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RESEARCH PAPER

Improved detection of multiple environmental antibiotics through an optimized sample extraction strategy in liquid chromatography-mass spectrometry analysis

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Abstract A solid-phase extraction/liquid chromatography/electrospray ionization/multi-stage mass spectrometry (SPE-LC-ESI-MS/MS) method was optimized in this study for sensitive and simultaneous detection of multiple antibiotics in urban surface waters and soils. Among the seven classes of tested antibiotics, extraction efficiencies of macrolides, lincosamide, chloramphenicol, and polyether antibiotics were significantly improved under optimized sample extraction pH. Instead of only using acidic extraction in many existing studies, the results indicated that antibiotics with low pK_a values (<7) were extracted more efficiently under acidic conditions and antibiotics with high pK_a values (>7) were extracted more efficiently under neutral conditions. The effects of pH were more obvious on polar compounds than those on non-polar compounds. Optimization of extraction pH resulted in significantly improved sample recovery and better detection limits. Compared with reported values in the literature, the average reduction of minimal detection limits obtained in this study was 87.6 % in surface waters (0.06–2.28 ng/L) and 67.1 % in soils (0.01–18.16 ng/g dry wt). This method was subsequently applied to detect antibiotics in environmental samples in a heavily populated urban city, and macrolides, sulfonamides, and lincomycin were frequently detected. Antibiotics with highest detected concentrations were sulfamethazine (82.5 ng/L) in surface waters and erythromycin (6.6 ng/g dry wt) in soils. The optimized sample extraction strategy can be used to improve the detection of a variety of antibiotics in environmental surface waters and soils.

Keywords Liquid chromatography-mass spectrometry · pK_a · Antibiotics · Extraction efficiency · Surface waters · Urban soils

Introduction

Trace levels of environmental antibiotics have generated great public health concerns in recent years because of their wide occurrence in urban surface waters [1] and biosolid-amended soils [2]. Environmental antibiotics may lead to adverse health effects in aquatic organisms or pose selective pressure to promote the growth of antibiotic-resistant bacteria that could indirectly affect human health, and therefore, environmental antibiotics should be accurately quantified for a comprehensive environmental risk assessment. However, accurate quantification of antibiotics in environmental samples is usually difficult because of the low antibiotic concentrations and the complexity of environmental matrices. The commonly used liquid chromatography-mass spectrometry standard method 1694 was developed by the US Environmental Protection Agency (US EPA) to analyze pharmaceuticals and personal care products [3] and has been used to analyze environmental antibiotics in waters and soils [4, 5]. In spite of its wide application,

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this method has not been fully optimized for efficient and sensitive detection of multiple environmental antibiotics due to its limitations in sample extraction of environmental antibiotics with different chemical characteristics, and previous studies have indicated that the accuracy of sample extraction for liquid chromatography/mass spectrometry (LC/MS) analysis may be affected by a few factors, such as matrix effect, solvent, pH, extraction technique, and cartridge.

EPA method 1694 has mainly been applied in the analysis of water samples, and the effects of sample extraction and proper internal standards for soil samples and related matrix effects have not been fully investigated. Previous studies of antibiotics in soils focused on agricultural soils, compost soils, reclaimed water-irrigated soils, and manure applied soils, but there are limited studies on antibiotics in urban soils. Antibiotics that have been frequently detected in soil environments include tetracyclines, sulfonamides, quinolones, macrolides, lincomycin, chloramphenicol, and ionophore [6–8], but these antibiotics were usually not analyzed in one method. Therefore, it is important to develop a sensitive LC/MS method to simultaneously detect multiple antibiotics in environmental soils and evaluate the matrix effect.

Extraction solvent may affect extraction efficiency as well. A combination of acidic buffer and organic solvent has been used to extract antibiotics from solid matrices in previous studies. Methanol, acetonitrile, and ethyl acetate are three commonly used organic solvents while phosphate buffer and McIlvaine buffer are two commonly used acidic buffers to extract antibiotics from solid matrices [9, 10]. Huang et al. obtained better extraction performance using acetonitrile and phosphate buffer with addition of ethylenediaminetetraacetic acid (EDTA) than using methanol and McIlvaine buffer with EDTA in the extraction of tetracycline, sulfonamides, quinolones, and roxithromycin [11]. Additionally, Ho et al. showed that the usage of acetonitrile and phosphate buffer, which is applied in the US EPA method 1694, gave higher recovery for compounds with low polarity while methanol and McIlvaine buffer performed better in extracting high-polarity analytes [12]. Although ethyl acetate had been used previously to extract tetracycline [13], macrolides, and ionophores [14] to reduce matrix effect due to the low solubility of humic and fulvic acids in ethyl acetate, ethyl acetate may reduce recovery of the analytes. Therefore, there is a need to identify appropriate isotope-labeled internal standards to evaluate sample recovery of extraction solvents.

Extraction pH is another important factor that could affect extraction efficiencies of antibiotics [15]. A wide range of pH values had been previously applied to extract antibiotics. Simultaneous extraction of erythromycin-H_2O and sulfamethoxazole was conducted at pH 2.0 and other pharmaceuticals were extracted at pH 13 by Vanderford et al. [16]. pH 3.0 was used to extract erythromycin, roxithromycin, tylosin, and seven sulfonamides by Ye et al. [17]. pH 4.0 was used to extract azithromycin, erythromycin, clarithromycin, roxithromycin, and five sulfonamides by Göbel et al. [18]. There were also studies using two pH values to extract sulfonamides and macrolides separately. For example, Miao and Spongberg used pH 3.0 to extract tetracyclines and sulfonamides and used pH 6.0 to extract macrolides [19, 20]. However, only acidic condition is used in EPA method 1694 for sample extraction, except for a few compounds that are extracted at basic conditions. Therefore, there is a critical need to quantitatively evaluate the effects of extraction pH on sample extraction efficiency.

