



Determination of pharmaceuticals in biosolids using accelerated solvent extraction and liquid chromatography/tandem mass spectrometry

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ABSTRACT

An analytical method was developed to quantitatively determine pharmaceuticals in biosolid (treated sewage sludge) from wastewater treatment plants (WWTPs). The collected biosolid samples were initially freeze dried, and grounded to obtain relatively homogenized powders. Pharmaceuticals were extracted using accelerated solvent extraction (ASE) under the optimized conditions. The optimal operation parameters, including extraction solvent, temperature, pressure, extraction time and cycles, were identified to be acetonitrile/water mixture (v/v 7:3) as extraction solvent with 3 extraction cycles (15 min for each cycle) at 100 °C and 100 bars. The extracts were cleaned up using solid-phase extraction followed by determination by liquid chromatography coupled with tandem mass spectrometry. For the 15 target pharmaceuticals commonly found in the environment, the overall method recoveries ranged from 49% to 68% for tetracyclines, 64% to 95% for sulfonamides, and 77% to 88% for other pharmaceuticals (i.e. acetaminophen, caffeine, carbamazepine, erythromycin, lincomycin and tylosin). The developed method was successfully validated and applied to the biosolid samples collected from WWTPs located in six cities in Michigan. Among the 15 target pharmaceuticals, 14 pharmaceuticals were detected in the collected biosolid samples. The average concentrations ranged from 2.6 µg/kg for lincomycin to 743.6 µg/kg for oxytetracycline. These results indicated that pharmaceuticals could survive wastewater treatment processes, and accumulate in sewage sludge and biosolids. Subsequent land application of the contaminated biosolids could lead to the dissemination of pharmaceuticals in soil and water environment, which poses potential threats to at-risk populations in the receiving ecosystems.

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1. Introduction

Administration of pharmaceuticals is the most common practice for human disease control and treatments. Large fractions of administered dosage are, in fact, not assimilated or metabolized in human body, but released into influents to municipal wastewater treatment plants (WWTPs) as either parent compounds or bioactive metabolites [1–3]. Unfortunately, currently operating WWTPs are not designed for effective removal of pharmaceuticals; as a result, a portion of pharmaceuticals could survive wastewater treatment processes, and disseminate to the environments via effluents from WWTPs in a dissolved form [4,5] or sorbed pharmaceuticals by sewage sludge [6]. Most sewage sludge is treated to produce biosolid and is subsequently applied to agricultural lands for its fertilizer value. This practice undoubtedly introduces sludge-born pharmaceuticals into soil and water environment where their

fate and risks remain largely unknown [7–12]. There is growing concern regarding the potential impacts of these pharmaceuticals on at-risk populations in the ecosystems.

The information on pharmaceutical compositions and concentrations in land-applied biosolids is critical to assessing the exposure and potential risks. Analytical methods have been developed to analyze pharmaceuticals in water [10,11,13,14], animal manure [15–18] and soil [15,19–21]. In several studies analytical methods have been also reported to identify and quantify pharmaceuticals in sewage sludge from WWTPs [10,22–26]. These methods are worthy of further examination for their applicability to multiple classes of pharmaceuticals in sewage sludge or biosolids [27]. The procedure of the analytical methodology usually consists of extraction of pharmaceuticals from sludge/biosolid, cleanup of extracts, and analysis using liquid or gas chromatography. Liquid–solid partitioning is the most common approach utilized in combination of ultrasonic-assisted extraction [15], microwave-assisted extraction [19] and accelerated solvent extraction (ASE). The extraction efficiency is dependent of sample matrices and characteristics of target analytes [28–30]. Among these three extraction tech-

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niques, ASE becomes more preferred because it provides apparent advantages such as less solvent consumption, automatic procedure for simultaneous extraction of multiple samples, short sample preparation time, and higher extraction recoveries. Solid-phase extraction (SPE) is usually selected as the cleanup tool for the sludge/biosolid extracts, and the corresponding cartridges commonly used included Waters Oasis hydrophilic–lipophilic balance (HLB), strong-anion exchange (SAX), strong cation-exchange (SCX) or their combinations [21,26,31]. The pharmaceuticals are then analyzed using liquid chromatography with fluorescence or ultraviolet detectors [25,32,33] or gas chromatography coupled to mass spectrometer [34]. Recently, liquid chromatography/tandem mass spectrometry (LC–MS/MS) became the most efficient tool for identifying and quantifying trace levels of pharmaceuticals in environmental samples [11,35–38].

The objective of this study was to develop a robust, sensitive and practical analytical method to simultaneously identify and quantify multiple classes of pharmaceuticals in biosolids. The ASE experimental parameters (i.e., extraction solvent, pressure, temperature, extraction time and cycles) were optimized to achieve the maximum extraction efficiencies for pharmaceuticals. SPE was used to pre-concentrate and clean up the ASE extracts. The amounts of pharmaceuticals were quantitated by a LC–MS/MS equipped with electrospray ionization (ESI) source. The method was validated, and applied to determine multiple classes of pharmaceuticals in biosolid samples collected from several WWTPs of different locations in Michigan, USA.

