Fully-Automated SPE Coupled to UPLC-MS/MS Method for Simultaneous Detection of Trace Sulfonamides, Quinolones, and Macrolide antibiotics in Water

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Research Article

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Abstract

A fully automated solid-phase extraction (SPE) coupled ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method was developed for analysis of antibiotics (sulfonamides, quinolones, and macrolide) in water matrices. Sample preparation optimization included the selection of the best SPE material and configuration (HLB disks), sample volume (500–1000 mL water sample (pH = 3)) with a flow rate at 1–2 mL min^{-1}, and an elution procedure with 2 · 6 mL methanol, 2 · 6 mL acetone-methanol (V/V = 1/1). Meanwhile, the parameters for UPLC-MS/S detection of each analyte was optimized, including LC retention time, and MS parameters. The instrumental limits of detection (LOD) and quantification (LOQ) of analytes ranged from 0.01–0.72 μg L^{-1} and 0.05–2.39 μg L^{-1}, respectively, with good linear correlation (R^2 > 0.995) and precision (< 9.9%). Matrix spike recoveries ranged between 63.3–99.2% in pure water, 60.8–91.3% in surface water (SW), and 59.9–102.8% in wastewater effluent (WWE) with relative standard deviations (RSD) below 11%. The matrix effects (MEs) observed for most of the analytes were ion suppression (0–25.8%) except for four compounds that had enhancement (0–8.0 %) in SW or WWE. This method can basically meet the needs of trace antibiotic residues detection in waters. Trace levels of sulfonamides, quinolones and macrolides using the developed antibiotic method were below LOQ (BQL) −94.47 ng L^{-1} in WWEs and BQL−15.47 ng L^{-1} in SW in the lower reaches of the Yangtze River Basin.

1. Introduction

The widespread occurrence of antibiotics in the aquatic environment triggered a great concern because it led to an alarming increase in antimicrobial resistance, thereby posing potential ecological and health risks (Danner et al. 2019; Roberts and Zembower 2020; Sharma et al. 2016; Zhang et al. 2015). Antibiotics that are classified in different categories including macrolides, quinolones, sulfonamides, β-lactams, tetracyclines, chloramphenicol, etc. (Daghrir and Drogui 2013; Luo et al. 2011; Pedrouzo et al. 2008), which have been widely used to prevent or treat bacterial infections in humans and animals, as well as promoting the animal growth (Kovalakova et al. 2020). Their continuous discharge of antibiotics in the environment has resulted in surface water concentrations ranging from ng L^{-1} to μg L^{-1}, leading to the emergence of antimicrobial resistance (Kovalakova et al. 2020; Roberts and Zembower 2020). The World Health Organization (WHO) has reported that the increase in antimicrobial resistance was one of the top ten threats to global health in 2019 (Roberts and Zembower 2020). Exposure to antibiotics in waters may induce systemic effects to the intestinal flora of humans, and cause many diseases related to immunity and metabolism (Zhang et al. 2015). Therefore, a reliable analytical method for the detection of antibiotics in waters is necessary to understand the fate of antibiotics in water matrices.

In recent years, a new trend in antibiotics analysis consisted of developing novel and reliable methods for simultaneous determination of multiple compounds in various matrices (environmental water, soil, food, animal tissues). The analytical techniques consist of sample preparation (collection and pretreatment) and detection method. Sample pretreatment including separation and enrichment largely determines the accuracy and repeatability of residue detection and analysis (Choi et al. 2007). Solid phase extraction (SPE) is one of the most widely used techniques for processing water samples and extracts in laboratories due to its simplicity, speed, low solvent consumption, good reproducibility, high recovery rates and high sensitivity

(Ramos 2012). However, the effect of separation and enrichment for SPE is mostly affected by the selectivity of the cartridge/disk type, in addition to the eluent, elution procedure, and sample pH among others. For instance, Zhi et al. (2020) reported that the recovery rates for tetracycline, sulfonamide, quinolone, macrolide and lactam antibiotics in fresh manure using Oasis Hydrophilic-Lipophilic Balance (HLB), PRiME HLB and PEP SPE were 58.5–103.6%, 38.8–135.2% and 0.0–100.6%, respectively.

Since many antibiotics are non-volatile and have high molecular weight, the most common methods for antibiotics detection utilizes liquid chromatography-mass spectrometry (LC-MS) (Hernandez et al. 2007; Panditi et al. 2013), with multiple reaction selection (MRM) as the preferred mode for increased analytical sensitivity and selectivity in complex matrices (Chiesa et al. 2015; Mokh et al. 2017; Zhi et al. 2020). Panditi et al. (2013) established an online SPE-LC-MS to simultaneously determine 31 antibiotics in drinking water, surface water (SW) and reclaimed water with recoveries between 50–150% in river and reclaimed water matrices. The method detection limits (MDL) ranged from 1.2–9.7, 2.2–15, and 5.5–63 ng L^{-1} in drinking water, SW and reclaimed waters, respectively. Li et al. (2016) used ultra-high performance LC-MS/MS (UPLC-MS/MS) to analyze the degree of antibiotic pollution in the Tiaoxi River Basin in China with an average recovery in the range of 75.5 to 88.9 %, and with the limit of quantification (LOQ) of 0.1 ~ 1.0 ng L^{-1}.

Achieving detection limits in the ng L^{-1} concentration or lower with simultaneous determination of multiple antibiotic compounds in natural water by LC-MS/MS is still a huge challenge due to the substantial differences in the physicochemical properties of antibiotics (such as polarity, solubility, pKa and stability), as well as the complexity of water matrices. Furthermore, during the development of the SPE-LC-MS/MS method for the detection of antibiotics, various factors such as pH value, extraction method, mobile phase composition, and mass spectrometry acquisition parameters need to be considered (Boix et al. 2014). The objective of this study was to develop a simple method for rapid analysis of 26 antibiotics including sulfonamides, quinolones and macrolides with a fully automated SPE procedure coupled to a UPLC–MS/MS. The selection of SPE cartridge/disk materials, elution solvent, elution volume, MS parameters, and LC separation were the key factors.

2. Experimental

2.1. Materials and reagents

Methanol (MeOH), dichloromethane (DCM), formic acid, and acetone of analytical grade were purchased from MERCK, Germany. Hydrochloric acid (superior grade) was purchased from Sigma, USA. EDTA-2Na (analytical grade, Chinese medicine, China) at 1.0 g L^{-1} was added to water samples to prevent photo-degradation and chelation with metal ions. Pure water (PW) was produced with a Milli-Q system (Millipore, USA).

