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Simultaneous determination of three classes of antibiotics in the suspended solids of swine wastewater by ultrasonic extraction, solid-phase extraction and liquid chromatography-mass spectrometry

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Abstract

This work describes a systematic approach to the development of a method for simultaneous determination of three classes of veterinary antibiotics in the suspended solids (SS) of swine wastewater, including five sulfonamides, three tetracyclines and one macrolide (tiamulin). The entire procedures for sample pretreatment, ultrasonic extraction (USE), solid-phase extraction (SPE), and liquid chromatography-mass spectrometry (LC-MS) quantification were examined and optimized. The recovery efficiencies were found to be 76%–104% for sulfonamides, 81%–112% for tetracyclines, and 51%–64% for tiamulin at three spiking levels. The intra-day and inter-day precisions, as expressed by the relative standard deviation (RSD), were below 17%. The method detection limits (MDLs) were between 0.14 and 7.14 μg/kg, depending on a specific antibiotic studied. The developed method was applied to field samples collected from three concentrated swine feeding plants located in Beijing, Shanghai and Shandong province of China. All the investigated antibiotics were detected in both SS and liquid phase of swine wastewater, with partition coefficients ($\log K_d$) ranging from 0.49 to 2.30. This study demonstrates that the SS can not be ignored when determining the concentrations of antibiotics in swine wastewater.

Key words: antibiotics; swine wastewater; suspended solids; ultrasonic extraction; solid-phase extraction; mass spectrometry

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Introduction

Concerns about the occurrence, transport and fate of veterinary antibiotics in the environment have been ever increasing in recent years. Antibiotics have been widely used to promote animal growth as well as prevent or treat microbial infections in livestock production industry, especially during the periods of a high risk for future infections such as after weaning or in transportation (Rabølle and Spliid, 2000; De Liguoro et al., 2003; Angenent et al., 2008). In China, most of veterinary antibiotics are applied as feed additives to promote the growth of animals. However, about 50%–90% of these antibiotics can not be directly absorbed by animals but excreted as their original forms or primary metabolites. Typically, concentrated animal feeding operations (CAFOs) house thousands of animals, and usually use several waste lagoons to store the urine and manure for later use as fertilizer. These waste lagoons are of particular concern because they contain a relatively high level of antibiotic residues (commonly at μg/L or mg/L levels), which either ultimately end up being spread on croplands and find their way into the food chain, or leach through soils and eventually reach groundwater, causing a genetic selection of resistant bacteria (Campagnolo et al., 2002; Ben et al., 2008).

In recent years, a variety of analytical methods have been developed for the detection of antibiotics in different matrices related to swine stockbreeding, such as wastewater effluent, manure, polluted soils and livestock tissues. In our previous work, an analytical method for determination of three classes of antibiotics in the liquid phase of swine wastewater was developed, and eight antibiotics including four sulfonamides, three tetracyclines and one macrolide were detected with concentrations ranging from 0.62 to 32.67 μg/L in the samples collected from three CAFOs in Beijing area (Ben et al., 2008). However, these data only presented the concentrations of dissolved antibiotics, rather than the total concentrations of antibiotics in swine wastewater because the suspended solids (SS) were commonly removed by high-speed centrifugation and membrane filtration during sample pretreatment process. It is worth noting that the swine wastewater contains a high SS concentration (commonly at g/L level), and a significant fraction of antibiotics or their bioactive metabolites

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could end up in the SS (Díaz-Cruz et al., 2003). To obtain the total concentration of antibiotics in swine wastewater, it is also important to further extract, enrich and detect antibiotics in the SS phase.

The suspended solids of swine wastewater is a special matrix which mostly consisted of undissolved organic materials (e.g., proteins, lipids) and nutrients. Ultrasonic extraction (USE) is normally applied to antibiotics in solid-phase matrices. Blackwell et al. (2004) extracted oxytetracycline, sulfachloropyridazine and tylosin from soil and pig slurry with the assistance of ultrasonic agitation, and used a mixture of methanol, EDTA and McIlvaine buffer at pH 7.0 as the extractant. Aust et al. (2008) and Karci and Balcioğlu (2009) adopted USE to extract tetracycline, sulfonamide, and fluoroquinolone antibiotics from animal manure and agricultural soils in Canada and Turkey prior to LC-MS-MS or HPLC analysis, respectively. Nowadays, pressurized liquid extraction (PLE) has also been used to accelerate the extraction process, which can reduce the extraction time, solvent consumption, and realize automation as well (Dabrowski et al., 2002; Schlüsener et al., 2003; Burkhardt et al., 2005; Jacobsen and Halling-Sørensen, 2006; Beck et al., 2008). However, the high cost of this apparatus somewhat prevents its popularization. Therefore, USE still plays an irreplaceable role in extracting antibiotics from solid-phase samples (López de Alda and Barceló, 2001; Löffler and Ternes, 2003).

This study was to develop an applicable method for simultaneous determination of three classes of veterinary antibiotics in the SS of swine wastewater. The antibiotics of interest included five sulfonamides (SAs), three tetracyclines (TCs), and one macrolide. These compounds have a wide range of physical-chemical properties, and have been extensively used for therapeutic or growth-promoting purposes in animal production in China. The developed method adopted a comprehensive sample preparation procedure including sample pretreatment, USE and solid-phase extraction (SPE) to achieve desired recovery efficiencies for all the nine investigated antibiotics. Then the extracted samples were analyzed by liquid chromatography-mass spectrometry (LC-MS). To demonstrate the feasibility and reproducibility of the developed method, field samples collected from three CAFOs were analyzed to determine the antibiotic concentrations in the SS of swine wastewater. Meanwhile, the antibiotic concentrations in the liquid phase of swine wastewater were analyzed in parallel according to our previous work (Ben et al., 2008). Based on the liquid and SS-phase concentrations, the partition coefficients ($\log K_d$) of the selected antibiotics were estimated.