2. Experimental

2.1. Chemicals and materials

Tetracycline, demeclocycline, chlortetracycline, oxytetracycline, meclocycline, sulfadiazine, sulfamerazine, sulfamethazine, sulfamethoxazole, carbamazepine, acetaminophen, caffeine, tylosin and lincomycin were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Doxycycline was obtained from Fisher Bioreagents (Pittsburgh, PA, USA). $^{13}\text{C}_6$ -sulfamethazine was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Sime-ton was purchased from AccuStandard (New Haven, CT, USA). Methanol, sodium ethylenediaminetetraacetate (EDTA), formic acid, and sulfuric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile was purchased from EMD Chemicals (Gibbstown, NJ, USA). Waters Oasis hydrophilic–lipophilic balance (HLB) cartridge was purchased from Waters Corporation (Milford, MA, USA).

2.2. Biosolid samples

During the winter season of 2009, six grab biosolid samples were collected from WWTPs of Lansing, East Lansing, St. Clair, Plainwell, Traverse City, and Imlay City, MI, USA. The biosolids collected from WWTPs of Traverse City, Plainwell, St. Clair, Imlay City, and Lansing were classified as class B biosolids which meet the criteria for land application. The biosolids collected from East Lansing WWTP was sent off to landfills. At each sampling site, one liter of biosolid was collected, and stored in a polypropylene narrow-necked bottle. The bottles were capped, placed in an iced cooler, and transported immediately to laboratory. The samples were then freeze-dried to obtain dry solids, ground using a mortar and pestle, passed through a 0.5 mm sieve, and stored in a refrigerator (-20°C) prior to use.

2.3. Sample extraction and cleanup

The dried biosolid sample was weighed (500.0 mg) into cellulose extraction thimble, and mixed with diatomaceous earth

(5.0 g) to prevent aggregation during extraction process. $^{13}\text{C}_6$ -sulfamethazine and meclocycline were used as surrogates for sulfonamide and tetracycline antibiotics, respectively. The collected biosolid samples had been checked not to contain meclocycline. 100 μL of $^{13}\text{C}_6$ -sulfamethazine (1.0 mg/L) and 200 μL of meclocycline (1.0 mg/L) were spiked into the biosolid samples, which were then placed on a rotator and mixed overnight. The thimbles were then placed into 22 mL stainless steel extraction cells, and extracted using a Dionex ASE 200 accelerated solvent extractor (Sunnyvale, CA, USA). The extraction solvent and operating conditions had been optimized in which acetonitrile and water mixture (v/v = 7:3) was used. The samples were preheated for 5 min, and then extracted at 100°C under 100 bars. The static extraction period was set 15 min with three extraction cycles. The flushing solvent was 100% of cell volume, and extraction cells were purged with N_2 for 120 s. The extracts were collected and transferred to volumetric flasks, and diluted to 100 mL with deionized water. The pH value of the solution was adjusted to ~ 3.0 using H_2SO_4 . An aliquot of 10.0 mL of the diluted solution passed through a preconditioned HLB solid-phase cartridge (Waters Corporation, Millford, MA, USA) at a rate of 1 mL/min. The analyte-loaded HLB cartridge was rinsed with 3 mL of deionized water, and eluted by 5 mL of methanol–water mixture (v/v = 1:1) containing 150 mg/L EDTA. The elute volume was reduced to 1.0 mL using a gentle N_2 flow to concentrate the samples, which took ~ 45 min. The prepared samples were transferred to amber LC autosampler vials, and 10 ng of sime-ton (internal standard) was added prior to LC–MS/MS analysis.

2.4. Liquid chromatography and tandem mass spectrometry

The prepared samples were analyzed using a LC–MS/MS system consisting of a Shimadzu high-performance liquid chromatography (HPLC, Columbia, MD, USA) fully integrated with an Applied Biosystems Sciex 3200 triple quadrupole mass spectrometer (Foster City, CA, USA). A PEAK Scientific gas generator (NM20ZA, Billerica, MA) was used to supply gases required for the mass spectrometer. Turbo IonSpray source of the mass spectrometer was operated with electrospray ionization source in positive mode, ionspray voltage at 5500 V and temperature at 600°C . Curtain gas pressure was 10 psi, collision gas pressure was 6 psi, and ion source gas pressure was 10 psi. The compound-dependent mass spectrometer parameters were optimized for each analyte (Table 1). Pharmaceutical concentrations were quantified using multiple-reaction monitoring mode. Two pairs of precursor/product ion transitions were simultaneously monitored during analysis in order to unambiguously identify the target pharmaceuticals in environmental samples [11,39]. The pair of precursor and product 1 ions were selected to quantify the analytes because the product ion 1 manifested a relatively greater response abundance than product ion 2.