Analytical standards of antibiotics (> 97% purity) were purchased from Dr. Ehrenstofer GmbH (Germany). The selection of 26 analytes was based on data acquired from provided literatures about the detection frequency, concentration, toxicology toxicological relevance, and availability of reliable analytical methods (Anumol and Snyder 2015; Han et al. 2015; Zhi et al. 2020). The selected antibiotics consists of three categories: sulfonamides, quinolones, and macrolides. Detailed information about all the target analytes is presented in Table 1. Isotopically-labeled surrogates (ISs) including sulfamethazine-$^{13}C_6$, sulfamethoxazole-$^{13}C_6$, difloxacin

hydrochloride-D_3, ofloxacin hydrochloride-D_3, ofloxacin hydrochloride-D_5, ciprofloxacin hydrochloride-D_8, and erythromycin-^{13}C-D_3 were purchased from Cambridge Isotope Labs (USA). Stock solutions of mixed analytical standards (1mg L^{-1}) and mixed IS standards (50 μg L^{-1}) were prepared in MeOH.

SPE cartridges/disks included Oasis HLB SPE cartridge (500 mg/6 mL, Waters, USA), C18 SPE cartridge (500 mg/6 mL, Lab Tech, China), C18 SPE cartridge (1 g/6 mL, IST, Sweden), Florisil SPE cartridge (500 mg/6 mL, Chromatography, USA), HLB SPE disk (ϕ 47 mm, Atlantic, USA), and C18 SPE disk (ϕ 47 mm, Atlantic, USA).

Table 1 Categories, names and molecular weights of target antibiotics.

Category	Compound	Molecular formula	Molecular weight	Abbreviation	Scope of use
Sulfonamides	Sulfadiazine	$C_{10}H_{10}N_4O_2S$	250.3	SD	Human & Animal
	Sulfamerazine	$C_{11}H_{12}N_4O_2S$	264.3	SDMD	Human & Animal
	Sulfamethazine	$C_{12}H_{14}N_4O_2S$	278.33	SM2	Human & Animal
	Sulfamethoxadiazole	$C_9H_{10}N_4O_2S_2$	270.33	SMT	Human & Animal
	Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	253.28	SMX	Human & Animal
	Sulfamethoxine	$C_{12}H_{14}N_4O_4S$	310.36	SDM	Human & Animal
Quinolones	Mabofloxacin	$C_{17}H_{19}FN_4O_4$	362.36	MAR	Animal
	Enrofloxacin	$C_{19}H_{22}FN_3O_3$	359.4	ENR	Animal
	Dafloxacin mesylate	$C_{20}H_{24}FN_3O_6S$	453.5	DAM	Animal
	Difloxacin	$C_{21}H_{19}F_2N_3O_3·HCl$	438.85	DFLX	Animal
	Ofloxacin	$C_{18}H_{20}FN_3O_4$	361.37	OFL	Human & Animal
	Norfloxacin	$C_{16}H_{18}FN_3O_3$	319.33	NOR	Human & Animal
	Lomefloxacin hydrochloride	$C_{17}H_{19}F_2N_3O_3·HCl$	387.81	LOM	Human & Animal
	Nalidixic acid	$C_{14}H_{12}FNO_3$	261.25	NAL	Human & Animal
	Flumequine	$C_{12}H_{12}N_2O_3$	232.24	FLU	Animal
	Ciprofloxacin hydrochloride	$C_{17}H_{18}FN_3O_3·HCl$	367.8	CIP	Human & Animal
Macrolides	(7S)-Lincomycin	$C_{18}H_{34}N_2O_6S$	406.54	LIN	Human & Animal

Spiramycin	$C_{43}H_{74}N_2O_{14}$	842.51	SPM	Human & Animal
Tilmicaoxin	$C_{46}H_{80}N_2O_{13}$	869.2	TILM	Animal
Tylosin	$C_{46}H_{77}NO_{17}$	916.1	TYL	Animal
Leucomycin A5	$C_{39}H_{65}NO_{14}$	771.93	LM	Human & Animal
Anhydroerythromycin A	$C_{37}H_{65}NO_{12}$	715.91	ETM-H_2O	Human & Animal
Clarithromycin	$C_{38}H_{69}NO_{13}$	747.96	CTM	Human & Animal
Azithromycin Dihydrate	$C_{38}H_{72}N_2O_{12}\cdot 2H_2O$	784.98	AZM□2H_2O	Human & Animal
Roxithromycin	$C_{41}H_{76}N_2O_{15}$	837.1	ROM	Human & Animal
Clindamycin hydrochloride	$C_{18}H_{33}ClN_2O_5S\cdot HCl\cdot H_2O$	479.46	CLI	Human & Animal

2.2. Sample collection and treatment

Triplicates of SW and wastewater effluent (WWE) samples (1.0 L) were collected in clean brown glass bottles from the Yangtze River (a depth of ~0.5 m below the water surface) and effluents of Bailonggang sewage treatment plant (STP) which adopts multi-mode anaerobic-anoxic-aerobic activated sludge process in Pudong area, Shanghai. Detailed information including the longitude and latitude are provided in Table S1. Samples were transported to the laboratory in iceboxes overnight, immediately filtered by 0.45 μm mixed fiber membrane to remove suspended solids, acidified by adding H_2SO_4 (pH 3.0), followed by Na_2EDTA (1.0 g L^{-1}) addition, and spiked with a mixture of all ISs to achieve a final concentration of 50-100 ng L^{-1} depending on the type of analyte and sample matrix.

The conditions of actual sample preparation were determined based on the results from experiments using PW consisting of suitable acidified condition (pH 3.0, 4.0 or 5.0), flow rate (1-5 mL min^{-1}), and volume of loading sample (0.10-1.00 L), whether preserved with 1.0 g L^{-1} of Na_2EDTA.

2.3. Fully-automatic solid phase extraction and enrichment

A fully automatic cartridge-disk universal SPE system (LabTech, China) coupled with a MultiVap-8 channel parallel concentrator (LabTech, China) (Figure S1) were used for sample extraction and enrichment of target antibiotics. The system automatically executed the activation, sample passing, cleaning, nitrogen drying, soaking, and elution of 12 SPE cartridges/disks, simultaneously.

The systems sequence first preconditioned the SPE cartridges/disks with 15 mL MeOH, 10 mL acetone-MeOH solution and 10 mL PW successively. Then, water samples (0.5 ~ 1 L) were passed through SPE cartridges/disks with a flow rate of 1~5 mL min^{-1}, followed by 2´5 mL acidified PW to rinse the SPE cartridges/disks. After samples loading, the cartridge/disk were dried for 10 mins with N_2-blowdown, and then eluted into 50-mL nitrogen blowpipe using eluent. The elution solvent was either MeOH, water, acetone, MeOH-DCM (V/V = 1/1), MeOH-acetone (V/V = 1/1) or DCM and the corresponding volume was set at 3´6 mL, 4´6 mL, or 5´6 mL. Finally, the collected eluent was dried with N_2-blowdown in nitrogen blowpipe submerged at 45℃ water, and then diluted to 2 mL using MeOH-water (LC eluent mobile phase), filtered through 0.2 μm nylon syringe filters, and transferred to a 1.0 mL injection vial for subsequent UPLC-MS/MS analysis.

In this study, determination of the SPE cartridge/disk type, eluent, and elution procedure for the entire analysis of target antibiotics were investigated with the PW and the absolute recoveries were compared to identify the most ideal method.

