JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Simultaneous Analysis of Free and Conjugated Estrogens, Sulfonamides, and Tetracyclines in Runoff Water and Soils Using Solid-Phase Extraction and Liquid Chromatography−Tandem Mass Spectrometry

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ABSTRACT: The ability to monitor multiple analytes from various classes of compounds in a single analysis can increase throughput and reduce cost when compared to traditional methods of analyses. This method for analyzing free (parent estrogen) and conjugated estrogens (metabolites) along with sulfonamides and tetracyclines utilizes a high pH (10.4) mobile phase with an ammonium hydroxide buffer for both positive- and negative-mode electrospray ionization. A single-step sample preparation by solid-phase extraction (SPE) was used to isolate and concentrate all analytes simultaneously. The analytical method was developed and validated for recoveries at 3 concentration levels for water and soil and produced recoveries of 42−123% and 21−105% respectively. Method detection limits ranged from 0.3 to 1.0 ng/L for water samples and 0.01 to 0.1 ng/g for soils. The method quantification limit ranged from 0.9 to 3.3 ng/L for water samples and 0.06 to 0.7 ng/g for soils. The single-point standard addition calibration procedure was validated across a linear range of MQL to 100 ng/L with ≥82% accuracy against a matrix matched standard curve. Furthermore, sorption of tetracyclines onto glassware was investigated and minimized by 10% using nitric acid-rinsed glassware, while separation parameters were further optimized based on retention time and signal responses. This method has been used for the quantification of estrogens, tetracyclines, and sulfonamides in soil and runoff waters with multiple compounds detected simultaneously in a single analysis.

KEYWORDS: tetracyclines, sulfonamides, estrogens, conjugated, steroids, wrong-way-round ionization, liquid chromatography, mass spectrometry, LC−MS/MS, hormones, antibiotics, metabolites

INTRODUCTION

The widespread use of antibiotics in livestock for growth promotion, in addition to disease prevention, has caused concerns due to the link between antibiotic use and proliferation of resistant bacteria in the environment.[1] As a matter of fact, nontherapeutic usage (as a growth promoter) of antibiotics accounts for the majority of usage in the agricultural industry and is about 90% higher than therapeutic use.[2] This has prompted many organizations such as the World Health Organization (WHO) and U.S. Food and Drug Administration (FDA) to issue recommendations or evaluations that nontherapeutic usages should be reduced.[3,4] One of the major concerns is the increased emergence of resistant pathogenic bacteria due to selective pressure, which in turn may affect humans through direct contact or via consumption of contaminated food and water.[5] In addition to the resistant bacteria, the antibiotic resistance genes (ARG) themselves have been shown to be persistent in the environment and to have the ability to grow into new cells even after the original host has died.[1] Therefore, many studies consider the ARG, which are closely linked to antibiotic usage, as a contaminant itself. Recent studies by Storteboom et al. using the molecular signature of sulfonamide and tetracycline ARG have suggested that ARG arise from discrete sources like wastewater treatment plants or animal feeding operations where elevated concentrations of antibiotics can persist.[6,7] This concern led European countries, such as Denmark, to invoke a voluntary ban on the use of antibiotics for nontherapeutic usage in 1999, which has resulted in a 60% drop in the overall usage of antibiotics in Denmark.[8]

The release of naturally occurring hormones into the environment through land application of manure is also a concern. The most potent class of steroidal hormones from livestock is the excreted natural estrogens and their metabolites. Estrogens are especially potent in aqueous environments, where sexual dependence of newly hatched salmon are highly dependent on the estrogen concentrations in water.[9] The ecotoxicity of estrogens in the environment has been well documented, and includes reports on feminization of male fish in streams exposed to poultry runoff,[10] and increased levels of vitellogenin in female turtles due to exposure to as low as 0.05 ng/L estrogens.[11] Furthermore, conjugated metabolites of estrogens, which are more abundant than the free estrogens (parent estrogens), are also a concern, despite being nonestrogenic when released into the environment, because these polar estrogen conjugates are more readily transported and may be transformed back into their parent estrogenic forms.[12]

Received: November 11, 2010
Accepted: January 31, 2011
Revised: January 29, 2011
Published: February 22, 2011

dx.doi.org/10.1021/jf104355x | *J. Agric. Food Chem.* 2011, 59, 2213–2222

Application of animal manure, such as poultry litter, to fertilize croplands is a common agricultural practice. Poultry litters are known to contain antibiotics and naturally excreted estrogens.[13,14] In order to truly understand the ecological risks of manure-borne hormones and antibiotics it is critical to determine the long-term fate, transport, and potency of these chemicals on a watershed scale. To accomplish such investigations, a reliable, fast, and cost-effective analytical method that can simultaneously determine multiple compounds of interest is needed.