The Shimadzu HPLC system consisted of two LC-20AD pumps, a SIL-20A autosampler and a DGU-20A degasser operated by a CBM-20A controller. The analytes were separated using a Phenomenex Luna C_{18} column (150 mm \times 4.6 mm, particle size: 3 μm , Torrance, CA, USA). A binary gradient mobile phase was applied with a flow rate of 350 $\mu\text{L}/\text{min}$ in which phase A (water) and phase B (acetonitrile) both contained 0.1% (v/v) formic acid. The mobile phase gradient was programmed as: at 0 min 100% phase A; 0–2.0 min phase B linearly increased to 12%; 2–20 min phase A and phase B held at the ratio of 88:12; 20–22 min phase B linearly increased to 25%; 22–35 min phase B increased to 40%; 35–40 min phase B increased to 80%, and held at this ratio until 42 min. From 42 to 43 min phase B reduced to 0%, and equilibrated in the flow system for 5 min before next injection. The injection volume was 10 μL . After each sample injection, the first three-minute elution was diverted to waste through a built-in valve, and then switched to the inlet of tandem mass spectrometer.

Table 1
Precursor, product ions and mass spectrometer parameters used to identify and quantify pharmaceuticals.

Pharmaceuticals	Precursor ion (<i>m/z</i>)	Product ion 1 (<i>m/z</i>)	Product ion 2 (<i>m/z</i>)	DP ^a (V)	EP ^b (V)	CEP ^c (V)	CE ^d (V)	CXP ^e (V)
Sulfadiazine	250.9	108.2	156.1	37	9	30	27	36
Sulfamerazine	264.9	155.7	172.4	45	4	20	22	10
Sulfamethazine	279.0	186.1	162.0	37	5	20	23	18
Sulfamethoxazole	254.1	156.2	107.8	38	8	15	22	14
Chlortetracycline	479.2	444.2	462.2	40	4	27	27	40
Demeclocycline	465.0	448.1	430.3	36	9	30	27	36
Doxycycline	445.4	428.2	339.3	33	9	50	31	45
Oxytetracycline	461.3	426.4	444.2	28	7	22	21	41
Tetracycline	445.4	428.2	339.3	33	9	50	31	45
Acetaminophen	152.0	110.0	93.0	40	10	150	23	31
Caffeine	195.0	138.0	110.0	49	5	20	29	16
Carbamazepine	237.1	194.3	165.4	50	3	14	23	18
Erythromycin	734.6	576.4	558.4	17	11	30	32	48
Lincomycin	407.3	126.3	359.2	56	5	22	33	11
Tylosin	916.5	174.0	771.9	63	9	36	47	14

^a Declustering potential.

^b Entrance potential.

^c Cell entrance potential.

^d Collision energy.

^e Collision cell exit potential.

2.5. Method validation

External standard solutions were prepared in methanol–water mixture (*v/v* = 1/1) containing 150 mg/L EDTA. The same background solution was also used to elute analytes from the HLB cartridge during the cleanup step of sample extracts. Standard curves were established with the concentrations spanning 2 orders of magnitude. The method sensitivity was determined by analyzing pharmaceuticals at ~20 µg/L for 10 times using the ASE extracts fortified with target compounds, and standard deviation of these measurements was calculated. Limit of detection (LOD) was estimated as three times the standard deviation, and limit of quantification (LOQ) was defined as ten times the standard deviation. To evaluate extraction efficiency and repeatability of the overall method, the selected target pharmaceuticals and two surrogates (i.e., ¹³C₆-sulfamethazine and meclocyline) were spiked into biosolid samples, and mixed together overnight. Eight replicates of spiked biosolid samples were prepared followed by accelerating solvent extraction, SPE cleanup and LC–MS/MS analysis (described above). Biosolid control samples without spiked pharmaceuticals were also prepared; the amounts of pharmaceuticals measured in the control were subtracted from the amounts measured in the spiked samples. The sample matrix effects on the responses of tandem mass spectrometer was evaluated via spiking known amounts of target pharmaceuticals into the extract of biosolid which was obtained by ASE followed by the SPE cleanup. The measured pharmaceutical concentrations in the spiked samples were subtracted by the background pharmaceutical amounts originally present in the biosolids, and compared with the amounts of the spiked analytes.

