)
Trimethoprim	103 (±6)	111 (±8)	107 (±3)	102 (±5)	102 (±6)
Sulfathiazole	99 (±4)	103 (±2)	105 (±2)	106 (±5)	104 (±4)
Sulfapyridine	101 (±3)	103 (±6)	98 (±5)	102 (±3)	100 (±5)
Sulfamerazine	98 (±6)	102 (±5)	99 (±2)	105 (±2)	103 (±6)
Sulfamethazine	104 (±3)	106 (±2)	99 (±4)	105 (±3)	105 (±3)
Sulfamethoxazole	102 (±5)	106 (±10)	102 (±3)	102 (±1)	102 (±2)
N_4-acetyl sulfamethoxazole	101 (±5)	102 (±5)	105 (±3)	98 (±8)	96 (±1)

Note: N_4-acetyl sulfamethazine standard and its isotope labelled standard were not available at the time of this test.

antibiotics in sludge were lower than MDLs reported in previous studies using pressurised liquid extraction or accelerated solvent extraction [18,22]. The use of greater amount of solvents (methanol and acetone) together with ultrasonication at 40 °C is a possible explanation for better recoveries of sulfonamide antibiotics extracted from the sludge. N_4-acetyl sulfamethoxazole was the least sensitive target compound with an MDL in wastewater and sludge of 5.9 ng/L and 1.5 ng/g, respectively. Besides possible poor ionisation efficiency, it seems likely that the nature of the chromatographic separation may have contributed to the increased MDLs for this analyte. Increased background noise was observed in the chromatogram around the period when N_4-acetyl sulfamethoxazole was eluted (100% methanol mobile phase). At this time, many matrix compounds are expected to be co-eluted from the LC column, potentially leading to ion suppression in the ESI. Method quantitation limits (MQLs) were conservatively determined by tripling the values of MDLs.

The determinations of MDLs for the analysis of trace organic compounds in many previous studies were based on the simple principle of identifying a concentration that could achieve a S/N of 3 [9,10,13,14]. However, for complex sample matrices, S/N was often observed to significantly vary between replicates of the same sample and even between multiple injections of the same sample. In contrast, the method applied to determine MDLs in this study ensures that the reported MDLs reflect the full limitations to the overall sensitivity derived from instrument sensitivity and sample matrices including the effectiveness of LC separation, SPE efficiency and repeatability, correlation of surrogate standards to their corresponding target compounds, and the degrees of consistency and accuracy of the operator in preparing, spiking samples and quantifying analyte peak areas.

3.2.4. Analysis of wastewater samples

Results of the analysis of wastewater samples are provided in Table 7. Trimethoprim, sulfamethoxazole, and sulfapyridine were detected in almost all samples at concentrations of hundreds of ng/L or higher. The presence of sulfamethoxazole and trimethoprim in domestic wastewater were expected since they are among the top 50 (by mass) dispensed pharmaceuticals for human treatment in Australia [7]. High concentrations of sulfapyridine found in wastewater influent and effluent samples (up to 4.26 μg/L) was initially surprising since this drug is not prescribed for direct human treatment in Australia. However, further investigations revealed that sulfapyridine is the major metabolite of sulfasalazine, which is a highly prescribed anti-inflammatory drug in Australia, dispensed in even greater masses than sulfamethoxazole or trimethoprim [7]. It has been reported that up to 60% of administered sulfasalazine is excreted as sulfapyridine [23]. Sulfadiazine,

Table 6
Instrument intraday and interday stability (RSD%), instrument detection limits (IDL) and method detection limits (MDL) of target compounds in different matrices.