In addition, three extraction techniques are commonly used to extract antibiotics from soil samples: (1) ultrasound-assisted extraction (UAE), which applies ultra-sonication to break down solid substances and assist the extraction of organic compounds by organic solvent [21]; (2) microwave-assisted extraction (MAE), which uses microwave energy to heat mixture of solvents and solid samples to promote partition of the analytes from sample matrix into the solvent [22]; and (3) pressurized liquid extraction (PLE), which maintains elevated temperature and pressure to enhance mass transfer and dissolving of analytes [23]. MAE and PLE are easier to operate due to the high degree of automation but require greater investment in expensive equipment. Conversely, UAE offers a simple extraction procedure but requires relatively long analysis time. In terms of extraction performance, one previous study demonstrated that these three methods required similar amount of solvents and exhibited similar sensitivity, selectivity, accuracy, and precision [24]. Therefore, selection of an extraction technique can be determined by cost factors rather than analytical factors [21]. The application of UAE for optimized detection of multiple antibiotics in urban environmental samples is still limited.

Extraction cartridge may also affect extraction efficiency. Despite the rapid development of online SPE/LC/MS/MS, TurboFlow™ chromatography/LC/MS/MS, and rapid resolution LC/MS/MS, conventional solid-phase extraction (SPE) method still remains as the most commonly used technique for LC/MS analysis due to the easy accessibility of extraction equipment and relatively low cost. Different types of SPE cartridges, including C_{18}, carbon, polymeric sorbents (ENV, ENV+, EN, PPL, hydrophilic/lipophilic balanced (HLB)), weak cation exchanger (CBA), and mixed-phase cation exchange cartridges, have been used to extract antibiotics, while HLB SPE cartridge remains the most favored option for sample extraction [25–27]. Waters Oasis HLB cartridges have been used in the majority of studies for the extraction of sulfonamides, macrolides, lincosamides, and β-lactams from environmental water matrices [28]. Strata X and Oasis MCX have been used to extract sulfonamides, macrolides, lincosamides, and β-lactams [28–30]. Monensin and chloramphenicol in environmental waters were extracted with Oasis HLB SPE cartridge [31–36] or Strata X [37]. Furazolidon was

extracted from water samples with a Isolut ENV+ SPE cartridge but with a recovery at only 36 % [38]. Supel™ Select HLB SPE comprises a hydrophobic component with hydrophilic modification, and can be used to retain a broad range of polar and non-polar antibiotics but has been rarely reported for the analysis of antibiotics from environmental water samples.

The objective of this study was to optimize a sample extraction strategy to improve detection of multiple environmental antibiotics through an optimized solid-phase extraction/liquid chromatography/electrospray ionization/multi-stage mass spectrometry (SPE/LC/ESI/MS/MS) analysis. The optimized method was subsequently used to measure the occurrence and concentrations of antibiotics in surface waters and soils without exposure to treated wastewater in a heavily populated urban city. A total of 13 antibiotics in seven classes were analyzed, which include four macrolide antibiotics (azithromycin, clarithromycin, erythromycin, tylosin), one lincosamide antibiotic (lincomycin), one chloramphenicol antibiotic (chloramphenicol), one polyether antibiotic (monensin), three sulfonamide antibiotics (sulfamerazine, sulfamethazine, sulfamethoxazole), two β-lactam antibiotics (amoxicillin, ceftiofur), and one nitrofuran antibiotic (furazolidon). These compounds were selected based on their wide usage, medical significance, and various chemical characteristics. Extraction efficiencies and various impacting factors were evaluated. The improved detection of environmental antibiotics could provide useful information for a comprehensive environmental risk assessment of environmental antibiotics.

Materials and methods

Chemicals and materials

Chemical structures and properties of the tested antibiotics are listed in Table S1 in the Electronic supplementary material (ESM). Antibiotics and Supel™ Select HLB SPE cartridges (60 mg, 3 mL) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amoxicillin·$3H_2O$ (phenyl-$^{13}C_6$) and sulfamerazine (benzene-d_4) were purchased from Alpha Analytical (Singapore), and erythromycin (*N*,*N*-dimethyl-$^{13}C_2$) and (±)-chloramphenicol (ring-d_4, benzyl-d_1) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Individual stocks and calibration solutions were prepared in methanol and stored at −20 °C. Ammonium acetate and high-performance liquid chromatography (HPLC)-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). EDTA tetrasodium dihydrate ($Na_4EDTA·2H_2O$) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Laboratory reagent water was prepared in an ELGA Labwater water purification system (Wycombe, UK).

Sample collection

Surface waters and soils were collected from four locations with different land use types in Singapore: a park, an agricultural area, an industrial area, and a residential area. Surface waters were collected from rivers and canals with two 2.5 L amber glass bottles rinsed two times with tap water, acetone, methanol, and deionized water before sampling. Soils were collected from river banks closely adjacent to the sampling points of waters. Top layer soils (<10 cm) were collected with grass and plant roots removed using a methanol-rinsed shovel and filled in methanol-rinsed wide mouth polyethylene bottles. At each sampling location, soil samples were collected from three different locations and homogeneously mixed. All samples were stored on ice and transferred to the laboratory in the dark within 2 h for further processing. Blanks were run along each sampling process to detect contamination during transportation or sample analysis. The properties of water and soil samples were presented in the ESM in Tables S2 and S3.

Sample extraction and preparation

A total of 500 mL surface water sample from each sampling location was filtered with Millipore glass-fiber filters with 1.0 μm pore size. Our preliminary experiments on the absolute recoveries of internal standards using 100 mL samples and 500 mL sample volume with deionized water spiked with internal standards indicated that there were no statistical differences in absolute recovery in four out of six tested isotope-labeled compounds, and the increased sample loading volume from 100 to 500 mL only caused a slight loss of the compounds (ESM Table S4); 500 mL sample loading volume provides the advantage to accommodate the heterogeneity of complicated environmental samples, and therefore was selected in this study. In addition, the loss of analytes can be well compensated by the usage of isotope-labeled internal standards.