1 Material and methods

1.1 Chemicals and standards

The studied antibiotics were purchased from the following sources: sulfathiazole (STZ, 99%), sulfamethoxazole (SMX), sulfamethizole (SML), sulfadimethoxine (SDM), tetracycline hydrochloride (TCN, 95%) and oxytetracycline hydrochloride (OTC, 95%) from Sigma-Aldrich, USA; sulfamethazine (SMN, 99%) and chlortetracycline hydrochloride (CTC, 90%) from Acros, USA; tiamulin fumarate (TIA, 98%) from Dr. Ehrenstorfer, Germany. The major physical-chemical properties of these antibiotics are presented in Table 1.

Acetonitrile, methanol and ethyl acetate (EtOAc) were obtained from Fisher Scientific, USA, and dichloromethane from J. T. Baker, the Netherlands. Formic acid, disodium ethylenediamine tetraacetate (Na_2EDTA), acetone and other chemicals were purchased from Beijing Chemical Reagents Company, China. All organic solvents used for sample preparation were of at least residue-analysis grade. HPLC-grade solvents were used for the analyses related to liquid chromatography.

The stock solution of five SAs was prepared as a mixture at an individual concentration of 100 mg/L in deionized (DI) water. It could keep stable for more than one month at 4°C. The stock solutions of TCs and TIA were freshly prepared at a concentration of 100 mg/L in DI water prior to each experiment to avoid hydrolysis. DI water was purposely used to dissolve the antibiotics instead of organic solvent in consideration that organic solvent may affect the interactions between the SS and the spiked antibiotics, thus distorting the reliability of spiking experiments. McIlvaine buffer solution was prepared by dissolving 21.0 g of citric acid monohydrate, 17.75 g of Na_2HPO_4, and 60.5 g of $Na_2EDTA{\cdot}2H_2O$ in 1.625 L of DI water (Koesukwiwat et al., 2007).

1.2 Glassware cleaning

The glass particles are bound together by interbedded silanol during the production process. Silanol, a kind of macromolecule with strong polarity, has a looser reticulate structure than silicate tetrahedron. It is easily combined with other macromolecules through hydrogen bond. TCs

Table 1 Major properties and detection parameters of selected antibiotics in the selected ion recording mode

ABs.	pKa[a]	logK_{ow}[b]	Precursor (qualifier) ions (m/z)	RT[c] (min)
SMX	1.85 ± 0.30 5.60 ± 0.04	0.90	254 (156)	22.8
STZ	2.01 ± 0.30 7.11 ± 0.09	0.72	256 (156)	7.2
SML	1.86 ± 0.30 5.92 ± 0.04	0.54	271 (156)	17.5
SMN	2.07 ± 0.30 7.49 ± 0.13	0.89	279 (204)	13.5
SDM	2.13 ± 0.30 6.08 ± 0.09	1.63	311 (156)	25.8
TCN	3.32 ± 0.30 7.78 ± 0.05	–1.13	445 (427)	12.3
OTC	3.22 ± 0.30 7.46 ± 0.03	–0.89	461 (443)	10.6
CTC	3.33 ± 0.30 7.55 ± 0.02	–0.36	479 (462)	19.4
TIA	7.6	–	494 (192)	24.6

[a] Ben et al., 2008; [b] Díaz-Cruz et al., 2006. [c] RT: retention time obtained from the LC-MS extracted-ion SIR chromatogram of selected antibiotics in the reference matrix.

are well known to form chelate complexes with silanol groups (Hamscher et al., 2002). Therefore, all glassware was heated for 2 hr at 450°C, cooled down to ambient temperature, rinsed with saturated methanolic Na_2EDTA solution, and air-dried prior to analysis of antibiotics.

1.3 Sample pretreatment

The swine wastewater, which was used as the reference matrix for method development, was collected from an anaerobic lagoon of a swine feeding plant located in Beijing. The swine wastewater was filtered through a 2-mm screen on-site to remove large debris and centrifuged at 7000 r/min (Beckmann, J2-HS, USA) in laboratory for 15 min within 24 hr. The SS at the bottom of centrifuge tubes were carefully taken out, freeze-dried under vacuum (Boyikang, FD-1-50, China) for at least 48 hr, homogenized using a mortar and pestle, sieved to obtain the desired particles (diameter ≤ 0.5 mm), and stored in amber glass bottles at 4°C.

After development, the analytical method was applied to determine the concentrations of selected antibiotics in the SS of swine wastewaters collected from three CAFOs in Beijing, Shanghai and Shandong Province. The major physical-chemical properties of these wastewaters are shown in Table 2. Chemical oxygen demand (COD), free ammonia (NH_3-N), total nitrogen (TN) and total phosphorus (TP) were determined by Hach methods 8000, 10031, 10072 and 10127, respectively, with a DR/5000 spectrophotometer (Hach, Loveland, USA). The concentration of SS was analyzed in accordance with the Standard Methods (APHA, 2005).