The analysis of free and conjugated estrogens, sulfonamides, and tetracyclines in the environment has traditionally required multiple analyses.[15−17] Typically, free and conjugated estrogens are detected by liquid chromatography with tandem mass spectrometry (LC−MS/MS) in negative mode electrospray ionization (−ESI).[18,19] To improve selectivity and sensitivity, some researchers have used +ESI LC−MS/MS or gas chromatography/mass spectrometry after deconjugation of estrogen metabolites, followed by derivatization.[12,20,21] For analysis of antibiotics, such as sulfonamides and tetracyclines, separate extraction and LC−MS/MS analysis are required. Not only are these analytical approaches tedious and costly, but more importantly, the integrity of unstable analytes can be compromised due to long analysis times.

The main objective of this study was to alleviate the limitations of current analytical techniques for estrogen and antibiotic analysis, which require two or more separate sample preparation and LC−MS/MS methods. We developed and optimized a strategy for sample preparation and analysis that can simultaneously detect and quantify 28 steroidal hormones, antibiotics, and their metabolites in runoff water and soil samples. This robust method has been fully validated for the analysis of selected hormones and members of the sulfonamide and tetracycline classes of antibiotics. This paper presents data that demonstrate the benefits of performing LC−MS/MS under high pH mobile phase, and the implications of analyte sorption onto glassware. Finally, the use of single-point standard addition to alleviate ionization interferences and sorption effects, for increased accuracy of analysis, is highlighted and discussed.

MATERIALS AND METHODS

Chemicals and Reagents. 17α-Ethinylestradiol (EE2), 17β-estradiol (17β-E2), 17α-estradiol (17α-E2), sulfamethazine (SMZ), sulfadimethoxine (SDM), sulfameter (SMT), sulfamethizole (SMI), sulfamerazine (SMR), sulfachloropyridazine (SCP), sulfadiazine (SPD), sulfamethoxazole (SMX), tetracycline (TC), and oxytetracyline (OTC) were obtained from Sigma-Aldrich (St. Louis, MO). Estrone (E1) was acquired from TCI (Tokyo, Japan), and 4-epitetracycline (ETC) was obtained from Spectrum Chemical Manufacturing Corporation (Gardena, CA). 17β-Estriol (E3) and sulfathiazole (STZ) were obtained from ICN Biomedicals Inc. (Aurora, OH). Conjugated estrogen metabolites, estrone-3-sulfate (E1-3S), estrone-3-glucuronide (E1-3G), 17α-ethinylestradiol-3-glucuronide (EE2-3G), 17β-estradiol-3-sulfate (17β-E2-3S), 17α-estradiol-3-sulfate (17α-E2-3S), 17β-estradiol-17-sulfate (E2-17S), and 17β-estradiol-3-glucuronide (E2-3G) were obtained from Steraloids Inc. (Newport, RI). Anhydrotetracycline hydrochloride (ATC), anhydrochlorotectracycline hydrochloride (ACTC), 4-epichlorotetracycline hydrochloride (ECTC), and chlorotetracycline hydrochloride (CTC) were obtained through Acros Organics (Morris Plains, NJ). Stable isotopes of 17β-estadiol-3-glucuronide-16,16,17-d_3 (E2-3G-d_3), 17β-estradiol-3-sulfate-2,4,16,16-d_4 (E2-3S-d_4), estrone-3-sulfate-2,4,16,16-d_4 (E1-3S-d_4), estrone-2,4,16,16-d_4 (E1-d_4), and 17β-estradiol-16,16,17-d_3 (E2-d_3) were obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec). Stable isotope of sulfamethoxazole-d_4 (SMX-d_4) was acquired from Toronto Research Chemicals, Inc. (North York, Ontario).