Sample preparation procedure for surface water samples and soil samples initially followed US EPA method 1694, where the extraction of azithromycin, clarithromycin, erythromycin, lincomycin, tylosin, sulfamerazine, sulfamethazine, and sulfamethoxazole was performed at acidic condition (pH=2). Neutral condition (pH=7) was used for sample extraction and sample extraction efficiencies under both acidic condition and neutral condition were compared. In addition, compounds not covered in EPA method 1694 (chloramphenicol, monensin, amoxicillin, ceftiofur, furazolidon) were included in this study. The SPE extraction efficiency was tested by spiking antibiotics into reagent water and calculating the recovery of each compound. Considering that our samples are urban soils without known anthropogenic inputs of antibiotics, naturally produced antibiotics including erythromycin, lincomycin, tylosin, chloramphenicol, and monensin are of

more interest in our analysis. As these compounds are characterized with relatively low polarity, we selected acetonitrile and phosphate buffer for sample extraction instead of ethyl acetate as the extraction solvent. We selected UAE because of the accessibility of the equipment and also the cost-effectiveness of UAE and believe this is a novel application for simultaneous detection of multiple antibiotics in environmental samples.

pH values of water samples were adjusted to 2.0 for acidic extraction, while no pH adjustment was performed for neutral extraction, as pH values of surface water samples were identified as neutral in preliminary experiments. Then water samples were spiked with 50 μL of internal standard solution (12.5 ng chloramphenicol-d_5, 12.5 ng erythromycin-$^{13}C_2$, 50 ng sulfamerazine-d_4, 50 ng sulfamethazine-$^{13}C_6$, 50 ng sulfamethoxazole-$^{13}C_6$, and 256 ng of amoxicillin·$3H_2O$-$^{13}C_6$) and 500 mg $Na_4EDTA{\cdot}2H_2O$, and extracted with Supel™ Select HLB SPE cartridges pre-conditioned with 10 mL methanol and 6 mL reagent water. For acidic extraction, additional 6 mL of reagent water at pH 2.0 was applied in the pre-conditioning. After loading the samples, SPE cartridges were dried under vacuum for 5 min. Final compounds were eluted with 6 mL methanol in amber glass vials and dried under a stream of nitrogen and reconstituted with 0.3 mL methanol and 0.2 mL 5 mM ammonium acetate.

Soil samples were freeze-dried, grinded, and homogenized with a mortar and pestle, which was pre-cleaned with tap water and rinsed with acetone and methanol. One gram of grinded soil sample was spiked with 100 μL of internal standard solution. For acidic extraction, soils were extracted three times using 30 min sonication in 20 mL acetonitrile and 15 mL phosphate buffer (0.14 M $NaH_2PO_4{\cdot}H_2O$/85 % H_3PO_4, pH=2). For neutral extraction, similar process was applied but 15 mL reagent water was used instead of 15 mL phosphate buffer. The extracts were concentrated by rotary evaporation at 40 °C and diluted in 200 mL reagent water and 500 mg $Na_4EDTA{\cdot}2H_2O$. Extracts were processed through SPE in a similar fashion as water samples and final compounds were reconstituted with 0.6 mL methanol and 0.4 mL 5 mM ammonium acetate. The final extracts were filtered with PTFE syringe filters with a pore size of 0.2 μm.

LC/ESI/MS/MS analysis

An Agilent 1290 Infinity HPLC system (Santa Clara, CA, USA) was used, and sample separations were performed on a C18 column with a 50×2.1 mm column packed with 2.7 μm particles (Agilent Poroshell 120 SB-C18). Mobile phases consisted of 5 mM ammonium acetate buffer (phase A) and 1:1 acetonitrile/methanol (phase B), and a flow-rate of 0.5 mL/min was applied. A gradient program was initially set up with 90 % phase A and 10 % phase B for 0.5 min, gradually increased to 40 % phase B over 3.5 min, and increased to 100 % phase B over 3 min, held at 100 % phase B for 3 min, and finally returned to initial condition over 0.1 min and held for 5 min. Mass analysis was carried out using an Agilent 6490 mass spectrometer (Santa Clara, CA, USA) equipped with Agilent Jet Stream electrospray technology. The triple quadrupole was operated in simultaneous positive and negative ionization modes. Multiple reaction monitoring (MRM) was used for quantitation. Two transitions, one quantifier and one qualifier, were monitored for all compounds. The operation parameters were set up as follows: capillary voltage, 4000 V; nebulizer pressure, 40 psig; drying gas flow, 11 L/min; gas temperature, 220 °C; sheath gas flow, 11 L/min; sheath gas temperature, 350 °C; and nozzle voltage, 0 V in positive and 1500 V in negative ion mode. Fragmentor voltages were set at 380 V. Collision energies and cell acceleration voltages were optimized for each compound (ESM Table S5).

Quantitation

Quantitation process mainly followed EPA method 1694, and a 7-point calibration with a calibration range of 0.5–350 ng/mL was used for all compounds, except that a calibration range of 10–3000 ng/mL was used for amoxicillin because of its low sensitivity. Internal standards were used with the following concentrations in each calibration point, 25 ng/mL chloramphenicol-d_5, 25 ng/mL erythromycin-$^{13}C_2$, 100 ng/mL sulfamerazine-d_4, 100 ng/mL sulfamethazine-$^{13}C_6$, 100 ng/mL sulfamethoxazole-$^{13}C_6$, and 512 ng/mL for amoxicillin·$3H_2O$-$^{13}C_6$. The labeled analog was used as internal standard for the native compound. If a labeled analog is not available, a compound that gives best recovery to a native compound was used as internal standard. Response factor (RF) was calculated for each native compound at each calibration point using Eq. (1):

$$\mathrm{RF} = A_\mathrm{n} \times C_\mathrm{is}/(A_\mathrm{is} \times C_\mathrm{n}) \quad (1)$$

where A_n is the area of the daughter m/z for the native compound, C_{is} is the concentration of the internal standard, A_{is} is the area of the daughter m/z of the internal standard, and C_{is} is the concentration of the internal standard (ng/mL) [3]. Linearity was accepted when the relative standard deviation (RSD) of RF was less than 20 %, which was stricter than the standard used by EPA method 1694 (RSD of RF <35 %). The concentration of each native compound in the final extract was calculated using Eq. (2):

$$C_\mathrm{ex}(\mathrm{ng/mL}) = A_\mathrm{n} \times C_\mathrm{is}/(A_\mathrm{is} \times \mathrm{RF}) \quad (2)$$

where C_{ex} is the concentration of a native compound in the final extract [3]. The quantitation was accepted when three criteria were met: (1) retention time was within ±15 s of the respective retention time in the most recent calibration standard, (2) qualifier/quantifier ion ratio was within ±20 % of that

obtained from the calibration standards, and (3) signal-to-noise ratio (S/N) at the LC peak value for each native compound at its daughter m/z must be greater than or equal to 3.0 for each compound, which is higher than the S/N ratio of 2.5 used in EPA method 1694.