3. Results and discussion

3.1. Selection of extraction solvent

The biosolid sample collected from Lansing WWTP was fortified with carbamazepine, sulfamethazine, sulfamethoxazole, tetracycline, chlortetracycline and oxytetracycline with concentrations ranging from 780 to 1862 µg/kg on dry matter basis, and subjected to ASE using water and organic solvent mixtures. The extracted concentrations of pharmaceuticals were subtracted by the measured concentrations of the corresponding compounds in the unfortified biosolid sample, and reported in Table 2. Extraction solvents used here were aqueous methanol or acetonitrile mixtures with volume

ratios of organic solvent to water 7:3, 5:5 and 3:7. Several previous studies indicated that polar organic solvents (e.g. acetonitrile, methanol) and water mixtures manifested the superior capability to extract pharmaceuticals from sewage sludge and soil [17,26,35]. Among the extraction solvents tested, it appeared that higher extraction efficiencies were achieved for the mixtures containing greater contents of organic solvent (Table 2). Acetonitrile/water mixture (*v/v* = 7:3) was more effective to remove pharmaceuticals from biosolids than methanol/water mixture. Therefore, acetonitrile/water mixture (*v/v* = 7:3) was chosen as the extraction solvent in this study. To evaluate the influence of pH on extraction efficiency, pH of acetonitrile/water mixture (*v/v* = 7:3) was adjusted to ~2.0 using hydrochloric acid, and then extracted the selected pharmaceuticals from the biosolid. The use of acidified extraction solvent, in fact, lowered extraction efficiency by 36% for tetracycline, 42% for chlortetracycline, and 75% for oxytetracycline compared to unacidified acetonitrile/water mixture; no apparent discrepancy was observed for extraction of carbamazepine and sulfonamides. Several previous studies indicated that acidified water–organic solvent mixture (e.g., pH < 2.5) enhanced extraction recoveries of pharmaceuticals, but the target pharmaceuticals were not the same as those in this study [25,26]. The acidification of extraction solvent protonates the acidic functional groups (e.g., carboxylic acids and phenols) in the organic fractions of sewage sludge/biosolids, and thereby reducing electrostatic interactions between sewage sludge/biosolid and cationic moiety of tetracyclines. In these previous studies, sand was used to mix with sewage sludge or soil samples prior to extraction [25,26,40]. Diatomaceous earth, used as the dispersant of biosolid in our study, could release a certain amount of inorganic cations (e.g., Na⁺, Mg²⁺, etc.) which could effectively suppress the interactions of biosolid with cationic speciation of tetracyclines hence resulting in a higher extraction efficiency.

3.2. Optimization of ASE operating parameters

High pressure is applied during ASE operation in order to keep extraction solvent in liquid state while temperature is held at or above the boiling point. The application of high pressure also facilitates the contact of extraction solvent with analytes via penetrating into the domains in biosolids where the extraction solvent could not enter under normal atmospheric pressure. In this study four levels of pressure (55, 80, 100, and 130 bars) were applied to the extraction, and the corresponding extracted concentrations are reported in Table 3. The pharmaceutical concentrations at different extrac-

Table 2Extracted concentrations of pharmaceuticals vs. spiked concentrations from biosolid by water–organic solvent mixtures using accelerating solvent extraction^a.

Pharmaceuticals	Spiked conc. (µg/kg)	Extraction solvents						
		Acetonitrile/water (7:3)	Acetonitrile/water (7:3), pH 2.0	Acetonitrile/water (5:5)	Acetonitrile/water (3:7)	Methanol/water (7:3)	Methanol/water (5:5)	Methanol/water (3:7)
Sulfamethazine	982	947 ± 21 ^b	920 ± 31	551 ± 25	393 ± 11	758 ± 24	475 ± 23	368 ± 28
Sulfamethoxazole	780	564 ± 29	710 ± 13	494 ± 17	266 ± 28	541 ± 27	459 ± 20	339 ± 17
Chlortetracycline	1512	786 ± 18	456 ± 5	540 ± 17	306 ± 23	708 ± 20	525 ± 24	373 ± 22
Oxytetracycline	1862	1008 ± 25	261 ± 21	627 ± 24	308 ± 12	776 ± 14	655 ± 10	360 ± 10
Tetracycline	1518	1065 ± 28	681 ± 76	538 ± 4	282 ± 39	628 ± 36	359 ± 20	302 ± 35
Carbamazepine	840	700 ± 32	705 ± 53	480 ± 34	272 ± 19	437 ± 27	260 ± 18	214 ± 15

^a Operation conditions: 100 bars, 100 °C, 3 × 15 min.^b measured concentration: mean ± standard deviation (n = 3).**Table 3**

Extraction efficiency of pharmaceuticals under varying accelerating solvent extraction parameters.