2.4. Liquid chromatography

LC separation was performed on an Agilent 1290 UPLC coupled with an Agilent ZORBAX Eclipse Plus C18 (2.1 mm × 100 mm, 1.8 μm) column. The column was maintained at 40 °C, and the injection volume was 2.0 μL. A dual eluent mobile phase consisted of MeOH with 0.05% formic acid (A) and water with 0.1% acetic acid (B) at 0.300 mL min^{-1}. The suitable gradient procedures were set up as follows: 0–3 min, 90–85 % A; 3–24 min, 85–50 % A; 24–30 min, 50–10% A, and post-time was 5 min. The separation effect of target standards is shown in Figure S2.

2.5. Mass spectrometry

Mass spectrometry was performed on an Agilent 6460 triple quadrupole mass spectrometer. Samples were analyzed in positive electron spray ionization (ESI+) with multiple reaction monitoring (MRM) mode. The ESI+ operating conditions of the source were as follows: collision nitrogen flow at 7 L min^{-1}, sheath nitrogen flow at 11 L min^{-1} at 350 °C, spray source pressure at 45 psi, desolation temperature at 300 °C, and capillary voltage at 3500 V. In the scanning range of 150-1000 m/z, the first-order MS spectrum of target antibiotics was presented as precursor ions at $[M+H]^+$ mode. The optimized MS/MS product ions, collision voltage (V), fragmentation voltage (V), retention time (Table S2), and the mass spectrum of each analyte is shown in Figure S3(a-z).

2.5. Method validation

The analytical method was evaluated through the estimation of the linearity, (absolute) recoveries, instrumental limits of detection (LOD) and quantification (LOQ), precision expressed as repeatability in terms of the relative standard deviation (RSD), and matrix effects (MEs) of the SW and WWE samples.

Linearity was verified by establishing matrix-matched calibration (MMC) curves (fitted as Eq.(1)) made using the peak area ratio of seven standards at 2-100 µg L^{-1} in relation to IS at 50 or 100 µg L^{-1}, which can compensate the matrix effects that influence the analytical response (Monteiro et al. 2016). The corresponding coefficient of determination (R^2) was > 0.990 and usually exceeded 0.995 for quantification (Ho et al. 2012). Accordingly, the concentrations for analytes were calculated by comparing standard-IS peak area ratio from calibration curve to analyte-IS peak area ratio in the analyzed samples using (Eq.(1)).

$$\frac{A_a}{A_{IS}} = a * C_a + b \qquad (1)$$

where A_a is the area of the analyte; A_{IS} is the area of the IS; the concentration of IS was 50 or 100 µg L^{-1}; C_a is the concentration of the analyte (µg L^{-1}); *a* and *b* are the fitting slope coefficient and intercept coefficient, respectively.

The instrumental LOD and LOQ were defined as the lowest concentrations with an analyte signal to noise ratio (SNR) equal to or greater than 3 and 10, respectively, using the matrix calibration curves (Mokh et al. 2017; Zhi et al. 2020). The absolute recoveries were calculated by comparing the peak areas of each analyte obtained in the matrix samples followed by SPE (A_{matrix}) and the standard followed by direct chromatographic injection mode ($A_{standard}$) in five replicates using Eq.(2). For each batch of sample analysis, one relevant blank matrix was spiked with ISs (50 ug L^{-1}) as quality control (QC) sample to check for the possible background of the analytes from the matrix samples. To assess which cartridge/disk was the most appropriate for SPE of the target analytes, the concentration of the target analyte was set at 100 µg L^{-1} in PW.

$$Absolute\ \ Recovery\ \ (\%) = \frac{A_{matrix} - A_{qc}}{A_{standard}} \times 100 \qquad (2)$$

Where A_{matrix} is the peak area of each analyte spiked in matrix samples prior SPE; $A_{standard}$ is the peak area of analyte standard; A_{qc} is the peak area of each analyte in QC sample.

The precision of the method was verified using the intra-day (in five hours) and inter-day (five days) reproducibility expressed by RSD through analyzing five replicate injections of a 100 µg L^{-1} standard by means of a one-way ANOVA.

The matrix spike recoveries calculated in PW, SW and WWE represented the isotope corrected recoveries (Eq. (3)) while absolute recoveries were usually lower (Anumol and Snyder 2015). The concentrations of target analytes and ISs were set at 50 µg L^{-1}. The ME value indicated the enhancement or reduction of the antibiotics signal in SW and WWE, which was calculated by comparing the difference ($P_{standard}$ -P_{matrix}) between the peak

areas obtained in the standard and matrix with the peak area obtained in the standard ($P_{standard}$) at 50 ng L^{-1}, using Eq. (4).

$$Matrix\ Spike\ Recoveries\ (\%) = \frac{C_{matrix} - C_{qc}}{C_{standard}} \times 100 \quad (3)$$

Where C_{matrix} is the concentration of each analyte spiked in matrix samples prior SPE; $C_{standard}$ is the concentration of analyte standard; C_{qc} is the concentration of each analyte in QC sample.

$$ME(\%) = \frac{P_{standard} - P_{matrix}}{P_{standard}} \times 100 \quad (4)$$

where $P_{standard}$ is the peak area in the standard and P_{matrix} is the peak area obtained in the matrix. A positive value of ME indicates signal suppression while a negative value indicates signal enhancement.

Quality control procedures were carried out, using batch samples, blank solvents, blank samples, and new MMC curves to evaluate whether there was carryover or background contamination, and to verify the performance of the method. Each analyte retained the same retention time as the corresponding calibration standard within 5%, and the same ion ratio less than 20%.

3. Results And Discussion

3.1. SPE optimization

3.1.1 Selection of SPE cartridges/disks

The extraction and elution effect of analytes were affected by SPE cartridge/disk type, eluent, elution procedure, sample pH etc., with the cartridge/disk type being the primary factor. The extraction efficiency of SPE cartridges/disks depends on its ability to retain target analytes from the aqueous phase and the ability to elute the analytes using the organic phase. C18 is the most widely used material for SPE, which can adsorb a variety of organic substances in the range of weak to moderate polarity; Florisil cartridges can extract polar compounds from non-polar solutions; HLB can retain acidic, basic and neutral compounds in a wide range of polarities (from polar to non-polar), and has a good enrichment effect on polar compounds in particular (Hennion 1999; Thurman and Mills 1998). In this study, six kinds of cartridges/disks were investigated to identify their enrichment and elution effects on target antibiotics. The absolute recoveries were calculated (Eq. (2)) for SPE of 1.0 L PW spiked with analytes at 100 μg L^{-1}. A total of 40 mL of elution solvent (MeOH-acetone (V/V = 1/1)) was used for the elution of these analytes.