Sample recovery

Spiking experiments were conducted to evaluate recovery in surface waters and soils; 500 mL waters were spiked with 200 ng/L amoxicillin and 20 ng/L other native compounds. One gram soil sample was spiked with 20 ng/g native compounds and 200 ng/g amoxicillin. Spiking was done by adding stock solution into surface water or grinded dry soil samples. The spiked samples were vigorously shaken to achieve homogeneous mixing and then left overnight before extraction. Concentrations of compounds in the original water samples (Eq. 3) and soil samples (Eq. 4) were calculated as follows:

$$\text{Concentration in aqueous phase (ng/L)} = C_{ex} \times V_{ex}/V_s \quad (3)$$

where C_{ex} is the concentration, V_{ex} is the volume of final water extract in milliliters, and V_s is the volume in liters.

$$\text{Concentration in solid sample (ng/g)} = C_{ex} \times V_{ex}/W_s \quad (4)$$

where W_s is the dry weight of soil sample in grams [3]. Relative recovery was calculated using Eq. (5):

$$\text{Relative recovery (\%)} = \text{Concentration found}/\text{Concentration spiked} \times 100 \quad (5)$$

The average and RSD of relative recoveries were calculated from triplicate spiking experiments. Conditions with relative recovery above 150 % or below 50 % or with RSD above 30 % were considered out of range (OOR) and unsuitable for the following LC/ESI/MS/MS analysis. Absolute recovery of internal standards was calculated using Eq. (6):

$$\text{Absolute recovery (\%)} = A_{is} \text{ in sample}/A_{is} \text{ in calibration standards} \times 100 \quad (6)$$

Matrix effect

Triplicate samples were used to evaluate matrix effects. Final extracts were separated into two parts with one part (extr) being kept unchanged and the other part (extr′) spiked with labeled compounds and native compounds at the concentration of CS7 (the seventh point of the calibration standard (calibr std.) with 3000 ng/mL amoxicillin and 350 ng/mL other compounds). Absolute recoveries from matrix effect were calculated using Eqs. (7) and (8):

$$\text{Absolute matrix recovery}_n(\%) = (A_n \text{ in extr}' - A_n \text{ in extr})/A_n \text{ in CS7} \times 100 \quad (7)$$

$$\text{Absolute matrix recovery}_{is}(\%) = (A_{is} \text{in extr'} - A_{is} \text{ in extr})/A_{is} \text{ in calibr std.} \times 100 \quad (8)$$

Signal suppression was calculated for both native compounds and isotope-labeled internal standard following Eq. (9). Relative recovery due to matrix effect was calculated using Eq. (10):

$$\text{Signal suppression (\%)} = 100 - \text{Absolute matrix recovery} \quad (9)$$

$$\text{Relative matrix recovery (\%)} = \text{Absolute recovery}_n/\text{Absolute recovery}_{is} \times 100 \quad (10)$$

Detection limits, linearity, method quantification limits, and repeatability

Instrument detection limits (IDLs) were calculated as three times the standard deviation of concentrations from seven injections of solvent blanks. Method detection limits (MDLs) were tested following the guideline in Appendix B of 40 CFR Part 136 developed by the US Environmental Protection Agency [39]. An estimated detection limit was made for each compound at concentration that generated an instrument S/N at 3. Antibiotics were spiked to reagent water and soil reference matrix at two times the estimated detection limit, and seven replicates of the spiked water and soil were processed through the entire analytical method. MDLs were calculated as three times the standard deviation of concentrations from the seven replicates. Soil reference matrix was prepared by washing playground soil repeatedly with acetonitrile and sonication [3].

Linearity range and method quantification limits (MQLs) were determined with reagent water and soil reference matrix spiked with analytes at five concentration levels ranging from 0.5 to 250 ng/L in waters and from 0.5 to 250 ng/g in soils, except that amoxicillin was spiked from 5 to 2500 ng/L in waters and from 5 to 2500 ng/g in soils. MQL was defined as the lowest concentration with an S/N higher than 10.

The repeatability was calculated as the relative standard deviation of repeated analyses in water and soil samples fortified with analytes at 10 ng/L and 10 ng/g, respectively. The intra-day precision was evaluated by analyzing ten times the same sample in 1 day. The inter-day precision was estimated by analyzing one sample on three different days in a week (each day the sample was injected three times in the LC/MS/MS system).

Results and discussion

Improved sample extraction strategy

Supel™ Select HLB SPE cartridge, which was rarely used for the extraction of antibiotics from urban environmental samples, was used in this study. We compared the Supel™ Select HLB and Waters Oasis HLB SPE cartridges to evaluate the effects of extraction cartridges on extraction efficiencies. The difference between these two types of HLB SPE cartridges is that the Waters Oasis HLB SPE cartridge consists of an adsorbent with an equal ratio of the hydrophilic nvinylpyrrolidone and the lipophilic divinylbenzene [40], while the Supel™ Select HLB SPE is a reversed-phase interaction predominated polymer with hydrophilic modification. The results indicated that Waters Oasis HLB SPE cartridge with a higher surface area retained all internal standards to 100 %, except for amoxicillin·$3H_2O$-$^{13}C_6$ at 95 %, sulfamethazine-$^{13}C_6$ at 83 %, and erythromycin-$^{13}C_2$ at 73 %. On the other hand, the relative recovery using Supel™ Select HLB cartridge reached 100 % for most of the analytes due to proper isotope-labeled internal standards. The results indicated that Supel™ Select HLB can be used for environmental antibiotic analysis.

The results of linearity test indicated that correlation coefficients (R^2) of linear regression analyses within the range of 1 and 250 ng/L, which covered the concentrations of the analytes in our environmental samples, were all above 0.99 (ESM Table S6), except for monensin, whose relative recovery decreased from 111±11 % at 1 ng/L spiking level to 24± 2 % at 250 ng/L spiking level. The intra-day precision was below 7.7 %, and the inter-day precision was below 14.4 % (ESM Table S7), suggesting that our method was relatively robust for complex environmental samples.