1.4 Ultrasonic extraction

One gram of above-pretreated SS was accurately weighed into a 50-mL centrifuge tube, and then 30 mL of 0.2 mol/L citric acid buffer with pH adjusted to 4.7 and 100 μL of 5% Na_2EDTA solution were added. The tube was vortexed for 30 sec, ultrasonicated for 30 min, and centrifuged at approximately 4000 r/min for another 10 min. The supernatant was decanted into a 250-mL glass bottle and the SS residue was extracted one more time with 10 mL of 1:1 (*V*/*V*) mixture of methanol : 0.2 mol/L citric acid buffer. Vortexing was further applied to re-suspend the SS after centrifugation. After extraction by two cycles, approximately 40 mL of supernatant was combined and diluted to approximately 200 mL with DI water to reduce the methanol content below 5%. At last, the diluted supernatant was filtered through 0.45-μm glass fiber filters to remove potential particulate impurities which may block cartridges in the following SPE step.

Table 2 Characteristics of swine wastewaters collected from three CAFOs

	Plant 1	Plant 2[a]	Plant 3
COD (mg/L)	16950	12520	9100
NH_3-N (mg/L)	1182	404	110
TN (mg/L)	1590	520	200
TP (mg/L)	762	84	210
SS (mg/L)	2180	2010	1970

[a] Reference matrix used for method development.
Plant 1: sampled from Shanghai area in Mar 2009; Plant 2: sampled from Beijing area in Jul 2008; Plant 3: sampled from Shandong province in Jul 2009.

1.5 Solid-phase extraction and matrix clean-up

SPE is the most commonly used concentration and clean-up technique for analysis of environmental samples which allows large sample volumes to be concentrated and purified in one step. In this study, an Oasis HLB cartridge (500 mg/6 mL, Waters) was pre-conditioned sequentially with 5 mL of methanol, 5 mL of 0.5 mol/L HCl and 5 mL of DI water. The diluted SS extract was then passed through the cartridge at a flow rate of 5 mL/min, and the cartridge was washed with 5 mL of 5% methanol solution and 5 mL of DI water. After the cartridge was dehydrated under airstream, 3 mL of *n*-hexane was loaded to further remove fat and fat-soluble impurities in the matrix, and then 10 mL of dichloromethane/acetone mixture (3:2, *V*/*V*) was applied to elute the antibiotics. Finally, the extract was dried under a gentle stream of nitrogen and re-dissolved in a mixture of 0.8 mL of methanol and 1.2 mL of DI water. In addition, we also evaluated the purification efficiency of an SAX cartridge (Supelco, USA), which was situated on the top of the HLB cartridge. After passing the diluted SS extract through the tandem cartridges, the SAX cartridge was washed with 2 mL of citric acid buffer (pH 4.0) and disconnected from the HLB cartridge.

1.6 LC-MS system

This work employed an LC-MS system consisting of Alliance 2695 LC and ZQ 4000 MS (Waters, USA). A Waters Symmetry C18 column (2.1 mm × 150 mm, 5-μm pore size), thermostated at 30°C, was used for the separation of antibiotics in combination with a guard column (2.1 mm × 10 mm) for protection purpose. Gradient elution was programmed with 0.2% formic acid in water (mobile phase A), methanol (mobile phase B) and acetonitrile (mobile phase C) to obtain simultaneous separation of the nine antibiotics. The mass spectrometer was equipped with an electrospray ionization (ESI) source and operated in the positive ion mode. The MS parameters were set as follows: capillary temperature 120°C, desolvation temperature 300°C, capillary voltage 3.5 kV, desolvation gas flow 300 L/hr, and cone voltage 20–30 V. For each antibiotic, the precursor ion (i.e., $[M+H]^+$) was used for quantification in the selected ion recording (SIR) mode, while one qualifier ion was used for confirmation purpose along with the retention time (Table 1). Other detailed information is available in our previous work (Ben et al., 2008). The LC-MS extracted-ion SIR chromatogram for the nine studied antibiotics prepared in the extracted reference matrix (i.e., SS) is shown in Fig. 1.

1.7 Method performance

The entire analytical procedures were meticulously checked for accuracy, precision, linearity, matrix effect and method detection limits (MDLs). The SS samples were fortified with SAs, TCs and TIA at three concentration levels (Table 5), regarding the maximum amounts like-

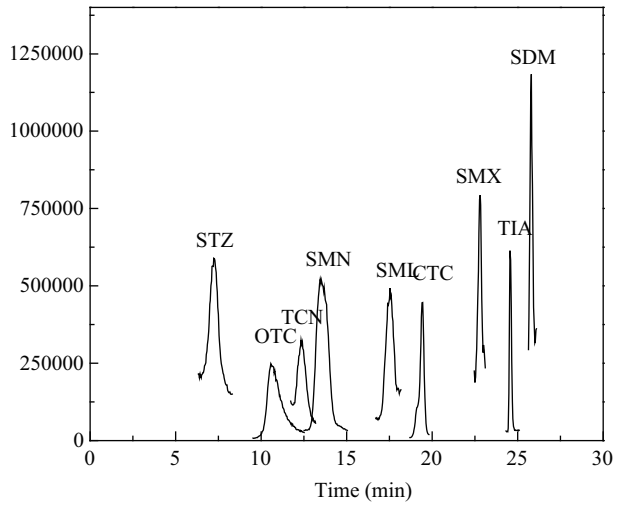

Fig. 1 Extracted-ion SIR chromatogram of selected antibiotics prepared in extracted reference matrix. The LC-MS conditions are described in Section 1.6. Spiked antibiotic concentrations: SAs 1 mg/kg, TCs 5 mg/kg, TIA 2 mg/kg.

ly to be found in solid samples. The fortified samples were extracted and analyzed through the entire procedures. Recoveries were determined by triplicate samples at each concentration level and the intra-day precision was assessed. The inter-day variation was determined by repeating the recovery experiments once a week with triplicate samples for three continuous weeks.