Compounds	Inter-day stability RSD % $n=10$	Intra-day stability RSD % $n=20$	IDL (pg) $n=10$	MDL (ng/L) in ultrapure water $n=7$	MDL (ng/L) in tap water $n=7$	MDL (ng/L) in MBR mixed liquor $n=7$	MDL (ng/L) in MBR effluent $n=7$	MDL (ng/g) in MBR sludge $n=7$
Sulfadiazine	1.7	2.4	0.2	0.2	0.7	4.4	1.6	0.9
Trimethoprim	3.3	3.8	0.1	0.3	0.3	0.9	1.1	0.6
Sulfathiazole	3.3	5.3	0.2	0.3	0.3	3.6	1.0	1.2
Sulfapyridine	2.5	3.8	0.5	0.3	0.6	3.5	1.0	0.5
Sulfamerazine	2.5	3.3	0.2	0.2	0.3	3.4	1.1	0.9
Sulfamethazine	1.9	4.2	0.2	0.3	0.4	3.3	0.9	0.5
Sulfamethoxazole	2.4	2.9	0.3	0.2	0.7	3.5	1.7	1.1
N_4-acetyl sulfamethazine	1.4	3.4	0.3	0.3	0.3	1.4	0.7	0.7
N_4-acetyl sulfamethoxazole	4.9	3.9	0.8	0.4	0.4	5.9	1.4	1.5

Table 7
Concentrations of target compounds detected in wastewater and sludge (±RSD %) ($n=3$).

Compounds	Wastewater treatment plant (capacity = 3800 EP)									
	Samples collected in July					Samples collected in November				
	Raw influent (ng/L)	MBR electro-chlorination effluent (ng/L)	Secondary clarifier effluent (ng/L)	UV-disinfected effluent (ng/L)	MBR sludge (ng/g)	Raw influent (ng/L)	MBR electro-chlorination effluent (ng/L)	Secondary clarifier effluent (ng/L)	UV-disinfected effluent (ng/L)	MBR sludge (ng/g)
Sulfadiazine	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Trimethoprim	583 (±17%)	339 (±2%)	349 (±1%)	16 (±3%)	71 (±4%)	858 (±1%)	47 (±3%)	438 (±4%)	331 (±3%)	32 (±3%)
Sulfathiazole	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Sulfapyridine	4260 (±17%)	739 (±6%)	794 (±10%)	226 (±4%)	62 (±7%)	48 (±2%)	253 (±3%)	395 (±4%)	382 (±2%)	35 (±2%)
Sulfamerazine	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Sulfamethazine	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Sulfamethoxazole	1740 (±3%)	542 (±1%)	736 (±9%)	206 (±2%)	51 (±5%)	735 (±4%)	300 (±12%)	912 (±4%)	755 (±1%)	28 (±3%)
N_4-acetyl sulfamethazine	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
N_4-acetyl sulfamethoxazole	445 (±2%)	35 (±3%)	66 (±2%)	47 (±4%)	<MQL	390 (±4%)	27 (±5%)	5 (±14%)	8 (±9%)	<MDL

MDL, method detection limit; MQL, method quantitation limit.

sulfamethazine, sulfamerazine, and sulfathiazole were not detected in any samples. This result was expected since these antibiotics are not used for human medicine in Australia, but restricted to agricultural and veterinary uses. Consistent with the identification of parent drugs, the sulfamethoxazole metabolite (N_4-acetyl sulfamethoxazole) was observed in the municipal wastewater samples, while the sulfamethazine metabolite (N_4-acetyl sulfamethazine) was not. Similarly, only sulfamethoxazole, sulfapyridine and trimethoprim were found in the MBR sludge with the concentrations ranging from 28 to 71 ng/g dry weight. N_4-acetyl sulfamethoxazole metabolite was only detected in sludge samples collected in July, but the concentrations were below the method quantification limit (<4.5 ng/g).

3.2.5. Matrix effect characterization

Since analogue isotope labelled standards were found to be effective to correct matrix effects for the corresponding compounds, the matrix effects (either signal enhancement or suppression) from real wastewater samples on unlabelled compounds were investigated by comparing the signal response of the isotope labelled standards in spiked real samples with those in spiked ultrapure water. The matrix effect on sulfapyridine could not be investigated since its analogue isotope labelled standard was not available. Real matrix samples, including screened raw sewage, electro-chlorinated MBR effluent, mixed liquor in MBR, secondary clarifier effluent and post UV effluent were collected in triplicate samples from the WWTP on two occasions in a month (Batch 1 and Batch 2). The matrix effects on the signal response – either suppressions or enhancements are summarized in Table 8. Signal suppression was observed for all wastewater matrices for all compounds except in the case of the post UV effluent sample for Batch 1, where the signal of N_4-acetyl sulfamethazine-d4 (representing N_4-acetyl sulfamethazine) was slightly enhanced by 1%. The magnitude of the mean signal suppression ranged from 6% to 86%

Table 8
Mean percent changes in signal response due to sample matrices with respect to signal response in ultrapure water (±SD) (negative = signal suppression; positive = signal enhancement).