The recovery results of isotope-labeled internal standards and native compounds using SPE extraction indicated that recovery of antibiotics was significantly affected by extraction pH. As shown in Table 1, the internal standards including amoxicillin·$3H_2O$-$^{13}C_6$, sulfamerazine-d_4, sulfamethazine-$^{13}C_6$, and sulfamethoxazole-$^{13}C_6$ were extracted efficiently under acidic conditions and recovery of erythromycin-$^{13}C_2$ was significantly improved under neutral extraction condition ($p<0.05$). Furthermore, the absolute recovery results of native compounds indicated that extraction efficiencies of erythromycin, tylosin, lincomycin, and monensin were significantly improved under neutral conditions ($p<0.05$). In contrast, extraction efficiencies of sulfonamides and β-lactams were significantly improved under acidic conditions ($p<0.05$), while similar recovery efficiencies were observed for azithromycin, clarithromycin, chloramphenicol, and furazolidon under two extraction pH values ($p>0.05$).

Table 1 SPE extraction efficiency of analytes and isotope-labeled internal standards

Category	Category	Compound	Average SPE extraction efficiency (%)[a]	
			pH=2	pH=7
Native compounds	Macrolides	Azithromycin	90 (15)	89 (15)
		Clarithromycin	80 (30)	92 (5)
		Erythromycin	6 (34)	*91 (9)*
		Tylosin	55 (30)	*89 (1)*
	Lincosamide	Lincomycin	1 (30)	*96 (5)*
	Chloramphenicol	Chloramphenicol	64 (7)	74 (3)
	Polyether	Monensin	22 (24)	*42 (6)*
	Sulfonamides	Sulfamerazine	*41 (17)*	1 (6)
		Sulfamethazine	*60 (17)*	8 (2)
		Sulfamethoxazole	*89 (5)*	2 (8)
	β-lactams	Amoxicillin	*42 (9)*	1 (13)
		Ceftiofur	*96 (5)*	32 (21)
	Nitrofuran	Furazolidon	37 (22)	41 (5)
Isotope-labeled internal standards		Erythromycin-$^{13}C_2$	7 (2)	*85 (4)*
		Chloramphenicol-d_5	64 (16)	70 (12)
		Sulfamerazine-d_4	*43 (9)*	1 (1)
		Sulfamethazine-$^{13}C_6$	*55 (12)*	7 (6)
		Sulfamethoxazole-$^{13}C_6$	*92 (9)*	2 (1)
		Amoxicillin·$3H_2O$-$^{13}C_6$	*41 (15)*	5 (3)

[a] Conditions with statistically better extraction efficiencies ($p<0.05$) are set in italics. Values in parentheses are standard deviations

The effect of pH on extraction efficiency observed in this study is generally consistent with several previous studies [15, 33, 41]. Brown et al. also reported that acidic pH gave better recoveries for acidic compounds while basic pH gave better recovery for basic compounds [42]. However, Hao et al. reported that the recovery of sulfamerazine and sulfamethazine under neutral conditions (68–74 %) were higher than those under acid conditions (23–26 %) in surface water samples collected in Grand River in Canada [40].

The optimized extraction conditions were further summarized in Table 2, and the results indicated that chemical properties, such as pK_a and octanol-water partition coefficient (K_{ow}), should be considered for the selection of appropriate internal standards. Our results indicated that the effects of pH values on extraction efficiencies of antibiotics can be explained by their acid dissociation constants (pK_a), i.e., antibiotics with low pK_a values were extracted more efficiently under acid conditions and antibiotics with neutral pK_a values were extracted more efficiently under neutral conditions. For example, the pK_a values of tylosin, lincomycin, erythromycin, and monensin range between 7.1 and 8.9 (Table 2), and they were more efficiently extracted under neutral conditions. Lincomycin with a pK_a of 7.6 contains a pyrrolidine ring, which is an organic base that is able to gain a proton (ESM Fig. S1). Under acidic conditions, lincomycin is in the form of its cation. With a pH increase, more lincomycin molecules change to neutral form, resulting in higher retention on the SPE cartridges, where hydrophobic interaction is the dominant retention mechanism. Conversely, a sulfonamide antibiotic contains one basic amine group ($-NH_2$) and one acidic amide group (−NH–) (ESM Fig. S2) that corresponds to two pK_a values. Sulfonamides tend to exist as cations at pH below their p$K_{a,\,1}$ values (1.9–2.3), and the increase of pH above 2.0 would increase the portion of sulfonamides to exist as neutral molecules. However, further increase of pH above their p$K_{a,\,2}$ values (5.4–7.5) would change the neutral forms of molecules to anions, which could result in low extraction in SPE cartridges. After the initial pH adjustment, the subsequently added chelating agent Na_4EDTA increased the pH (from 2.0 to 2.8 and from 7.0 to 10.0 as tested) by uptaking protons as conjugate base. The extraction efficiencies of chloramphenicol at both acid and neutral conditions were similar, which can be explained by the fact that the pK_a value of chloramphenicol is 11.03 and therefore both acidic and neutral conditions do not make a difference. The β-lactams form carboxyl acids [−COOH] under acid condition and carboxyl groups [$-COO^-$] under neutral conditions. The pK_a values of amoxicillin (2.4) and ceftiofur (3.7) are both below 7, suggesting that they can be better extracted under acidic conditions, which is consistent with our results.

However, the effects of pH on polar and non-polar compounds are different. The change in pH affects polar compounds in a more obvious way than it affects non-polar compounds. For example, amoxicillin (log K_{ow}=0.87), ceftiofur (log K_{ow}=1.6), sulfamerazine (log K_{ow}=0.14), sulfamethazine (log K_{ow}=0.89), sulfamethoxazole (log K_{ow}=0.89), lincomycin (log K_{ow}=0.56), and tylosin (log K_{ow}=1.63) are relatively more polar than the other compounds and their recoveries between pH 2 and pH 7 showed significant difference