Methanol, acetonitrile, and ethyl acetate were of LC−MS and HPLC grade and obtained through Burdick & Jackson (Morristown, NJ). Glacial acetic acid, sulfuric acid, nitric acid, and ammonium hydroxide were of ACS grade and obtained through J.T. Baker (Phillipsburg, NJ). Disodium ethylenediamine tetraacetate (EDTA) was obtained from Fisher Chemical (Fairlawn, NJ). Sodium chloride was purchased from EM Science (Gibbstown, NJ). Hydromatrix used in soil extractions were acquired from Varian (Palo Alto, CA). NANOpure™ water was used in all experiments from Barnstead International (Dubuque, IA). Oasis hydrophilic−lipophilic balance (HLB) SPE 500 mg and 6 mL cartridges were purchased through Waters (Mildford, MA).

Glassware Treatment. All glassware used were treated with a 10% nitric acid bath for 8 h, then rinsed with water, and baked at 250 °C to reduce adsorption of tetracyclines onto glassware.

Standard Preparation and Stability. Primary standard solutions of analytes were prepared at 1 mg/mL concentrations in methanol and stored at −40 °C. An exception was SPD, due to its limited solubility stock standards were prepared in 1% ammonium hydroxide in methanol. From the primary standard solutions, 3 individual calibration standard solutions at 10 μg/mL were prepared (e.g., estrogens, sulfonamides, and tetracyclines). Stability of primary and calibration solutions were tested against freshly prepared standards. Estrogen solutions are stable for at least 1 year. Sulfonamides solutions are stable for at least 2 months. Tetracycline solutions are stable for at least 3 months.

Spiking standards used for standard addition spiking were prepared each day from calibration standard solutions stored at −40 °C at 500 ng/mL. Quality control standards were prepared at 10 ng/mL from the spiking solution in water/methanol (95/5, v/v) with 0.1% glacial acetic acid to check for precision throughout an analysis.

Aqueous Extraction and Cleanup. A 0.5 L aqueous sample was collected in an amber glass bottle and acidified to pH of 2 ± 0.2 using sulfuric acid. The aqueous sample was filtered sequentially through 1.5 and 0.7 μm Whatman glass filters (Clifton, NJ) and spiked with 100 μL of surrogate standards (250 ng/mL) of SMX-d_4, E2-3G-d_3, 17β-E2-3S-d_4, E1-3S-d_4, 17β-E2-d_3, and E1-d_4 and immediately stored at 4 °C. Prior to extraction, 12.5% EDTA was added to the 0.5 L aqueous sample to obtain 0.1% EDTA in the sample. The pH was adjusted to 4 with either sulfuric acid or ammonium hydroxide, which was based on the study from Pailler et al.[17] showing that pH 4 is ideal for all compounds. The sample was then loaded onto an Oasis HLB SPE cartridge for the extraction of all analytes. The HLB SPE was conditioned using 6 mL of methanol followed by 10 mL of 0.1% EDTA water. The sample was loaded onto the HLB SPE cartridge at approximately 5 to 10 mL/min. After loading, the HLB SPE cartridge was rinsed with 10 mL of water/methanol (95/5, v/v) and allowed to dry under vacuum for approximately 30 min.

The first elution using 10 mL of an ethyl acetate/methanol (9/1, v/v) mixture was collected in an amber vial. Then, the SPE cartridge was washed sequentially with 10 mL of acid-wash solution (5% methanol with 2% acetic acid by volume), and 10 mL of base-wash solution (5% methanol with 2% ammonium hydroxide by volume), which were discarded. The HLB SPE cartridges were then dried by maintaining the vacuum for approximately 30 min. The remaining analytes in the SPE cartridge were eluted with a second solvent consisting of 10 mL of methanol + 2% ammonium hydroxide; this elution was collected in a separate vial. The collected extracts were evaporated to less than 1 mL under a stream of nitrogen at 30 °C. The two separate extracts were combined into a graduated tube and evaporated to approximately 0.2 mL. The combined extract was then brought to a 1 mL volume with