Figure 1(a) shows the range of absolute recoveries obtained for 26 target analytes tested with each cartridge/disk. A recovery between 70–130% was considered acceptable and used as the criteria for cartridge selection (Anumol and Snyder 2015). The C18 and HLB disk had 10 analytes in this range and good reproducibility (RSD < 10%, Table S3), followed by five for HLB cartridge, three for C18 cartridge (IST), one for

C18 cartridge (Lab tech), and even zero for Florisil cartridge. Meanwhile, only HLB disk was able to extract all 26 analytes (recoveries > 0%), followed by the HLB cartridge and C18 disk with 24, the C18 cartridge (IST) with 23, and the C18 cartridge (Lab tech) and Florisil cartridge with 15 (Table S3). The HLB disk showed the best recovery since it can adequately enrich and elute all analytes, regardless of its polarity or it being acidic, basic, or neutral. Meanwhile, the C18 disk performed well for the sulfonamide antibiotics. The HLB cartridge and C18 cartridge (IST) eluted most antibiotics, but the elution efficiency was not as good as HLB disk since the numbers of analytes that were not recovered or had a recovery > 130% was ≥ 4 (Fig. 1(a)). The poor recoveries of the C18 cartridge (LabTech) and the Florisil cartridge was due to their poor elution effect with a solvent of MeOH-acetone. Thus, HLB disk was the most suitable choice. Notably, the recovery was greater than 130% for FLU using C18 disk, FLU and TYL using HLB cartage, and FLU, SDM and TYL using C18 cartridge (IST) (Table S3). This may be attributed to carryover from one injection to the next (Anumol and Snyder 2015).

Furthermore, Fig. 1(b) shows the distribution of absolute recoveries, for which the total mean values and standard deviations (SD) were calculated. The absolute recoveries for the HLB disk had a relatively concentrated distribution and the mean was relatively close to 100%. The C18 disk, HLB cartridge, and C18 had acceptable mean values, while the distribution was relatively dispersed with a SD of 46.6, 54.1 and 50.3%, respectively. The C18 cartridge (Lab tech) and Florisil cartridge had a smaller SD while the mean recoveries were quite low with values at 17.2% and 11.5%, respectively. It is always desirable to obtain the maximum recoveries with the best sensitivity but with respect to a highly diverse analyte list trade-offs are inevitable. Therefore, the HLB disk was selected for subsequent analyses.

3.1.2 Selection of the eluent and the optimum volume using HLB disk

The MeOH, acetone or DCM are the most widely used elution solvents for solid-phase extraction of antibiotics based on similar physicochemical polarity (from large to small) (Behera et al. 2011; Ghosh et al. 2009). In this paper, MeOH, acetone, MeOH-DCM (V/V = 1/1), MeOH-acetone (V/V = 1/1) and DCM with a 6 mL volume were selected as elution solvents to investigate, and the optimal solvent for each analyte was shown in Table S4 (recoveries not shown). The number of compounds that were eluted with the optimal tested solvent is shown in Fig. 2(a). MeOH and acetone exhibited better elution results for eight analytes, followed by that of DCM (four analytes), MeOH-DCM (three analytes), and MeOH-acetone (three analytes). Thus, both MeOH and acetone were selected as elution solvents for target antibiotics. Accordingly, the effect of the eluent's volume (6 mL ·3, ·4, and ·5) on the elution effect was studied (recoveries not shown), and the results are summarized in Table S5 and Fig. 2 (b). The maximum number of analytes with the optimal elution occurred when the volume was set at 4 · 6 mL for both MeOH and acetone, while a smaller elution volume (3 ·6 mL) could not completely elute all of the targeted antibiotics, and an excessive volume (5 · 6 mL) lead to the loss of recovery of some antibiotics, especially for sulfonamides (Table S5). Therefore, MeOH was selected as the main eluent with the supplement eluent of MeOH-acetone and an elution order of 6 mL MeOH, 6 mL MeOH, 6 mL acetone-MeOH, and 6 mL acetone-MeOH.

3.1.3 Determination of pH, flow rate, and volume of water samples and EDTA-2Na addition

The various pH values of water samples may result in different recoveries for the analytes. According to EPA1694 (Englert 2007), the extraction of antibiotic from the water matrix is usually performed under acidic conditions. In this paper, the pH of 1.0 L of PW was adjusted from an initial 6.8 to a final 3.0, 4.0 and 5.0 using H_2SO_4 (Figure S4). The results indicate that sulfonamides and quinolones had higher recoveries under acidic conditions, while macrolides had lower recoveries, which is probably due to some macrolides being as weakly basic base compounds such as pKa = 7.9 of SPM, pKa = 8.99 of CTM, and pKa = 8.5 of AZM–$2H_2O$ (DrugBank ; DrugBank ; DrugBank ; Wishart et al. 2018). When trying to achieve good recovery for the 26 antibiotics, it is recommended the water sample to be acidified to a pH of 3.0 before performing SPE.

According to the instructions for the HLB disk provided by the manufacturer, the flow rate of the water sample through this sorbent should not exceed 5 mL min^{-1}. However, previous literatures (Monteiro et al. 2016; Zhi et al. 2020) have shown that this can vary depending on the various characteristics of the water samples. For example, water samples with complex matrices such as STP samples should have a lower sample loading speed. Thus, the flow rate through the disk in this experiment was controlled at 1–2 mL min^{-1}.

When analyzing antibiotics in water matrices, the sample must be reasonably concentrated during pretreatment while removing the interfering substances as much as possible. The concentration factor was limited by the breakthrough volume of the SPE cartridge/disk, i.e., the maximum loading volume that can be percolated through the SPE cartridge/disk without losing the analytes. Considering the concentration of analytes and the influence of impurities in actual waters, the ideal loading volume was determined to be 1000 mL of water sample for PW, and surface water, and 500 mL for WWE.

Metal ions that are naturally present in certain water sample may form antibiotic-metal complexes with antibiotics (Aristilde and Sposito 2008; Cuprys et al. 2018; Pulicharla et al. 2017), which results in a reduction in the recoveries of target analytes. Antibiotics with more electron-rich groups containing N and O may lead to a stronger complexation with metal ions. Macrolides owned the strongest complexing ability, followed by quinolones and sulfonamides. This is because macrolides are more complex in structure and usually have 12–16 carbon lactone rings, while quinolones contain -COOH and –C = O, as well as piperazine groups, and sulfonamides containing N-functional groups are involved in the coordination. Considering the water sample and published literatures (Mokh et al. 2017; Zhi et al. 2020), EDTA-2Na at 1.0 g L^{-1} was added to water samples to reduce metal ion interference in water, while the amount of EDTA-2Na will be increased for metal polluted sample to ensure the removal of metal ion interference.