Table 2 Optimized extraction conditions and internal standards

Category	Compound	pK_a[a]	Log K_{ow}[a]	Surface waters		Soils	
				Extraction condition	Internal standards	Extraction condition	Internal standards
Macrolides	Azithromycin	8.74	4.02	Neutral	Erythromycin-$^{13}C_2$	Neutral	Erythromycin-$^{13}C_2$
	Clarithromycin	8.99	3.16	Neutral	Erythromycin-$^{13}C_2$	Neutral	Erythromycin-$^{13}C_2$
	Erythromycin	8.9	3.06	Neutral	Erythromycin-$^{13}C_2$	Neutral	Erythromycin-$^{13}C_2$
	Tylosin	7.73	1.63	Neutral	Erythromycin-$^{13}C_2$	Neutral	Erythromycin-$^{13}C_2$
Lincosamide	Lincomycin	7.6	0.20	Neutral	Erythromycin-$^{13}C_2$	Neutral	Chloramphenicol-d_5
Chloramphenicol	Chloramphenicol	11.03 [43]	1.14	Neutral	Chloramphenicol-d_5	Neutral	Chloramphenicol-d_5
Polyether	Monensin	7.95 [44]	5.43	Neutral	Chloramphenicol-d_5	Neutral	Chloramphenicol-d_5
Sulfonamides	Sulfamerazine	2.06/6.90 [45]	0.14	Acidic	Sulfamerazine-d_4	Acidic	Sulfamerazine-d_4
	Sulfamethazine	2.65/7.65	0.14	Acidic	Sulfamethazine-$^{13}C_6$	Acidic	Sulfamethazine-$^{13}C_6$
	Sulfamethoxazole	1.6/5.7	0.89	Acidic	Sulfamethoxazole -$^{13}C_6$	Acidic	Sulfamethoxazole-$^{13}C_6$
β-lactams	Amoxicillin	2.4/7.4/9.6 [46]	0.87	Acidic	Amoxicillin·$3H_2O$-$^{13}C_6$	Acidic	Amoxicillin·$3H_2O$-$^{13}C_6$
	Ceftiofur	3.7	1.60	Acidic	Sulfamethoxazole -$^{13}C_6$	Acidic	Sulfamethoxazole -$^{13}C_6$
Nitrofuran	Furazolidon	NA	−0.04	Acidic	Sulfamethazine-$^{13}C_6$	Acidic	Sulfamethazine-$^{13}C_6$

NA not available

[a] Unless otherwise specified, pK_a values are from Hazardous Substances Data Bank from the National Library of Medicine (http://toxnet.nlm.nih.gov/newtoxnet/hsdb.htm)

($p<0.05$) according to their pK_a values. In comparison, azithromycin (log K_{ow}=4.02) and clarithromycin (log K_{ow}= 3.16) are less polar and their recoveries under pH 2 and pH 7 are similar. An exception is erythromycin, whose log K_{ow} value and pK_a value are similar to azithromycin and clarithromycin, has significantly different recovery under these two pH conditions, which might be explained by the fact that erythromycin is very instable in acidic conditions. Fiese et al. reported that erythromycin underwent 10 % decay in only 3.7 s at 37 °C and pH 2, while for azithromycin, the $T_{1/10}$ was 20.1 min under the same conditions [47]. Another exception is monensin (log K_{ow}=5.43), which is the most non-polar antibiotic among the 13 tested antibiotics. Monensin showed a significant difference ($p<0.05$) in recovery under pH 2 (28±24 %) and pH 7 (52± 6 %). Similar results were observed for monensin by Hao et al., i.e., the recovery for monensin was 4 % at pH 2.5 and 75 % at pH 7.2 by an Oasis HLB SPE cartridge, and the authors explained that the low recovery of monensin at acidic pH was due to its low solubility [40].

K_{ow} is another important factor that should be considered for the selection of internal standards. An isotope-labeled analog is usually the best choice to be used as internal standards. For example, amoxicillin, erythromycin, chloramphenicol, sulfamerazine, sulfamethazine, and sulfamethoxazole were efficiently compensated with their own isotope-labeled compounds. However, the application of isotope-labeled compounds is limited due to their low availability and high costs. Therefore, alternatively internal standards with similar chemical properties are usually used when a labeled analog is not available. For example, erythromycin-$^{13}C_2$ was used as an internal standard for all macrolides in our study because they belong to the same antibiotic class and have similar chemical characteristics. Nonetheless, quantification of lincomycin in soil was more accurate when calibrated with chloramphenicol-d_5 than with erythromycin-$^{13}C_2$. Such a result may be explained by their different extent of polarity, which can be described with K_{ow}. Our results in ESM Table S4 indicated that compounds with comparatively higher polarity, such as amoxicillin·$3H_2O$-$^{13}C_6$, sulfamerazine-d_4, and sulfamethazine-$^{13}C_6$, had lower absolute recovery than the non-polar compounds, such as chloramphenicol-d_5 and erythromycin-$^{13}C_2$. Erythromycin has higher log K_{ow} value (3.06) than lincomycin (0.56) and can be adsorbed to soil particles more strongly, resulting in lower recovery than lincomycin, while the log K_{ow} value of chloramphenicol (1.14) is similar to that of lincomycin, which may explain the better recovery of lincomycin when chloramphenicol-d_5 is used as an internal standard. Sulfamethoxazole-$^{13}C_6$ and sulfamethazine-$^{13}C_6$ were used as internal standards for furazolidone and ceftiofur respectively, given that they are all comparatively polar compounds. However, chloramphenicol-d_5 instead of erythromycin-$^{13}C_2$ was used as an internal standard for monensin due to the fact that erythromycin-$^{13}C_2$ is more susceptible to signal suppression than monensin, and thus may over-quantify the concentration of monensin. Table 3 shows that antibiotics were all well compensated with

Table 3 Selection of isotope-labeled internal standard and relative recovery

Category	Compound	Isotope-labeled internal standards	Average recovery (%)[a]			
			Surface waters		Soils	
			pH=2	pH=7	pH=2	pH=7
Macrolides	Azithromycin	Erythromycin-$^{13}C_2$	OOR	*125 (24)*	OOR	*97 (21)*
	Clarithromycin	Erythromycin-$^{13}C_2$	OOR	*132 (30)*	OOR	*115 (5)*
	Erythromycin	Erythromycin-$^{13}C_2$	85 (17)	99 (10)	116 (23)	116 (10)
	Tylosin	Erythromycin-$^{13}C_2$	OOR	*100 (30)*	OOR	*135 (20)*
Lincosamide	Lincomycin	Erythromycin-$^{13}C_2$	OOR	*103.3 (20)*	OOR	OOR
		Chloramphenicol-d_5	OOR	*91 (40)*	OOR	*118 (27)*
Chloramphenicol	Chloramphenicol	Chloramphenicol-d_5	100 (6)	106 (1)	128 (27)	129 (16)
Polyether	Monensin	Chloramphenicol-d_5	OOR	*70 (25)*	OOR	*75 (24)*
Sulfonamides	Sulfamerazine	Sulfamerazine-d_4	89 (6)	93 (24)	128 (11)	123 (2)
	Sulfamethazine	Sulfamethazine-$^{13}C_6$	95 (7)	92 (14)	105 (6)	104 (12)
	Sulfamethoxazole	Sulfamethoxazole-$^{13}C_6$	98 (9)	94 (13)	*94 (4)*	89 (15)
β-lactams	Amoxicillin	Amoxicillin·$3H_2O$-$^{13}C_6$	*57 (16)*	OOR	*64 (17)*	OOR
	Ceftiofur	Sulfamethoxazole-$^{13}C_6$	*74 (14)*	OOR	*78 (13)*	OOR
Nitrofuran	Furazolidon	Sulfamethazine-$^{13}C_6$	*77 (18)*	OOR	*75 (26)*	OOR