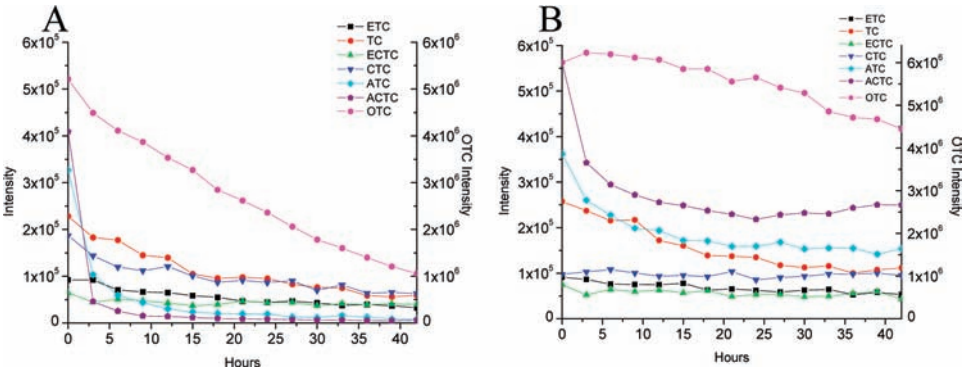

Figure 1. Sorption of 7 different tetracyclines tested at 3 h intervals over 42 h: (A) glassware with no treatment and (B) glassware treated with 10% nitric acid.

water/acetonitrile (95/5, v/v) + 0.1% acetic acid solution and vortexed. An aliquot of 0.4 mL was pipeted and spiked with 20 μL of 500 ng/mL spiking solution. This spiked sample was used as the single-point standard addition for quantification of the sample. The samples (nonspiked and spiked) were then centrifuged at 7000*g* for 5 min to remove any particles from the extract. Both aliquots (nonspiked and spiked) were analyzed by LC−MS/MS. A water blank was prepared using only water as the sample and treated identically to the samples.

Soil Extraction and Cleanup. Soils were extracted using a Dionex 200 (Sunnyvale, CA) accelerated solvent extraction (ASE) system. A 5 ± 0.5 g sample of soil was weighed and spiked with 100 μL of surrogate standards (250 ng/mL) and allowed to air-dry for approximately 30 min. The spiked soil was mixed with hydromatrix and loaded into a 33 mL stainless steel ASE tube sandwiched between hydromatrix to occupy dead volume in the tube. The soil method uses the capabilities of ASE to pressurize each tube to 100 bar at room temperature, hold 10 min, flush volume of 60%, and purge volume of 60% for extraction of all analytes. Each soil extraction was performed for 2 cycles using water/methanol/acetone (50/25/25, v/v/v) containing 25 mM EDTA, 2% ammonium hydroxide, and 0.6 M sodium chloride. The extract from ASE was diluted with enough water to reduce the organic strength to less than 5% by volume in the solution. The solution was adjusted to a pH of 4 with sulfuric acid. Samples were then treated identically to the aqueous sample procedure as described in the previous section. A soil blank was prepared with hydromatrix in lieu of soil and treated identically to samples.

LC−MS/MS. Analysis of estrogens, sulfonamides, tetracyclines, and their metabolites was performed using an Agilent 6410 triple quadrupole MS equipped with an 1100 HPLC system (Palo Alto, CA). A 10 μL aliquot was used for sample injection. Separation was achieved on a Thermo Scientific (Fullerton, CA) Betabasic C_{18} 2.1 × 100 mm, 3 μm particle size column, at a flow rate of 200 μL/min. A guard column with the same packing material as the analytical column was used. The mobile phase consisted of (A) water/methanol (96/4, v/v) with 5 mM ammonium hydroxide and (B) water/methanol/acetonitrile (10/10/80, v/v/v) with 5 mM ammonium hydroxide. The gradient profile consisted of 100% mobile phase (A) held for 2 min isocratically, then ramped to 30% B within 5 min, followed by a 2 min hold at 30% B, then a ramp to 100% B within 10 min, and holding at 100% B for 3 min. Finally, the mobile phase was returned back to 100% A within 1 min. The total run time for each injection was 30 min.