The MDL_{Inst} was determined by analyzing seven spiked DI water samples at an antibiotic concentration level which could approximately provide a signal-to-noise ratio (S/N) of three. Because some investigated antibiotics were usually present at a relatively high inward concentration in the reference matrix, it was almost impossible to find a reference matrix that did not contain any of the investigated antibiotics. As a result, the MDL_{SS} could not be directly determined. Instead, it was calculated from the MDL_{Inst}, the percent matrix effect (%ME) for each antibiotic, and the conversion coefficient of the extracted reference matrix to per gram of SS. The %ME was experimentally determined according to the following equation (Matuszewski et al., 2003):

$$\%ME = \frac{\text{Signal response of spiked post-Extracted reference matrix sample}}{\text{Signal response of spiked DI water sample}} \times 100$$

The spiked concentrations were 100, 500 and 200 μg/L for SAs, TCs and TIA, respectively. The value of 100%ME means no matrix interference with the antibiotic signal.

2 Results and discussion

2.1 Optimization of ultrasonic extraction

2.1.1 Extraction solvents

The extraction efficiencies of six different solvents, which were frequently applied to extracting antibiotics from solid samples, were comparatively investigated. The tested solvents included: (A) 0.2 mol/L citric acid buffer (pH 4.7); (B) 1:1 mixture of methanol and 0.2 mol/L citric acid buffer (pH 4.7); (C) McIlvaine buffer (pH 4.0); (D) 1:5 mixture of 1.0 mol/L citric acid buffer (pH 4.7) and EtOAc; (E) 85:15 mixture of buffered water (pH 9.0) and acetonitrile; (F) 1:1 mixture of methanol and acetone. The SS samples were extracted in one single step with 30 mL of each solvent under ultrasonication for 30 min. The EtOAc in solvent D, due to its water-insoluble nature, was first dried under a gentle stream of nitrogen post USE. The extract was re-dissolved with 10 mL of methanol, and then treated using the same procedures as detailed in Section 1.4.

Recovery was calculated as the percentage of the signal area of a spiked sample minus that of a blank sample in comparison to the area obtained from a corresponding standard solution prepared in DI water. Figure 2 indicates that except solvent F that failed to extract any TCs, all the other five solvents could simultaneously extract the nine antibiotics from the SS at varied degrees. Solvents A, B, C and D extracted the five SAs with a quite high recovery (51%–91%), while solvents E and F extracted the SAs much less effectively with a largely fluctuating recovery (9%–107%). In regard to TCs, only solvent A showed a quite high recovery (62%–67%), while the recoveries obtained by the other five solvents were all below 51%. The macrolide, TIA, was most effectively extracted by solvent B with a recovery of 45%, while the recoveries obtained by the other five solvents were all below 37%. In brief, solvent B was most effective for SAs and TIA, while solvent A most favored TCs. Thus, different extraction cycle combinations based on solvents A and B were examined to improve the recovery efficiency of selected antibiotics in the subsequent experiments.

2.1.2 Extraction cycle combinations

There extraction cycles were adopted as follows: (A) 30 mL of 0.2 mol/L citric acid buffer with 30 min ultrasonication; (A') 10 mL of 0.2 mol/L citric acid buffer with 10 min ultrasonication; (B) 10 mL of 1:1 mixture of methanol and 0.2 mol/L citric acid buffer with 10 min ultrasonication. Three different combinations of the above extraction cycles (i.e., A+A', A+B and A+B+B) were examined to optimize the recovery efficiency of selected antibiotics. Figure 3 shows the first combination (A+A') could notably improve the recovery of all the selected antibiotics, particularly STZ, SML, SDM and all TCs, as compared to the single-step extraction with A (Fig. 2). The second combination (A+B), which combined the relatively high recoveries of solvent A for TCs and solvent B for SAs and TIA, could increase the recovery of SAs by about 3%–30% and that of TIA by 9% in comparison to the first combination. Although the recovery of TCs was somewhat reduced by 2%–12%, it was still in the favorable range of 92%–101%. The third combination (A+B+B) increased the recoveries of STZ, SMN, TCN and CTC by 9%–14%, but yielded a relatively insignificant effect on the recovery of other antibiotics, as compared to the second combination. The addition of one more extraction cycle of B did not notably improve the recovery of selected antibiotics, implying that the extraction of antibiotics from the SS samples was controlled by distribution rather than