OOR out of range, i.e., relative recovery is either above 150 % or below 50 % or relative standard deviation is above 30 %

[a] Conditions with statistically better extraction efficiencies ($p<0.05$) are set in italics. No statistical differences were found for erythromycin, chloramphenicol, and sulfamerazine under two extraction pH values ($p>0.05$). Values in parentheses are standard deviations

the proper selection of internal standards, with relative recoveries ranging from 57±17 to 132±30 % in water and from 64 ±17 to 135±20 % in soil.

Matrix effect

In addition to extraction conditions, matrix effect is commonly encountered in LC/MS analysis with environmental extracts and should be minimized if possible. The results of matrix effect (ESM Table S8) showed that acidic extracts exhibited more severe signal suppression (average of 42 %) than neutral extracts (average of 33 %) in water extracts ($p<0.05$). Similarly, acidic extracts exhibited more severe signal suppression (average of 30 %) than neutral extracts (average of 23 %) in soil extracts ($p<0.05$). Strong signal suppression was observed for amoxicillin, macrolides, and lincomycin. Previous studies also reported signal suppression with acidic extraction [17, 40], which was likely caused by natural organic matter retained on the polymeric sorbent of SPE cartridge at acidic condition [45]. The 58 % signal suppression of erythromycin-$^{13}C_2$ indicated that matrix effect, instead of procedure loss, was the main cause of signal loss. Conversely, 7 % signal suppression of sulfonamides suggests that major loss of sulfonamides was from procedure loss. Instead of extracting samples only under acid conditions in EPA standard 1694, the results of this study suggest that sample extraction for select groups of antibiotics could be optimized under neutral conditions to minimize signal loss due to matrix effect.

Matrix effects between water extracts and soil extracts were also compared. Soil matrix is usually rich in complex organic matters that could be co-extracted with target compounds and cause severe matrix effect. However, the results in this study showed that soil extracts exhibited less signal suppression than water extracts under both conditions ($p<0.05$). Trends of signal suppression for different compounds in soils were similar to those in water samples, e.g., amoxicillin was observed with strongest signal suppression (59 %) in acidic extracts, macrolides were subject to strong signal suppression in both acidic (average of 38 %) and neutral (average of 41 %) extracts, and sulfonamides and furazolidon exhibited least signal suppression.

Detection limits

The detection limits and method quantification limits developed in this study after optimizing sample extraction were compared with reported values in the literature (Table 4). Detection limits of antibiotics in sediments and biosolids were also included from existing literature due to limited data of selected antibiotics in soils. The MDLs for all compounds in surface waters in this study were either lower than or in the lower range of reported detection limits in the literature, except for monensin, of which the MDL were higher than those reported in the literature. MDLs of furazolidon in surface water and soils were also calculated, while no information is available in the literature. For the antibiotics whose MDLs were lower in our study, the average reductions of MDLs were

Table 4 Instrument detection limits (IDLs), method quantification limits (MQL), and method detection limits (MDLs)

Category	Compound	IDLs (pg)	Surface waters (ng/L)[a]				Soils (ng/g)[a]			
			MQL	MDL			MQL	MDL		
				This study	Literature	Reduced by (%)		This study	Literature	Reduced by (%)
Macrolides	Azithromycin	3.20	0.50	0.18	0.02–7 [1, 48–50]		5.00	*0.61*	1.6–5.6 [51]	61.8
	Clarithromycin	0.20	0.50	0.16	0.001–1.44 [1, 48–50, 52]		0.50	0.08	0.08–9.3 [1, 5, 51–53]	
	Erythromycin	0.88	0.50	0.31	0.03–6 [48–50, 52]		0.50	*0.18*	0.5–2.7 [5, 52]	64.0
	Tylosin	0.60	0.50	0.15	0.06–6 [1, 48, 52, 54]		0.50	*0.23*	0.5–8.1 [5, 52]	54.0
Lincosamide	Lincomycin	0.48	0.50	0.22	0.16–6.7 [1, 48, 52]		0.50	*0.01*	0.26–4.7 [5, 52]	96.2
Chloramphenicol	Chloramphenicol	1.12	0.50	*0.06*	0.77–3.8 [52, 54]	92.2	0.50	*0.01*	0.49 [52]	98.0
Polyether	Monensin	48.83	1.00	0.36	0.36 [52]		50.0	18.2	0.91 [52]	
Sulfonamides	Sulfamerazine	1.60	0.50	*0.08*	0.45–16 [1, 48, 49, 54, 55]	82.2	0.50	*0.09*	0.5–1.4 [1, 5]	82.0
	Sulfamethazine	3.84	0.50	*0.17*	0.7–1.8 [1, 48, 49, 54, 55]	75.7	1.00	0.25	0.31–1.22 [1, 5, 52]	19.4
	Sulfamethoxazole	2.72	0.50	*0.32*	2.7–1.24 [26, 50]	88.1	0.50	0.14	0.24–2.9 [1, 5, 51–53]	41.7
β-lactams	Amoxicillin	68.99	10.0	2.28	2.08–20 [41, 56]		10.0	*7.03*	NA	>100
	Ceftiofur	1.24	10.0	1.33	1.15–3 [52, 57]		10.0	*1.39*	1.75 [52]	20.6
Nitrofuran	Furazolidon	1.10	1.00	*0.73*	NA	>100	1.00	*0.42*	NA	>100

NA not available due to limited studies targeting these compounds

[a] Detection limits in this study are set in italics if they are lower than the reported values in the literature

87.6 % in surface waters and 67.1 % in soils. The sensitivity was generally consistent with extraction efficiency (Table 3), which was resulted from optimized extraction conditions and appropriate internal standards.