Pharmaceuticals	Spiked conc. (µg/kg)	Pressurized liquid extraction conditions										
		Pressure (bar)				Extraction time (min)				Temperature (°C)		
		55	80	100	130	2 × 15	3 × 15	2 × 25	3 × 25	50	75	100
Sulfadiazine	466	270 ± 6	246 ± 6	293 ± 13	243 ± 16	192 ± 12	264 ± 28	193 ± 18	250 ± 27	300 ± 23	342 ± 18	338 ± 24
Sulfamerazine	414	249 ± 10	222 ± 17	289 ± 7	242 ± 15	200 ± 28	269 ± 22	188 ± 23	240 ± 15	277 ± 14	326 ± 33	308 ± 29
Sulfamethazine	491	437 ± 12	385 ± 11	456 ± 11	396 ± 26	439 ± 24	453 ± 25	420 ± 10	444 ± 35	380 ± 19	422 ± 26	446 ± 32
Sulfamethoxazole	390	274 ± 17	256 ± 33	279 ± 11	264 ± 26	203 ± 34	285 ± 27	174 ± 12	312 ± 22	243 ± 11	248 ± 15	266 ± 24
Chlortetracycline	756	413 ± 22	488 ± 14	434 ± 29	365 ± 20	393 ± 44	397 ± 19	401 ± 52	408 ± 60	579 ± 42	556 ± 20	572 ± 48
Demeclocycline	507	316 ± 10	151 ± 8	304 ± 11	300 ± 12	193 ± 29	311 ± 43	193 ± 23	272 ± 51	323 ± 19	313 ± 40	404 ± 32
Doxycycline	509	335 ± 7	266 ± 5	355 ± 12	328 ± 6	335 ± 26	342 ± 36	367 ± 26	298 ± 42	266 ± 24	328 ± 32	348 ± 45
Oxytetracycline	931	496 ± 43	483 ± 54	523 ± 60	580 ± 20	457 ± 41	491 ± 52	438 ± 30	505 ± 21	503 ± 52	614 ± 51	604 ± 85
Tetracycline	759	398 ± 52	315 ± 37	475 ± 50	340 ± 49	377 ± 30	394 ± 24	466 ± 45	432 ± 38	431 ± 42	456 ± 46	481 ± 35
Acetaminophen	788	617 ± 21	579 ± 12	761 ± 32	724 ± 10	432 ± 30	592 ± 35	464 ± 34	583 ± 69	648 ± 43	571 ± 26	653 ± 39
Caffeine	391	406 ± 8	397 ± 12	309 ± 10	308 ± 10	259 ± 24	315 ± 12	248 ± 24	318 ± 11	289 ± 31	299 ± 17	333 ± 26
Carbamazepine	420	377 ± 29	365 ± 38	389 ± 45	347 ± 28	281 ± 25	365 ± 26	321 ± 14	349 ± 28	371 ± 36	384 ± 14	378 ± 13
Erythromycin	636	231 ± 22	266 ± 28	408 ± 28	358 ± 26	432 ± 28	541 ± 35	450 ± 20	509 ± 26	394 ± 38	474 ± 48	498 ± 53
Lincomycin	538	424 ± 11	480 ± 21	449 ± 22	385 ± 9	356 ± 15	382 ± 10	347 ± 15	385 ± 23	377 ± 38	388 ± 35	406 ± 39
Tylosin	484	363 ± 14	359 ± 26	365 ± 25	277 ± 14	366 ± 22	407 ± 54	371 ± 33	403 ± 19	380 ± 36	396 ± 27	428 ± 21

tion pressures were compared using a linear-mixed model (SAS 9.1 software, SAS Institute Inc., Cary, NC, USA). Significant level was set at $p < 0.05$ with 95% confidence. The results indicate that the extraction efficiencies at pressure of 100 bars were significantly higher than those conducted at other pressures ($p = 0.0049$). This extraction pressure falls within the similar range reported in several previous studies [25,26].

Static extraction with 2 or 3 consecutive cycles was conducted at 100 °C and under 100 bars. Two periods of the static extraction time (15 min vs. 25 min) were tested for extraction efficiency (Table 3). In general, prolonging extraction time and more extraction cycles achieve greater extraction efficiency [41,42]. For the static time of 15 min vs. 25 min with the same numbers of extraction cycles, the analysis of the linear-fixed model results indicated no significant difference ($p = 0.897$) of extraction efficiencies. This suggests that 15 min of static period is sufficient for the analytes to approach partition equilibrium between biosolid and solvent. However, increase of consecutive extraction cycles from 2 to 3 significantly enhanced the extraction efficiencies for all pharmaceuticals tested ($p = 0.0002$). In each extraction cycle the induction of fresh solvent into the sample cell reestablishes new partition equilibrium for the analytes between the added solvent and the biosolid residues. This partitioning process drives the release of biosolid-sorbed pharmaceuticals to extraction solvent phase. Taken together, the observation of enhanced extraction efficiencies of 3 consecutive cycles (vs. 2 cycles), and no apparent difference with prolonged static time (25 min vs. 15 min), warrant that the optimal operative condition consists of 3 extraction cycles with 15 min static time in each cycle.