3.2. Blank, linearity, LOD, LOQ, and precision

In this study, none of target analytes was detected in the blanks through detection of blanks (PW) with and without isotopically labeled standards. Good linearity was found for 26 antibiotics by analyzing the seven concentrations calibration curves of standards (2–100 μgL^{-1}) with all compounds having an $R^2 > 0.995$. The calculated LODs (SNR > 3) and LOQs (SNR > 10) for the 26 antibiotic analytes ranged from 0.01–0.72 μg L^{-1} and 0.05–2.39 μg L^{-1}, respectively, which was comparable to those reported in a previous study using the same calculation method (Mokh et al. 2017). Detailed linearity, LODs, and LOQs are summarized in Table 2. The precision of the analytical method was verified using the intra-day and inter-day reproducibility, calculated as RSD (%) which were determined by analyzing five replicates of a 100 μg L^{-1} standard. The intra-day RSD

ranged from 1.0 to 9.9% and the inter-day RSD ranged from 1.5 to 8.7%, indicating a good repeatability and reliability of the method.

Table 2
Linear equation, correlation coefficients and detection limits.

Category	Compound	Linear equation	Correlation coefficients (R^2)	LOD (μg L^{-1})	LOQ (μg L^{-1})	Intra-day variability (n = 5) RSD (%)	Inter-day variability (n = 5) RSD (%)
Sulfonamides	SD	y = 0.0078x + 0.0042	0.9988	0.04	0.14	5.6	7.4
	SDMD	y = 0.0074x + 0.0022	0.9965	0.32	1.08	3.9	4.0
	SM2	y = 0.01x-0.0038	0.9985	0.72	2.39	5.3	5.0
	SMT	y = 0.0068x-0.0057	0.9983	0.38	1.26	2.0	3.3
	SMX	y = 0.0238x-0.0104	0.9991	0.15	0.49	1.9	5.0
	SDM	y = 0.0758x + 0.0338	0.9983	0.03	0.09	5.2	2.5
Quinolones	MAR	y = 0.0259x-0.0103	0.9995	0.04	0.12	2.4	2.2
	ENR	y = 0.0215x + 0.0055	0.9994	0.09	0.30	3.3	3.1
	DAM	y = 0.0043x-0.0117	0.9958	0.10	0.33	2.1	1.5
	DFLX	y = 0.0136x-0.0068	0.9998	0.05	0.18	5.9	8.7
	OFL	y = 0.0234x-0.0022	0.9992	0.05	0.15	1.9	2.7
	NOR	y = 0.0033x-0.0044	0.9955	0.59	1.98	2.4	3.6

LOD, limit of detection; LOQ, limit of quantitation; RSD, relative standard deviation. Standards of 2–100 μgL^{-1} were set for linearity evaluated. Standards of 100 μg L^{-1} was set for intra-day and inter-day reproducibility evaluated.

Category	Compound	Linear equation	Correlation coefficients (R^2)	LOD (μg L^{-1})	LOQ (μg L^{-1})	Intra-day variability (n = 5) RSD (%)	Inter-day variability (n = 5) RSD (%)
	LOM	y = 0.008x-0.0041	0.9995	0.09	0.31	7.6	6.3
	NAL	y = 0.0229x + 0.8748	0.9979	0.08	0.27	2.1	2.0
	FLU	y = 0.0123x-0.0043	0.9995	0.16	0.54	3.3	4.1
	CIP	y = 0.014x-0.0224	0.9974	0.69	2.29	5.6	6.5
Macrolides	LIN	y = 0.035x + 0.0092	0.9994	0.01	0.04	3.4	3.8
	SPM	y = 0.0008x-0.0001	0.9990	0.13	0.43	1.0	1.7
	TILM	y = 0.0043x + 0.0002	0.9988	0.09	0.29	3.5	1.8
	TYL	y = 0.0036x-0.0017	0.9994	0.07	0.23	3.3	4.5
	LM	y = 0.0035x-0.0038	0.9950	0.15	0.50	2.8	4.5
	ETM-H_2O	y = 0.01881x-0.001	0.9998	0.03	0.10	3.3	1.7
	CTM	y = 0.0460x + 0.004	0.9998	0.01	0.04	3.3	3.0
	AZM–$2H_2O$	y = 0.0071x + 0.0004	0.9995	0.07	0.22	9.9	3.7
	ROM	y = 0.0161x + 0.0026	0.9994	0.02	0.05	1.9	3.5

LOD, limit of detection; LOQ, limit of quantitation; RSD, relative standard deviation. Standards of 2–100 μgL^{-1} were set for linearity evaluated. Standards of 100 μg L^{-1} was set for intra-day and inter-day reproducibility evaluated.

Category	Compound	Linear equation	Correlation coefficients (R^2)	LOD (µg L^{-1})	LOQ (µg L^{-1})	Intra-day variability (n = 5) RSD (%)	Inter-day variability (n = 5) RSD (%)
	CLI	y = 0.1006x + 0.0226	0.9997	0.02	0.05	1.6	1.7

LOD, limit of detection; LOQ, limit of quantitation; RSD, relative standard deviation. Standards of 2–100 µgL^{-1} were set for linearity evaluated. Standards of 100 µg L^{-1} was set for intra-day and inter-day reproducibility evaluated.

3.3. Matrix spike recoveries and matrix effect assessment

The viability of the analytical method was verified by the recovery of analyte spiked in water samples from three different matrices (PW, SW, and WWE). Water samples were spiked with a mix of the analytical standards (50 µg L^{-1}) and ISs (50 µg L^{-1}), followed by SPE pretreatment. The matrix spike recoveries (Eq. (3)) and ME (Eq. (4)) were calculated and shown in Table S6 and Fig. 3. The matrix spike recoveries of analytes in PW was acceptable (70–130% (Anumol and Snyder 2015)) except for TILM (69.4 %), TYL (64.4 %), LM (63.3 %), and CLI (69.1%). The reproducibility (RSD) (Table S6) for all analytes was < 10% except for CIP (11%) (Fig. 3(a) and Table S6). In SW, the recoveries for all analytes ranged from 60.8–91.3%, but ten compounds were within 60–70%. The reproducibility for all compounds was good with respect to RSDs < 10.2% (Table S6). For the WWE samples, all matrix spike recoveries were within 59.9–102.8% with nine compounds outside the acceptable range, while RSDs for all analytes were less than 10% (Table S6). However, the recoveries for all analytes obtained in this paper were within EPA 1694 (Englert 2007) range of 5-200% for water matrices. This wide range is due to the special structure of antibiotics and their physicochemical properties. A previous investigation reported that the recoveries for tetracyclines and quinolones were 88–112% in river water samples, 41–87% in well water samples, and 23–103% in wastewater influent and effluent using SPE-LC-MS/MS (Reverté et al. 2003). The recoveries for 63 antibiotics in PW were in the range of 70.7–133.9% (Mokh et al. 2017). Furthermore, the ME had an insignificant influence on the RSD as most compounds had a similar RSD < 10% (Table S6), which was also previously reported (Mokh et al. 2017).