Application to environmental samples

The optimized SPE/LC/ESI/MS/MS method was then used to analyze urban environmental samples, and trace levels of antibiotics were detected in both urban surface waters and soils (Fig. 1). Amoxicillin, ceftiofur, furazolidon, and monensin were all below MDLs in all samples. Macrolides were the most frequently detected antibiotics in water samples, followed by sulfamethazine, sulfamethoxazole, chloramphenicol, lincomycin, and sulfamerazine. The highest concentration was observed for sulfamethazine (82.5 ng/L), followed by sulfamethoxazole (29.5 ng/L), sulfamerazine (16.3 ng/L), clarithromycin (13.1 ng/L), and erythromycin (3.0 ng/L). The wide occurrence of antibiotics was consistent with previous results that macrolides and sulfonamides were detected in urban waters [48, 54, 58, 59]. Compared with water samples, only erythromycin, clarithromycin, and lincomycin were detected in soils. Erythromycin was detected in all four locations with concentrations ranging from 1.0 to 6.7 ng/g dry wt. Clarithromycin was detected in two soil samples (0.4 and 0.6 ng/g dry wt). Lincomycin was detected in three samples with concentration ranging from 0.03 to 0.1 ng/g dry wt. A direct comparison with existing literature is difficult as there is not enough information available on the occurrence of antibiotics in urban soils.

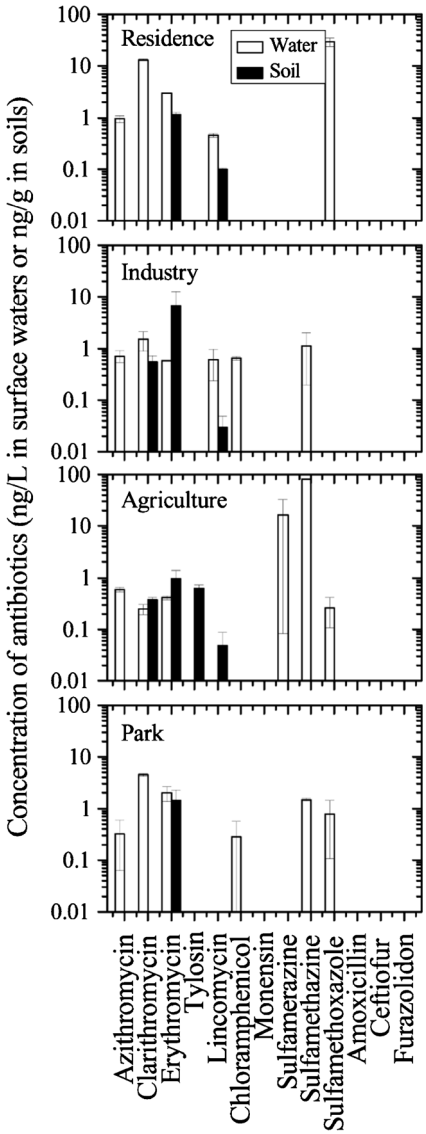

Fig. 1 Occurrence and concentrations of antibiotics in surface waters and soils

The concentrations of antibiotics may be explained by the affinity of antibiotics to soil particles, which can be described by their K_{ow} values. The log K_{ow} value of lincomycin (0.2) was lower than those of erythromycin (log K_{ow}=3.06) and clarithromycin (log K_{ow}=3.16), and therefore lincomycin has a low tendency to adsorb to soil particles and could be relatively easily washed off from soils and result in a relatively low concentration.

The sources of detected antibiotics still need further investigation. No correlation was identified between the detected antibiotic concentrations and land use types, suggesting that other sources could exist in urban environmental samples tested in this study. Usually, only sampling locations receiving land application of animal manure or biosolids were studied in the literature [10, 60, 61], as these locations are under direct point-source pollution and antibiotics could be transferred from these pollution sources. Urban surface waters and soils in this study were free of point-source contamination as treated wastewater was discharged to the sea, and therefore their detected concentrations were generally low. However, the wide occurrence of low level antibiotics suggests that other sources may contribute to the detected antibiotics and one possibility is natural production of antibiotics [10, 62, 63]. Both erythromycin and lincomycin could be naturally produced by *Streptomyces* species in soils [64, 65], which might be used to explain their wide occurrence in this study. Another study reported that chloramphenicol could be naturally produced by soil bacteria and subsequently uptaken by crops [63]. Further studies are needed to explore the natural production of antibiotics and evaluate their potential health risks to humans. As urban environment is a complex system, studies are needed to evaluate the origin and fate of trace levels of environmental antibiotics.

Conclusion

This study provides an optimized analytical protocol for sensitive and simultaneous quantitation of multiple classes of

antibiotics in urban surface waters and soils. The results indicated that extraction efficiencies of macrolides, lincosamide, chloramphenicol, and polyether antibiotics were significantly improved under neutral extraction pH. The results indicated that antibiotics with low pK_a values were extracted more efficiently under acidic conditions, and antibiotics with neutral pK_a values were extracted more efficiently under neutral conditions. The optimized method resulted in improved sample recovery and detection limits for antibiotics in surface waters (0.06–2.28 ng/L) and soils (0.01–18.16 ng/g dry wt). Future SPE/LC/ESI/MS/MS analysis procedure could be optimized if chemical parameters, such as pK_a, can be evaluated and a best strategy is adopted for sample extraction.

Compared with previous literature, the increased sensitivity of antibiotic measurement, especially in urban soils, enabled detection of antibiotics at low or even sub-nanogram-per-gram levels and revealed for the first time the wide occurrence of erythromycin, lincomycin, tylosin, and clarithromycin in urban soils without known anthropogenic input of antibiotics. However, no correlation was identified between land use type and the occurrence of antibiotics, suggesting other sources could contribute to the occurrence of environmental antibiotics. The optimized sample extraction conditions can be used to improve the detection of antibiotics in environmental samples for a comprehensive environmental risk assessment.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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