Increasing temperature in ASE operation resulted in enhanced extraction of the pharmaceuticals from biosolid (Table 3). The extraction temperature was tested at 50, 75, and 100 °C with the pressure of 100 bars, 3 extraction cycles and static period of 15 min in each cycle. The extraction efficiencies were observed to significantly increase with increasing temperature ($p < 0.0001$). Therefore, higher temperature is preferred in the ASE operation. However, considering thermal stability of analytes, the extraction was not conducted at temperature > 100 °C. In this study, the temperature of 100 °C was selected as optimal operating condition. The increase of temperature enhances dissolution of pharmaceuticals in extraction solvent and hence increasing extraction efficiencies. At the same time, the viscosity of extraction solvent and surface tension on biosolid surfaces decrease as temperature increases, which also facilitates the solvent to access the domains where the analytes are present. In addition, the enhanced extraction efficiency could be due to accelerating mass transfer rate from sample matrices to solvent at increased temperature.

3.3. Method validation

The linear ranges of standard curves and correlation coefficients, along with method LOD and LOQ values, are reported in Table 4. The standard curves were prepared with two order of magnitude, and demonstrated an excellent linearity with the correlation coefficients (r^2) > 0.999 . The described method was tested for simultaneous extraction and determination of 15 pharmaceuticals in biosolid, which manifested varying levels of LOD and LOQ. For the class of sulfonamides the LOD ranged from 0.6 $\mu\text{g}/\text{kg}$ (for sulfamethazine) to 15.0 $\mu\text{g}/\text{kg}$ (for sulfadiazine) on dry weight basis of biosolid. This method was less sensitive for tetracycline antibiotics as shown with the LOD ranging from 4.6 $\mu\text{g}/\text{kg}$ (for doxycycline) to 146 $\mu\text{g}/\text{kg}$ (for tetracycline). The overall method recoveries reported were the average of eight replicates of spiked samples ranging from 49.3% to 94.6%. The standard deviations of the recoveries ($n = 8$) were $< 10\%$ indicating the reported method achieved satisfactory repeatability. It was noted that tetracycline antibiotics manifested the relatively lower recoveries ranging from 49% to 68% (Table 4). This range of recoveries fell within the low end of the recovery range (i.e., 47.2–125%) reported by Jacobsen and Halling-Sorensen [17] in which tetracyclines were analyzed in swine manure using the similar analytical approach. However, the extraction recovery of tetracycline (53.8%) measured in this study is significantly greater than (~27%) reported by Lillenberg et al. [26]. The overall recoveries for sulfonamides (from 63.6% to 94.6%) are comparable with the results in several previous studies [17,26,37]. For instance, sulfamethazine manifested the highest recovery (94.6%) in this study; the similar results were also reported for extraction of sulfamethazine from swine manure (84.1% to 97.8%) [17], and from sewage sludge (104%) [37].

The manifestation of relatively low recoveries (e.g. ~50%) of the overall method for a certain class of pharmaceuticals such as tetracyclines could be attributed to inefficient extraction of ASE operation, analyte loss during SPE cleanup, and/or matrix effects on mass spectrometer responses from extracts. The loss of target analytes during SPE cleanup step was $< 11\%$ (describe below). To examine sample matrix effects on mass spectrometer responses, standard addition method was used in which the analytes were spiked into biosolid extracts after ASE treatment and SPE cleanup. The spiked pharmaceutical concentrations were tested at two levels: 10–23 $\mu\text{g}/\text{L}$ and 100–232 $\mu\text{g}/\text{L}$. The ratios of measured concentrations to the corresponding spiked concentrations ranged from 0.90 to 1.15, indicating matrix effects caused a minimal impact to the overall method recovery. Therefore, the low recoveries measured in this study result primarily from inefficient extraction of

Table 4
Linear range and correlation coefficient (r^2) of standard curves, method limit of detection (LOD), limit of quantification (LOQ), and recovery.

Pharmaceuticals	Linear range ($\mu\text{g}/\text{L}$)	r^2	LOD ($\mu\text{g}/\text{kg}$)	LOQ ($\mu\text{g}/\text{kg}$)	Recovery (%) ($n = 8$)
Sulfadiazine	5.8–580	0.9992	15.0	50.6	63.6 \pm 5.2
Sulfamerazine	5.2–518	0.9998	5.5	18.2	71.4 \pm 5.3
Sulfamethazine	6.1–614	0.9999	0.6	1.9	94.6 \pm 2.7
Sulfamethoxazole	4.9–488	0.9997	1.0	3.3	77.6 \pm 8.0
Chlortetracycline	9.4–945	0.9995	37.2	124	49.3 \pm 5.4
Demeclocycline	6.3–634	0.9996	25.9	86.3	55.0 \pm 5.0
Doxycycline	6.4–636	0.9993	4.6	15.5	68.3 \pm 1.1
Oxytetracycline	11.6–1160	0.9997	13.7	45.8	52.4 \pm 5.3
Tetracycline	9.5–1960	0.9997	146	488	53.8 \pm 8.7
Acetaminophen	9.8–986	0.9994	30.7	102	84.7 \pm 10
Caffeine	4.9–488	0.9999	8.4	28.3	79.8 \pm 3.8
Carbamazepine	5.2–525	0.9997	2.9	9.7	88.1 \pm 3.1
Erythromycin	8.0–795	0.9993	6.6	21.8	79.0 \pm 9.8
Lincomycin	6.7–673	0.9999	0.8	2.7	83.6 \pm 9.9
Tylosin	6.0–605	0.9992	5.4	18.1	77.3 \pm 5.4

Table 5
Recoveries of solid-phase extraction for pharmaceuticals spiked into biosolid extracts after accelerating solvent extraction.