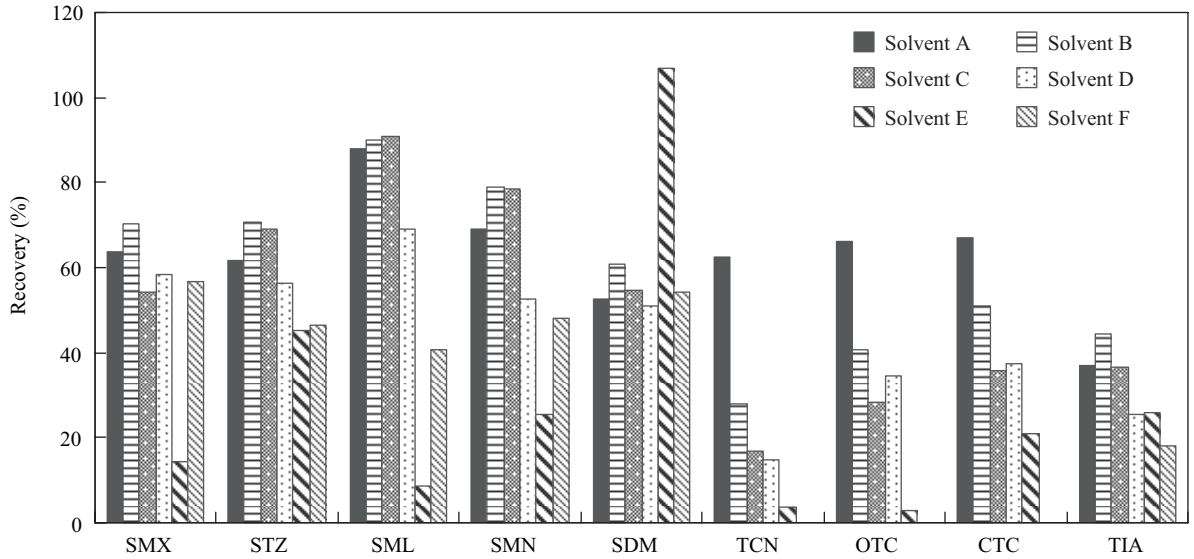

Fig. 2 Effect of different extraction solvents on the recovery of selected antibiotics. Solvents A: 0.2 mol/L citric acid buffer (pH 4.7); B: 1:1 mixture of methanol and 0.2 mol/L citric acid buffer (pH 4.7); C: McIlvaine buffer (pH 4.0); D: 1:5 mixture of 1.0 mol/L citric acid buffer (pH 4.7) and EtOAc; E: 85:15 mixture of buffered water (pH 9.0) and acetonitrile; F: 1:1 mixture of methanol and acetone. Spiked antibiotic concentrations were the same as in Fig. 1. The standard deviation for all data points was below 6% (triplicate experiments).

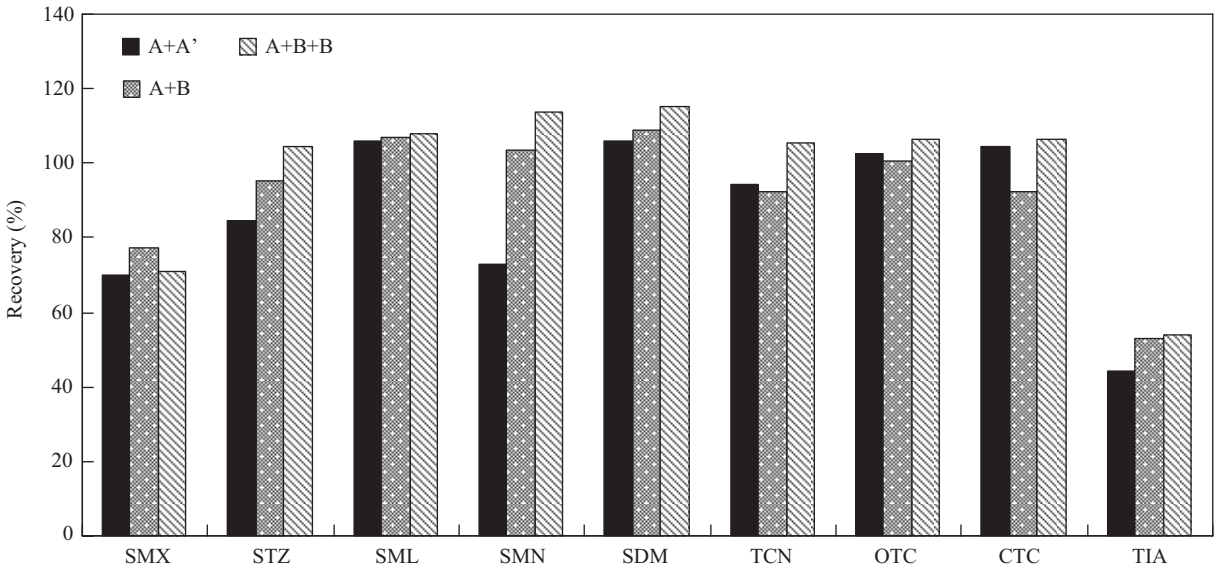

Fig. 3 Effect of different extraction cycle combinations on the recovery of selected antibiotics. Extraction cycles of A: 30 mL of 0.2 mol/L citric acid buffer with 30 min ultrasonication; A': 10 mL of 0.2 mol/L citric acid buffer with 10 min ultrasonication; B: 10 mL of 1:1 mixture of methanol and 0.2 mol/L citric acid buffer with 10 min ultrasonication. The pH of citric acid buffer was 4.7. Spiked antibiotic concentrations were the same as in Fig. 1. The standard deviation for all data points was below 7% (triplicate experiments).

desorption (Haller et al., 2002). To balance the tradeoff between the extraction recovery and solvent consumption, the second combination was selected as the optimal one, which could achieve the recovery efficiencies of 77%–108% for SAs, 92%–101% for TCs, and 53% for TIA.