Ionization was achieved through positive and negative ESI with a spray voltage of 4 kV, situated at a 90° angle to the entrance. The initial 10.5 min of the LC−MS/MS run was under +ESI, and thereafter, all ionization was performed in the −ESI mode. The highest sensitivity was achieved when using a drying gas temperature of 350 °C, a nebulizer pressure (N_2) of 22 psi, and drying gas (N_2) of 11 L/min. All analytes were monitored using two product ions in multiple reaction monitoring (MRM). The fragmentor and collision voltages were tuned for each

Table 1. Percent Accuracy ± Standard Deviation of Single-Point Standard Addition Compared to a Matrix Matched Calibration Curve in Surface Water Matrix (n = 2)

analyte	MQL	30 ng/L	100 ng/L
	Sulfonamides		
SPD	87% ± 7%	91% ± 7%	99% ± 6%
SMT	83% ± 2%	101% ± 1%	93% ± 11%
SMX	95% ± 3%	99% ± 1%	99% ± 5%
SMR	93% ± 1%	99% ± 1%	96% ± 10%
STZ	100% ± 3%	90% ± 3%	92% ± 3%
SMI	118% ± 6%	84% ± 8%	83% ± 3%
SCP	88% ± 8%	97% ± 4%	96% ± 9%
SDM	84% ± 5%	95% ± 1%	101% ± 0%
SMZ	84% ± 2%	99% ± 0%	98% ± 5%
	Tetracyclines		
OTC	88% ± 22%	91% ± 0%	84% ± 3%
ATC	110% ± 5%	90% ± 1%	84% ± 12%
ETC	110% ± 2%	96% ± 3%	78% ± 4%
TC	105% ± 2%	101% ± 2%	106% ± 5%
ECTC	91% ± 3%	100% ± 0%	82% ± 8%
CTC	100% ± 1%	98% ± 1%	84% ± 5%
ACTC	102% ± 2%	86% ± 1%	104% ± 3%
	Estrogens		
E2-3G	89% ± 3%	102% ± 3%	89% ± 2%
E1-3G	82% ± 3%	101% ± 0%	92% ± 4%
EE2-3G	105% ± 0%	104% ± 1%	100% ± 1%
E2-17S	108% ± 7%	109% ± 1%	102% ± 2%
17β-E2-3S	82% ± 9%	108% ± 1%	105% ± 2%
17α-E2-3S	112% ± 1%	112% ± 2%	101% ± 1%
E1-3S	109% ± 5%	113% ± 1%	114% ± 0%
E3	102% ± 2%	101% ± 2%	93% ± 5%
17β-E2	105% ± 6%	106% ± 2%	106% ± 2%
17α-E2	94% ± 9%	101% ± 2%	92% ± 4%
EE2	105% ± 7%	113% ± 12%	89% ± 14%
E1	84% ± 0%	106% ± 1%	93% ± 0%

*Percent accuracy =100 − [(single point concentration − matrix-matched concentration)/matrix-matched calibration curve concentration × 100].

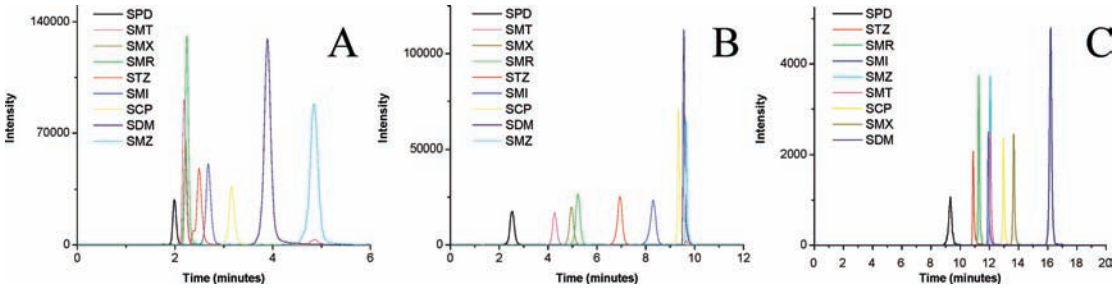

Figure 2. Shifts in sulfonamide retention times by varying the pH of the mobile phase: (A) pH at 10.4, (B) pH at 9.5, and (C) pH 4.5.

specific analyte to achieve the optimum signal intensity as described previously by Tso et al.[22] Data collection and analysis were performed using Agilent Technologies MassHunter Software Version B (Palo Alto, CA).