The ME caused by interferences of co-eluting constituents in the matrix can lead to a loss of sensitivity and reproducibility for trace quantification of analytes using ESI-LC–MS/MS (Anumol and Snyder 2015). The MEs for target analytes in SW and WWE (Fig. 3(b) and Table S6) indicate that all analytes were affected by suppression or enhancement while the magnitude of effect was vastly different. The suppression of 0.0–25.8% was observed for most analytes in SW, with the exception of DAM and ROM, which showed slight enhancement of 8.0 and 3.3 %, respectively. The MEs in WWE were comparable to those in SW, where most compounds had a suppression of 0–24%, whereas SD, SMX, DAM, and CLI experienced slight enhancement of 0.2–7.4%. This phenomenon may be due to the matrix complexity of the SW and WWE (Zhi et al. 2020) and the dependence of ESI methods on matrix effects (Anumol and Snyder 2015).

Notably, the matrix spike recoveries shown in Fig. 3(a) for the isotope corrected recoveries were lower than the absolute recoveries (Figure S5), which was also reported previously (Anumol and Snyder 2015). Moreover, the

suppression and enhancement signals could be minimized using the analytical standards and internal standards addition method (Mokh et al. 2017).

3.4. Analysis of real samples

This validated method was applied for the multiresidue analysis of 26 antibiotics in WWE and SW samples from the scaled Yangtze River and Bailonggang STP, respectively, during the summer. Two WWE samples were collected from two different outlets of the Bailonggang STP (WWE1 and WWE2), and three SW samples were collected from the Yangtze River near STP outlets (SW1), 5 km upstream of SW1 (SW2), and 4 km downstream of SW1(SW3) (Table S1). As shown in Table 3, the concentrations of the target antibiotics detected in the WWE samples ranged from below LOQ (BQL)–60.52 ng L^{-1} for WWE1 and BQL–94.47 ng L^{-1} for WWE2, with 21 and 22 of the antibiotics quantitatively detected, respectively. The fluoroquinolones (BQL–94.47 ng L^{-1}) were the most frequently detected group of antibiotics, where OFL and NOR was detected at the highest concentration of 60.52–94.47 ng L^{-1} and 31.37–37.62 ng L^{-1} in the two WWE samples. A previous investigation also proved that the quinolones were the most frequently detected compounds in six STPs around the Pudong New Area of Shanghai, China. The concentrations of OFL and NOR were in the range of 8.84–246.76 ng L^{-1} and 17.16–66.53 ng L^{-1}, respectively (Pan et al. 2020). OFL and NOR were hardly removed by STP using traditional activated sludge method. Gao et al. (2012) reported 36–130 ng L^{-1} of NOR and 58–75 ng L^{-1} of OFL in the WWEs using anaerobic/anoxic/oxic (A^2/O) activated sludge process, 45 ng L^{-1} of NOR and 72 of OFL ng L^{-1} using oxidation ditch (OD), 9.4 ng L^{-1} and 150 ng L^{-1} of OFL using anoxic/oxic (A/O) activated sludge process, and 56 ng L^{-1} NOR and 120 ng L^{-1} of OFL using A^2/O combined cilium nutrient removal (CNR) technology. As to macrolides, over eight differ compounds were detected in the WWE samples, and ETM-H_2O was the highest concentration antibiotic detected (41.32–52.35 ng L^{-1}). ETM-H_2O was also widely detected in the USA (mean concentrations, 76–110 ng L^{-1}) (Nelson et al. 2011), United Kingdom (mean concentration, 696–1385 ng L^{-1}) (Kasprzyk-Hordern et al. 2009), Singapore (maximum concentration, 267.5–381 ng L^{-1}) (Tran et al. 2016) and other areas of China (mean concentration, 358–2980 ng L^{-1}) (Leung et al. 2012). Table 3 displays four of the six sulfonamides detected. SMX was detected at the highest concentration at 29.67 ng L^{-1} in WWE2. SMX has been detected in WWEs in many countries, e.g., with mean concentrations at 18–910 ng L^{-1} in USA (Kostich et al. 2014; Nelson et al. 2011), 519 ng L^{-1} in Canada (Basiuk et al. 2017), and 153–3375 ng L^{-1} in France (Dinh et al. 2017).

There were 19 antibiotics detected in SW1 near the STP, 19 antibiotics detected 5 km upstream of Yangtze (SW2) and 22 detected 4 km downstream of Yangtze (SW3). Their concentrations ranged between BQL–15.47 ng L^{-1}. The detected antibiotics with a higher concentration in SWs were SMX (8.20–11.68 ng L^{-1}) for sulfonamides, NOR (9.06–15.47 ng L^{-1}) for quinolones, and ETM-H_2O (5.58–6.37 ng L^{-1}) for macrolides, which is similar to those in the WWEs. However, the total concentrations of antibiotics in SWs (9.08–15.47 ng L^{-1}) were significantly lower than those in WWEs (60.52–94.47 ng L^{-1}). This is likely due to the unremoved antibiotics from the STPs being diluted upon entering the river. Moreover, the concentrations of antibiotics in river near the STP were not significantly higher than those upstream of the STP, as well as the downstream sampling location, which is probably due to the rapid dilution by large amounts of water from the river and the presence of background antibiotics from other sources. The frequently detected SMX (a sulfonamide), NOR

and OFL (quinolones), and ETM-H_2O (a macrolide) have been reported in rivers in different countries (Danner et al. 2019; Kovalakova et al. 2020), with mean concentrations of four compounds up to such as 1209 ng L^{-1} in France (Dinh et al. 2017), 443 ng L^{-1} in the USA (Massey et al. 2010), and 172 ng L^{-1} in Lebanon (Mokh et al. 2017).

Table 3

Occurrence and concentration levels of antibiotics in different water matrices (ng L^{-1}) (n = 5).

Category	Compound	WWEs		SWs		
		WWE 1	WWE 2	SW1	SW2	SW3
Sulfonamides	SD	4.54	6.15	3.79	7.10	5.41
	SDMD	8.29	4.20	1.59	2.45	1.63
	SM2	BQL	BQL	2.30	6.81	6.02
	SMT	8.86	8.82	4.33	6.82	4.36
	SMX	4.00	29.67	8.20	11.30	11.68
	SDM	BQL	BQL	BQL	BQL	BQL
Quinolones	MAR	4.97	2.73	1.43	1.60	1.40
	ENR	3.97	0.46	BQL	BQL	BQL
	DAM	BQL	18.57	2.09	13.81	1.40
	DFLX	6.49	3.74	2.36	2.99	2.15
	OFL	60.52	94.47	9.08	3.97	3.20
	NOR	31.37	37.62	9.06	13.95	15.47
	LOM	BQL	8.81	6.05	2.78	6.55
	NAL	4.00	5.51	2.54	2.53	2.77
	FLU	4.79	4.52	2.49	3.21	2.83
	CIP	9.85	5.89	2.19	4.27	4.26
Macrolides	LIN	14.20	19.31	0.94	1.56	1.18
	SPM	4.86	BQL	BQL	BQL	BQL
	TILM	0.12	0.08	BQL	BQL	0.03
	TYL	2.98	BQL	BQL	BQL	0.99
	LM	4.63	4.52	BQL	BQL	2.28
	ETM-H_2O	52.35	41.32	5.58	6.37	6.26
	CTM	23.08	27.02	3.75	3.89	3.48
	AZM–$2H_2O$	24.12	28.07	4.02	4.39	3.86
	ROM	17.08	18.26	3.41	3.49	3.13

SWs: surface waters; WWE: wastewater effluents; BQD: Below limit of quantitation (LOQ).