It is noted that the recovery of TIA was notably lower than those of SAs and TCs. This is most likely related to the pH condition of the extraction solvent. The first and second ionization constants (i.e., $pK_{a,1}$ and $pK_{a,2}$) of the studied SAs and TCs are in the respective ranges of 1.80–2.15 and 5.30–7.78, while the weak base TIA has only one pK_a value (i.e., 7.6) (Table 1). When the extraction pH condition was governed by the citric acid buffer (pH 4.7), the SAs and TCs were mainly present in the neutral form, thus exhibiting a higher affinity for the extraction solvent (Pavlović et al., 2007). In contrast, TIA was predominately present in the cationic form (through protonating the amine moiety), and as a consequence, was extracted less effectively.

2.2 Optimization of solid-phase extraction

Many published methods used absolute methanol to elute target compounds in the SPE procedure (Löffler and Ternes, 2003; Jacobsen et al., 2004; Jacobsen and Halling-Sørensen, 2006). However, it was found that the antibiotics partially conjugated with some organic compounds present in the matrix and were washed out by methanol, leading to a notably reduced recovery. Therefore, a mixture of dichloromethane and acetone (3:2, *V*/*V*) was selected as the eluent in this work.

Table 3 Recoveries of selected antibiotics with and without SAX cartridge

ABs.	Without SAX cartridge		With SAX cartridge	
	Recovery[a] (%)	RSD[b] (%)	Recovery[a] (%)	RSD[b] (%)
SMX	83.9	5.5	92.8	2.7
STZ	77.4	10.2	75.9	8.5
SML	75.3	1.6	76.3	5.5
SMN	101.2	1.5	100.7	5.3
SDM	112.1	3.5	104.5	0.8
TCN	101.5	20.5	106.3	2.9
OTC	88.4	4.3	98.8	2.5
CTC	108.6	6.6	113.7	2.2
TIA	50.1	2.5	41.7	1.5

[a] Spiked antibiotic concentrations: SAs 1 mg/kg; TCs 5 mg/kg; TIA 2 mg/kg; [b] relative standard deviation (n = 3).

An SAX cartridge is generally applied to alleviate the matrix effect through adsorptive removal of anionic interfering compounds, thus minimizing the contamination, blocking and overloading of the subsequent HLB cartridge. At pH 4.7, the studied antibiotics were mainly present in the neutral and cationic forms and therefore would not be retained by the SAX cartridge, while the polymer-based HLB cartridge could effectively retain these antibiotics. Table 3 shows that the application of the SAX cartridge could somewhat reduce the relative standard deviations (RSD) of the recoveries of most antibiotics studied, implying that the matrix effect was suppressed to some extent. However, the SAX cartridge could not evidently improve the recoveries of SAs and TCs, but decrease the recovery of TIA from 50% to 42%. Therefore, the SAX cartridge was not adopted in this work.

2.3 Method validation

2.3.1 Standard calibration curve and linearity

External standard calibration method was employed to quantify the selected antibiotics in the SS of swine wastewater. The calibration curves of selected antibiotics included five points each and were established with a series of mixed standards prepared in DI water. The linearity ranges, slopes, y-intercepts and correlation coefficients of the standard calibration curves are listed in Table 4. Results indicate that a good linearity was achieved for the calibration curves of all the selected antibiotics, with correlation coefficients above 0.99.

2.3.2 Matrix effect and method detection limit

The percent matrix effect (i.e., %ME) for the investigated antibiotics ranged from 84% to 106% at the levels studied (Table 4). It indicates that after USE and SPE, the interfering compounds were well cleaned up. The MDL_{SS} of the developed method towards antibiotics increased with the order of TIA (0.14 μg/kg), SAs (0.23–1.84 μg/kg), and TCs (3.05–7.14 μg/kg). It is seen that the highest sensitivity was for TIA, while the lowest sensitivity was for OTC.

2.3.3 Recoveries and precisions

To investigate the recoveries of antibiotics, three concentration levels of all selected antibiotics were fortified in the reference SS. After spiking, the SS samples were shaken thoroughly to obtain an even distribution of antibiotics and then freeze-dried for 12 hr. For the blank SS sample, a same amount of DI water was spiked instead of the stock solution of antibiotics. All samples were subject to the USE and SPE extraction procedures as described above.

Table 5 shows that the recoveries of all SAs and TCs ranged from 76% to 112% at the three different spiking levels, which were within the U.S. EPA recommended range of 70%–120%. With respect to TIA, its recovery increased from 51 to 64% as the spiking level decreased from 4 to 0.2 mg/kg. Though the recovery of TIA was somewhat below 70%, its good repeatability, as reflected by the low RSD value (< 3%), made the developed method still applicable to the detection of TIA.

The intra-day precision was assessed by analyzing the triplicate reference SS samples during routine use of the developed method, and the inter-day precision was assessed by repeating the intra-day analysis once a week over three continuous weeks. Both inter- and intra-day precisions were expressed by the RSD value of repeated analyses. Table 5 shows that the intra-day repeatability and the inter-day reproducibility were all below 17% (RSD). These results were comparable to those of published methods for detection of antibiotics in solid matrices such as manure and soil, etc (Schlüsener et al., 2003; Blackwell et al., 2004; Jacobsen and Halling-Sørensen, 2006). US EPA recommends that a method be considered precise when the RSD is no more than 20%, thus the precisions in this study met the requirement for method development.