Data Analysis. Quantification of hormones and antibiotics in water or soil was performed by single-point standard addition using the following equation:[23]

$$\frac{C_{sam}}{C_{std} + dC_{sam}} = \frac{S_{sam}}{S_{std+sam}}$$

where

C_{sam} is the unknown concentration of analyte
C_{std} is the known concentration of analyte added
d is the dilution factor made by adding standard
S_{sam} is the signal intensity without the addition of standard, and
$S_{std+sam}$ is the signal intensity with standard addition

Method Performance Parameters. The method quantification limit (MQL) and method detection limit (MDL) were determined for soil and water by following published procedures by Gros et al.[16] The MDL and MQL were defined as the minimum detectable amount of analyte with signal-to-noise (S/N) ratios of 3 and 10, respectively. All compounds were spiked ($n = 3$) into soil or water and taken through the entire preparation procedures described in previous sections. Spike recoveries were performed using 500 mL of surface water collected in Tonawanda Creek (Amherst, NY) to represent an environmental water matrix previously described by Batt et al.[24] and 5 g of University at Buffalo's Greenhouse soil to represent a soil matrix which has been previously described by O'Connor et al.[25]

Quality Assurance Parameters. Blank injections were made at the beginning, at the end, and before each quality control standard to check for carry over. Quality control standards were injected after 10 samples, and percent recovery should be within ±20% from the beginning of the analysis. Surrogate spikes were only used to monitor for recoveries in all samples.

RESULTS AND DISCUSSION

Minimizing Tetracycline Sorption to Glassware. The sorption of tetracyclines onto glassware was a concern due to the ability for the tetracyclines to interact with silanols[26] or interact with organic or metal contaminants. This is especially relevant when large sets of samples are extracted, which require longer processing time, hence longer contact time of analytes with glassware. In order to minimize tetracycline sorption, a 10% nitric acid in water bath was used to rinse and remove any organic or metals, thus reducing the interactions between the tetracyclines and the glass surface by stripping all free metals ions and organics off of the surface of the glass.

Water samples were collected in acid rinsed glassware and pH was adjusted to 2 to minimize microbial activity. Prior to any SPE loading, EDTA was added to water samples as a chelating agent to prevent tetracyclines from interacting with metal ions.[16,27−29] Gros et al.[16] showed that, without addition of EDTA, the tetracyclines would interact with the metal ions causing a significant drop in recoveries. The decrease in tetracycline recoveries were mainly due to the tetracyclines interacting with free metal ions that could be present in the sample or glassware, thus greatly reducing the interaction between the HLB sorbent.[16] In this study, the pH of the samples was maintained at 4 because it was the optimal pH for recovery of tetracyclines without affecting recoveries for other analytes,[17] and EDTA was added at a final concentration of 2.7 mM.

A mixture of standard solutions of all analytes at 10 ng/mL was prepared and immediately injected (within 5 min) in 3 h intervals. The comparison of treated glassware versus no treatment revealed differences from the initial injection (based on signal intensity) as early as the first 3 h interval injection with decreases ranging from 0 to 89% (no treatment) and 0−39% (treated) as shown in Figure 1. After an extended time study of 42 h the decrease in tetracycline signals ranged from 38 to 97% (no treatment) and 2 to 57% (treatment). Despite the glassware pretreatment with 10% nitric acid, a decrease was still observed over 42 h, but the rate of sorption was minimized when compared to no treatment. Minimizing the rate of sorption allows a longer waiting period for samples in a queue, between the time the extracts have been prepared in the vials and the time the samples are injected in the LC−MS/MS. For sulfonamides and estrogens some loss was observed over 42 h, but with either treatment or no treatment of glassware the differences between treatments were still within ±13% (data not shown).

Quantification by Single-Point Standard Addition. Validation of the quantification method of single-point standard addition using 50 ng/L was performed by spiking surface water extract samples with the analyte mixture at 3 levels of concentrations: equivalent to the MQL for each analyte, 30 ng/L, and 100 ng/L. As presented in Table 1 all analytes can be accurately determined using the 50 ng/L single-point standard addition The single-point standard addition technique eliminates bias associated with using a surrogate matrix calibration curve because quantification and calibration are performed on the identical sample, and thus this procedure decreases overall analysis time.[30,31] While the use of a matrix-matched calibration curve is ideal for quantification, a single-point standard addition is more cost-effective and is an efficient technique because of the reduced number of injections.