Pharmaceuticals	Spiked concentration ($\mu\text{g/L}$)	Recovery (%) ($n=3$)	Spiked concentration ($\mu\text{g/L}$)	Recovery (%) ($n=3$)
Sulfadiazine	11.6	110.9 \pm 9.8	116.4	102.6 \pm 8.0
Sulfamerazine	10.4	86.8 \pm 6.5	103.5	102.5 \pm 8.3
Sulfamethazine	12.3	91.0 \pm 8.5	122.8	100.9 \pm 6.4
Sulfamethoxazole	9.8	115.2 \pm 8.6	97.5	97.7 \pm 7.1
Chlortetracycline	18.9	95.6 \pm 7.5	189.0	89.1 \pm 9.4
Demeclocycline	12.7	155.5 \pm 16.5	126.8	118.1 \pm 14.2
Doxycycline	18.9	116.9 \pm 9.1	189.8	111.4 \pm 11.3
Oxytetracycline	23.3	98.4 \pm 6.1	232.7	121.1 \pm 10.9
Tetracycline	12.7	108.5 \pm 12.8	127.2	119.4 \pm 9.0
Acetaminophen	19.7	102.1 \pm 8.2	197.1	113.5 \pm 8.9
Caffeine	9.8	103.7 \pm 4.6	97.7	110.0 \pm 5.2
Carbamazepine	10.5	100.5 \pm 8.5	105.0	101.4 \pm 8.0
Erythromycin	15.9	112.4 \pm 11.1	159.0	107.3 \pm 8.1
Lincomycin	13.5	129.2 \pm 13.4	134.6	176.5 \pm 18.9
Tylosin	12.1	98.2 \pm 7.5	120.9	96.8 \pm 8.8

pharmaceuticals from ASE procedure, particularly for the class of tetracycline antibiotics which usually demonstrate a great affinity with sorbents.

3.4. SPE cleanup

In order to evaluate the extent of pharmaceutical loss during SPE cleanup step, the target compounds were spiked into biosolid extracts after ASE treatment. The solution passed through the preconditioned HLB cartridge, followed by an elution with methanol–water mixture ($v/v=1:1$) containing 150 mg/L EDTA and analyzed by LC–MS/MS. Two concentration levels (10–23 $\mu\text{g/L}$ and 98–232 $\mu\text{g/L}$) of pharmaceuticals were spiked into the biosolid extracts (i.e., after ASE treatment). The measured pharmaceutical concentrations were subtracted by the amount of pharmaceuticals originally present in the samples, and compared to the spiked concentrations (Table 5). For most pharmaceuticals, the recoveries of spiked amount ranged from 86.8% to 120%, indicating that the loss of analytes during SPE cleanup step was minimal. Demeclocycline and lincomycin manifested relatively higher recovery (i.e., 155% for demeclocycline at 12.7 $\mu\text{g/L}$, and 176% for lincomycin at 134.6 $\mu\text{g/L}$), which was attributed to the experimental variations since reasonable recoveries were obtained for these two compounds at another concentration. Therefore, the relatively low recoveries of the overall method for some analytes (e.g., tetracyclines) could be attributed to the inefficient extraction from biosolids during the ASE step.

3.5. Application to biosolid samples

Biosolid samples were collected at WWTPs of six cities (Lansing, East Lansing, St Clair, Plainwell, Traverse City, and Imlay City) in Michigan, USA. These samples were extracted using the optimized method described above. The samples were conducted in triplicates. The measured concentrations and the corresponding standard deviations ($n=3$) are presented in Table 6. Among these samples, 14 out of the 15 target pharmaceuticals were found except tylosin. The average concentrations of the pharmaceuticals manifested a relatively wide range from 2.6 to 743.6 $\mu\text{g/kg}$ (Table 6). The calculated standard deviations for all the measurements were <17%, indicating a reasonable repeatability of the analysis. Tetracycline antibiotics were frequently detected with the concentrations ranging from 36.6 to 743.6 $\mu\text{g/kg}$. Doxycycline was detected in all 6 biosolid samples with concentrations ranging from 159.9 to 292.4 $\mu\text{g/kg}$. Relatively higher concentrations of tetracycline (281.9 $\mu\text{g/kg}$) and chlortetracycline (346.6 $\mu\text{g/kg}$) were found in the sample of Lansing WWTP compared to the samples collected from other five WWTPs. Oxytetracycline was detected in four biosolid samples from 51.9 to 743.6 $\mu\text{g/kg}$. Trace amounts of demeclocycline were found in three biosolid samples (i.e., 36.6, 98.2 and 131.2 $\mu\text{g/kg}$) from WWTPs in Plainwell, East Lansing, and St. Clair. Sulfonamides were also frequently detected, and the corresponding concentrations ranged from 4.8 (sulfamethoxazole) to 668.9 $\mu\text{g/kg}$ (sulfamerazine). For other six pharmaceuticals (acetaminophen, carbamazepine, caffeine, erythromycin, lincomycin and tylosin), the measured concentrations spanned from 2.6 to 370.4 $\mu\text{g/kg}$. Caf-