Ultimately, a highly sensitive, selective and robust method has been developed for simultaneous detection of

Table 4 Linear regression parameters and MDLs of selected antibiotics (each calibration curve includes five points)

ABs.	Standard calibration curves				MDL		
	Linearity range (μg/L)	Slope	y-intercept	r^2	MDL_{Inst} (μg/L)	%ME	MDL_{SS} (μg/kg)
SMX	10–1000	289934	–4248	0.9987	0.31	103	0.61
STZ	10–1000	195591	–3711	0.9974	0.75	93	1.60
SML	10–1000	170297	–1097	0.9998	0.85	93	1.84
SMN	10–1000	349383	–3924	0.9992	0.74	95	1.56
SDM	10–1000	592955	–4410	0.9996	0.10	84	0.23
TCN	50–5000	79760	–3651	0.9994	1.6	105	3.05
OTC	50–5000	62046	–2149	0.9997	3.4	96	7.14
CTC	50–5000	79848	–711	0.9999	2.3	106	4.26
TIA	20–2000	1014985	–4109	0.9991	0.06	89	0.14

Table 5 Recoveries and precisions of selected antibiotics spiked at three concentration levels (mg/kg)

ABs.	High level SAs: 2; TCs: 10; TIA: 4		Middle level SAs: 1; TCs: 5; TIA: 2		Low level SAs: 0.1; TCs: 0.5; TIA: 0.2		RSD[b] (%)
	Recovery (%)	RSD[a] (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	
SMX	80.4	0.6	87.9	1.5	75.7	2.0	5.5
STZ	101.9	1.2	93.9	2.7	81.3	6.6	8.7
SML	88.4	2.0	91.0	4.2	81.4	5.6	9.0
SMN	99.9	3.8	80.5	16.7	104.3	5.6	13.2
SDM	95.1	0.6	93.4	12.6	80.0	1.3	9.5
TCN	81.1	3.7	90.7	0.9	95.4	5.2	9.3
OTC	92.6	1.1	99.5	3.3	98.1	1.2	13.5
CTC	111.9	0.5	94.1	3.9	103.4	3.5	7.8
TIA	51.0	2.3	55.6	1.5	63.7	2.3	2.8

[a] intra-day repeatability (n = 3); [b] inter-day reproducibility (n = 3).
Spiked antibiotic level: SAs 1 mg/kg, TCs 5 mg/kg, TIA 2 mg/kg (middle level).

three classes of antibiotics in the SS of swine wastewater with all procedures optimized, including sample pretreatment, USE, SPE and LC-MS quantification.

2.4 Method application

This developed method was applied to measure the concentrations of selected antibiotics in the SS of swine wastewater samples collected from three typical CAFOs located in Beijing, Shanghai and Shandong Province. Besides, the antibiotic concentrations in the liquid phase of swine wastewater were also analyzed in parallel according to our previously developed method (Ben et al., 2008). Each sample was analyzed in duplicate with the standard deviation (SD) of antibiotic concentrations calculated.

Table 6 shows that all the nine antibiotics were detected in the SS and liquid phase of swine wastewater samples, exhibiting a broad concentration range. SML and SDM were only detected in Beijing sample, while STZ was only found in Shanghai sample. SMX was detected in all three samples. The concentration of individual SA ranged from 1.1 to 61.7 μg/L in the liquid phase, and from 12.4 to 2505.0 μg/kg in the SS of swine wastewater. TCs were ubiquitously detected in all the three samples, with concentrations ranging from 0.8 to 387.1 μg/L in the liquid phase and 130.6 to 36,271.3 μg/kg in the SS. TIA was also detected in both liquid phase and SS of all the three samples within a concentration range of 0.1–1.4 μg/L and 5.5–21.7 μg/kg, respectively. These data may reveal the current application status of veterinary antibiotics in the studied areas of China.

Based on the antibiotic concentrations measured in all swine wastewater samples collected from three CAFOs, the average distribution percentages of selected antibiotics between the SS and liquid phase could be readily calculated, as shown in Fig. 4. Results indicate that SAs and TIA were mainly present in the liquid phase, with a distribution percentage of 85%–99% and 94%, respectively. More TCs trended to end up in the SS than SAs and TIA. The distribution percentages of TCN, OTC and CTC in the liquid phase were 84%, 82% and 69%, respectively. Though TCs have lower $\log K_{ow}$ values than SAs (Table 1), their chemical structures facilitate the formation of complexes with mineral cations or organic matter of solid particles (Thiele-Bruhn, 2003). It is seen that the SS can not be ignored when detecting the concentrations of antibiotics in swine wastewater, particularly with respect to TCs.