CLI	BQL	29.94	BQL	BQL	BQL

SWs: surface waters; WWE: wastewater effluents; BQD: Below limit of quantitation (LOQ).

4. Conclusions

A method using automated SPE coupled to UPLC–MS/MS under MRM mode was developed and validated for the analysis of 26 antibiotics in water matrices. The optimized sample preparation for the best extraction and effective elution includes applying a HLB SPE disk, 500–1000 mL water sample (pH = 3) with a flow rate at 1–2 mL min^{-1}, and elution procedure (6 mL · 2 MeOH, and 6 mL · 2 acetone-MeOH (1/1)). The recoveries for all analytes were between 63.3–99.2% in PW, 60.8–91.3% in SW, and 59.9–102.8% in WWE with the corresponding reproducibility within 11%. The ME observed for most analytes was ion suppression (0–25.8%) with slight enhancement (0–8.0 %) in SW and WWE. Furthermore, the instrumental LODs and LOQs of analytes were < 1 μg L^{-1} and 2.39 μg L^{-1} respectively, with good linear correlation (R^2 > 0.995) and precision (RSD < 9.9 %). Water samples from WWEs and SWs were analyzed to evaluate the effectiveness of the proposed method. The concentrations of sulfonamides, quinolones and macrolides ranged between BQL–94.47 ng L^{-1} in WWEs and BQL–15.47 ng L^{-1} in SWs. SMX, OFL, and ETM-H_2O were detected at the highest concentration for the three types of antibiotics analyzed in the WWEs, and SMX, NOR and ETM-H_2O were present in the highest residual concentration among the tested antibiotics in the Yangtze River. The establishment and verification of this method enables the determination of antibiotic residues in actual water matrices for further research.

Declarations

Authors' contributions

Ming Zheng: Conceptualization, Investigation, Writing - original draft, review & editing, Funding acquisition. Suwen Tang: Writing - original draft, Investigation, Data curation. Yangyang Bao: Investigation, Resources, Methodology. Kevin D. Daniels: Data curation, Writing - review & editing. Zuo Tong How: Writing - review & editing. Mohamed Gamal El-Din: Writing - review & editing. Liang Tang: Supervision, Resources.

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Data availability

All data generated or analyzed during this study are included in this article (and its supplementary material).

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable

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Figures

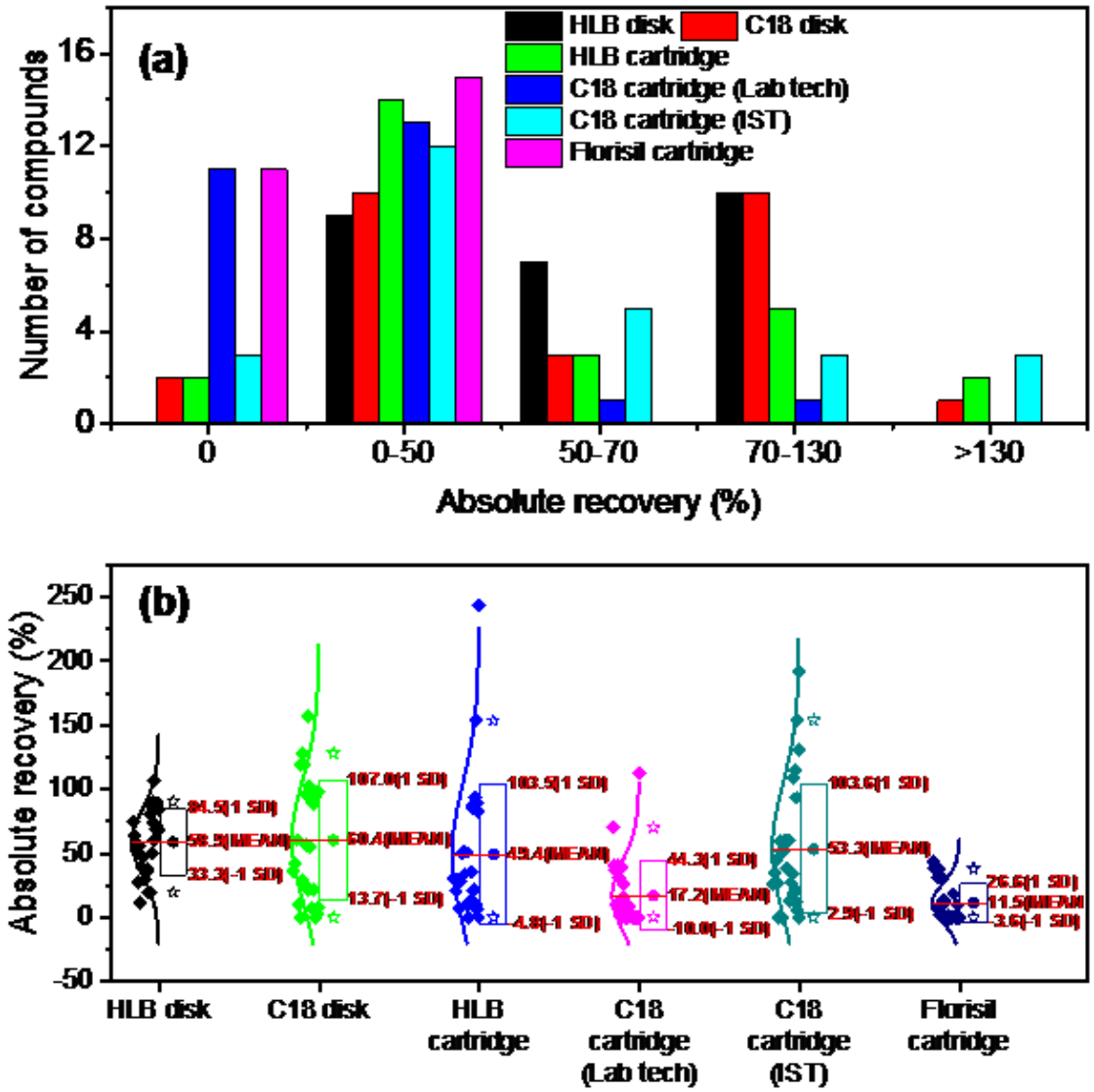

Figure 1

(a) Range of absolute recoveries and (b) box plot of absolute recoveries for target analytes with 6 different SPE cartridges/disks. (Analytes of 100 µg L-1 was set; elution solvent: MeOH-acetone (V/V = 1/1)).