	Screened raw influent		MBR mixed liquor		MBR-electrochlorination effluent		UV-disinfected effluent		Secondary clarifier effluent	
	Batch 1 ($n=3$)	Batch 2 ($n=3$)	Batch 1 ($n=3$)	Batch 2 ($n=3$)	Batch 1 ($n=3$)	Batch 2 ($n=3$)	Batch 1 ($n=3$)	Batch 2 ($n=3$)	Batch 1 ($n=3$)	Batch 2 ($n=3$)
Sulfadiazine-d4	−63 (±2)	−59 (±8)	−65 (±2)	−70 (±1)	−79 (±12)	−71 (±1)	−56 (±2)	−65 (±1)	−65 (±3)	−65 (±2)
Trimethoprim-d9	−80 (±2)	−77 (±6)	−55 (±1)	−28 (±1)	−57 (±2)	−32 (±1)	−51 (±1)	−40 (±2)	−54 (±2)	−31 (±3)
Sulfathiazole-d4	−66 (±2)	−63 (±7)	−66 (±3)	−75 (±2)	−81 (±11)	−77 (±1)	−58 (±2)	−68 (±1)	−66 (±3)	−69 (±2)
Sulfamerazine-d4	−52 (±2)	−55 (±5)	−49 (±4)	−59 (±3)	−69 (±16)	−64 (±2)	−40 (±3)	−56 (±1)	−52 (±4)	−55 (±2)
Sulfamethazine-d4	−42 (±3)	−54 (±9)	−44 (±4)	−63 (±1)	−68 (±18)	−67 (±1)	−31 (±2)	−63 (±1)	−43 (±5)	−64 (±2)
Sulfamethoxazole-d4	−69 (±1)	−65 (±7)	−71 (±2)	−68 (±2)	−81 (±12)	−69 (±1)	−62 (±1)	−65 (±1)	−68 (±3)	−63 (±1)
N4-acetyl sulfamethazine-d4	−36 (±1)	−42 (±4)	−15 (±3)	−10 (±2)	−11 (±10)	−14 (±3)	1 (±3)	−24 (±2)	−5 (±5)	−15 (±9)
N4-acetyl sulfamethoxazole-d5	−86 (±2)	−56 (±1)	−73 (±1)	−54 (±3)	−70 (±2)	−56 (±1)	−64 (±1)	−56 (±4)	−66 (±2)	−49 (±4)

depending on the analytes and the sample matrices. While the standard deviations for replicate extractions were relatively small, the variability between corresponding samples of Batch 1 and Batch 2 was significant. This variable behaviour demonstrates the general inadequacy of any efforts to pre-characterize the matrix effects in order to apply a standard correction factor. This result confirms the crucial role of isotope label standards for reliable trace analysis of pharmaceuticals in complex matrices such as wastewater influent or sludge.

4. Conclusions

A highly sensitive method for the analysis of six sulfonamide antibiotics, two sulfonamide metabolites (N_4-acetyl sulfamethazine and N_4-acetyl sulfamethoxazole) and trimethoprim was developed for the analysis of complex sewage samples both in aqueous and solid components. This method has been comprehensively validated in a variety of sewage matrices collected after varying levels of treatment. MDLs of all target compounds were between 0.2 and 0.4 ng/mL for ultrapure water, 0.4 and 0.7 ng/mL for tap water, 1.4 and 5.9 ng/mL for laboratory-scale MBR mixed liquor, 0.7 and 1.7 ng/mL for biologically treated effluent and 0.5 and 1.5 ng/g dry weight for MBR sludge. Analytical matrix effects leading to significant and largely unpredictable signal suppression in variably complex matrices compared to an ultrapure water matrix were demonstrated. The results of this investigation confirm the importance of accounting for such matrix effects for accurate quantification. The use of isotope labelled standards is effective to correct for compound loss and matrix interferences during the analysis.

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