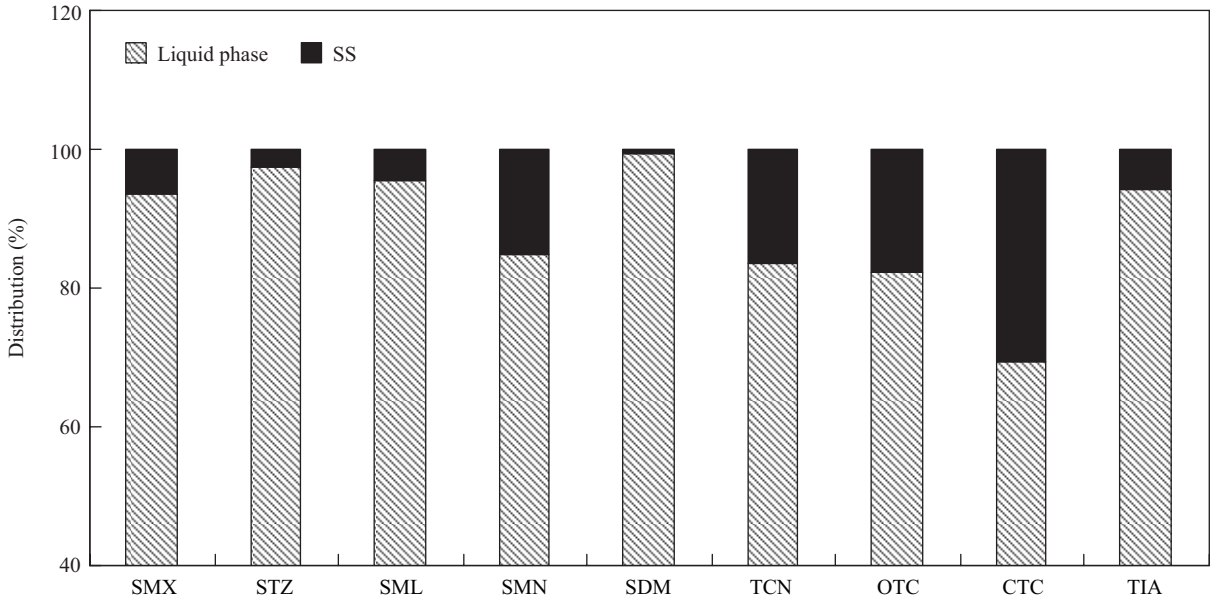

Fig. 4 Distribution percentages of selected antibiotics between the SS and liquid phase averaged over all swine wastewater samples collected from three CAFOs.

Table 6 Antibiotic concentrations detected in swine wastewaters collected from three CAFOs

ABs.	Plant 1		Plant 2		Plant 3		$\log K_d$ (mean ± SD)
	C_{SS} (μg/kg)	C_L (μg/L)	C_{SS} (μg/kg)	C_L (μg/L)	C_{SS} (μg/kg)	C_L (μg/L)	
SMX	83.5 ± 1.9	1.1 ± 0.1	151.5 ± 0.7	14.3 ± 0.1	316.5 ± 9.7	20.1 ± 0.2	1.37 ± 0.45
STZ	88.0 ± 8.3	6.9 ± 0.3	–	–	–	–	1.10
SML	–	–	214.7 ± 14.3	9.0 ± 0.2	–	–	1.38
SMN	342.4 ± 31.9	2.5 ± 0.1	2505.0 ± 77.8	61.7 ± 1.4	–	–	1.87 ± 0.38
SDM	–	–	12.4 ± 0.3	4.0 ± 0.5	–	–	0.49
TCN	3619.2 ± 170.6	41.2 ± 4.4	1610.0 ± 14.1	38.2 ± 2.9	130.6 ± 9.6	0.8 ± 0.0	1.93 ± 0.30
OTC	32044.9 ± 1394.3	387.1 ± 44.7	365.0 ± 42.4	3.5 ± 1.2	2419.4 ± 76.7	17.9 ± 0.5	2.02 ± 0.11
CTC	36271.3 ± 2216.3	138.8 ± 9.8	5180.0 ± 198.0	66.1 ± 7.2	1143.8 ± 31.1	3.0 ± 0.1	2.30 ± 0.36
TIA	12.3 ± 0.7	0.7 ± 0.2	21.7 ± 6.5	1.4 ± 0.3	5.5 ± 0.1	0.1 ± 0.0	1.41 ± 0.33

Data are presented as mean ± SD (n = 2); "–" means not detected;
Plant 1: sampled from Shanghai area in Mar 2009; Plant 2: pampled from Beijing area in Jul 2008; Plant 3: sampled from Shandong Province in Jul 2009; C_{SS}: antibiotic concentration in suspended solid; C_L: antibiotic concentration in liquid phase.

Since all the investigated antibiotics were detected in both SS and liquid phase, their partition coefficients (K_d), defined as the ratio of an antibiotic concentration in the SS (C_{SS}, μg/kg) to that in the liquid phase (C_L, μg/L) at equilibrium, were estimated. The $\log K_d$ values, averaged over all the detected field samples, were in the range of 0.49–1.87 for SAs, 1.93–2.30 for TCs, and 1.41 for TIA (Table 6). It was reported that the $\log K_d$ values of sulfadiazine ranged from 0.89 to 1.60 in the soils amended with swine manure (Sukul et al., 2008), and OTC had $\log K_d$ values ranging from 1.87 to 1.96 when partitioning between the solid and aqueous phases of swine manure (Loke et al., 2002).

3 Conclusions

In this study, a sensitive, selective and robust method for simultaneous detection of five SAs (SMX, STZ, SML, SMN and SDM), three TCs (TC, CTC and OTC), and one macrolide (TIA) in the SS of swine wastewater was developed. The systematic approach for method development was optimized including sample pretreatment, USE, SPE, and LC-MS analysis. Satisfactory recoveries were obtained for SAs and TCs. Although the recovery of TIA was comparatively lower, the good repeatability made it still applicable. The analysis of field swine wastewater samples clearly indicates that the SS can not be ignored. Hence, the establishment of detection methods for antibiotics in both SS and liquid phase of swine wastewater makes it possible for further studies on the occurrence, fate and control of antibiotics at concentrated swine feeding plants in a more comprehensive manner.

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