Table 6
Measured pharmaceutical concentrations and standard deviations ($\mu\text{g/kg}$ dry matter) in the collected biosolid samples.

Pharmaceuticals	Sampled wastewater treatment plants					
	Lansing	East Lansing	St. Clair	Plainwell	Traverse City	Imlay City
Sulfadiazine	nd ^a	nd	nd	nd	562.2 \pm 61.4	nd
Sulfamerazine	112.0 \pm 6.3	668.9 \pm 45.5	nd	nd	nd	nd
Sulfamethazine	nd	127.8 \pm 15.8	124.8 \pm 11.8	nd	nd	131.8 \pm 13.0
Sulfamethoxazole	4.8 \pm 0.5	26.1 \pm 3.0	8.8 \pm 1.2	35.9 \pm 4.4	nd	nd
Chlortetracycline	346.6 \pm 22.1	nd	nd	nd	90.2 \pm 10.0 ^b	69.6 \pm 7.6 ^b
Demeclocycline	nd	98.2 \pm 6.2	131.2 \pm 11.3	36.6 \pm 4.0 ^b	nd	nd
Doxycycline	291.2 \pm 22.0	224.3 \pm 30.8	149.6 \pm 11.0	292.4 \pm 48.5	234.0 \pm 26.8	159.9 \pm 19.1
Oxytetracycline	nd	51.9 \pm 4.4	nd	743.6 \pm 21.7	201.4 \pm 17.8	174.2 \pm 4.6
Tetracycline	281.9 \pm 42.3 ^b	nd	nd	nd	nd	nd
Acetaminophen	370.4 \pm 12.9	101.1 \pm 4.7	88.6 \pm 7.6 ^b	nd	nd	nd
Caffeine	47.9 \pm 5.2	47.1 \pm 6.1	46.9 \pm 3.7	75.5 \pm 4.1	33.9 \pm 3.7	nd
Carbamazepine	6.2 \pm 1.2 ^b	4.6 \pm 0.5 ^b	5.1 \pm 0.3 ^b	16.4 \pm 0.9	22.3 \pm 5.9	nd
Erythromycin	62.8 \pm 5.8	nd	10.4 \pm 0.8	16.8 \pm 1.3	nd	nd
Lincomycin	nd	6.2 \pm 1.1	8.7 \pm 1.4	nd	6.5 \pm 2.0	2.6 \pm 0.2
Tylosin	nd	nd	nd	nd	nd	nd

^a Not detected.

^b Detected but less than limit of quantification.

feine and carbamazepine, both of which are solely used by humans, were detected in the biosolids from five cities except Imlay City. Interestingly, Imlay City has the least populations among the six cities; this might be the reason responsible for the least occurrence of human medicine in the biosolid of Imlay City WWTP [43]. Tylosin is commonly used as veterinary pharmaceutical to control livestock infection and promote livestock growth. It is reasonable that no samples were found to contain tylosin in municipal WWTP biosolids. Acetaminophen was detected in three biosolid samples from Lansing, East Lansing and St. Clair with concentrations of 88.6–370.4 $\mu\text{g}/\text{kg}$. In fact, these three cities have more populations than Plainwell, Traverse City, and Imlay City.

4. Conclusions

This study describes an efficient and reproducible analytical approach for simultaneous determination of multiple classes of pharmaceuticals in biosolids. The experimental steps consisted of accelerated solvent extraction of freeze-dry biosolid sample, and cleanup by solid-phase extraction followed by simultaneous analysis by liquid chromatography/tandem mass spectrometry. The extraction parameters of ASE including extraction solvent, temperature, pressure, static period and extraction cycle were optimized to achieve satisfactory extraction efficiency. Overall, this method offers less laborious work, automatic extraction procedure, less consumption of organic solvent and relatively high extraction efficiency. The method has been tested successfully for simultaneously determining multiple classes of pharmaceuticals in WWTP biosolids collected from different locations. The results also suggest that many pharmaceuticals could survive wastewater treatment processes and accumulate in solid phase (e.g., biosolids). Land application of the contaminated biosolids could result in the dissemination of pharmaceuticals in soil and water environment. Chronic exposure to low levels of antibiotics might exert selection pressure on development of antibiotic resistant bacterial strains in the environment.

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