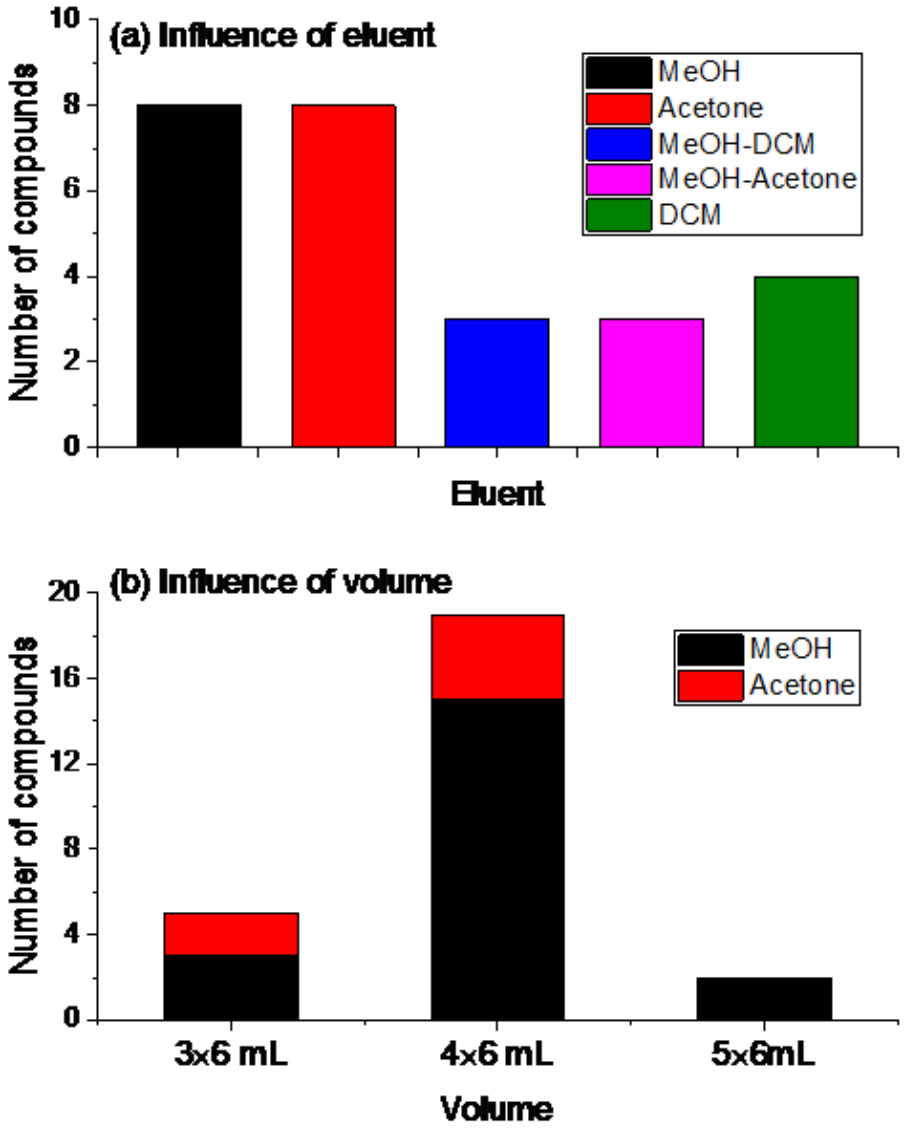

Figure 2

Numbers of compounds with optimal recovery (closest to 100%) using (a) different eluents (volume was set at 6 mL), and (b) different volumes of MeOH and acetone (each volume was 6 mL). (Analytes of 100 μg L-1 was set).

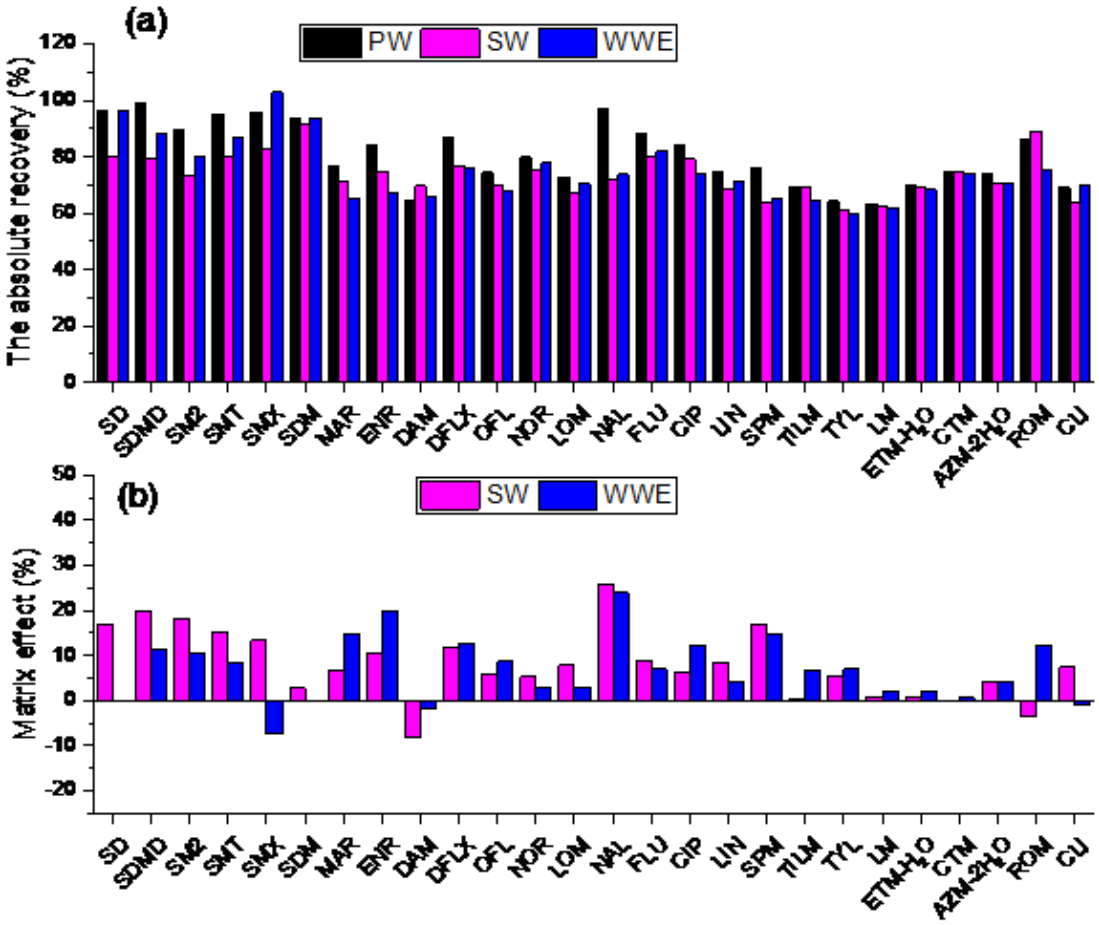

Figure 3

(a) Compound matrix spike recoveries in pure water (PW), surface water (SW), and wastewater effluent (WWE); (b) matrix effect (%) in SW and WWE. (Target analytes and isotopically-labeled surrogates (ISs) were both set at 50 μg L-1).

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