The ability to eliminate matrix interferences by standard addition is quite apparent. However, standard addition also

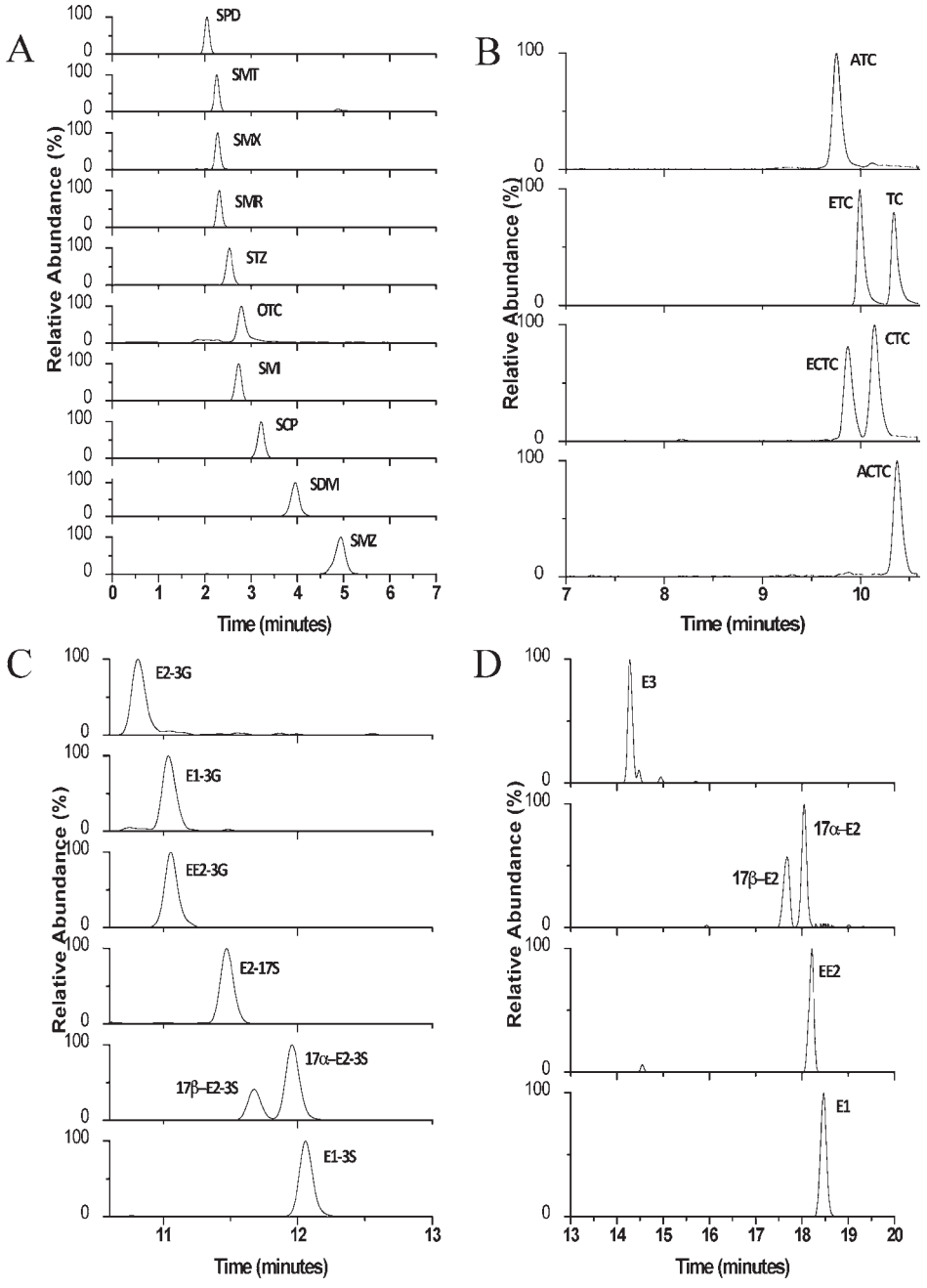

Figure 3. MRM chromatogram of 10 ng/mL standard spiked into surface water: (A) sulfonamides, (B) tetracyclines, (C) conjugated estrogens and (D) free estrogens demonstrate separation and detection in ±ESI using a high pH mobile phase.

compensates for loss in signal intensity due to sorption onto the glassware, which was most evident with tetracyclines. For instance, even with treated glassware, the highest loss observed for ACTC was 57% relative to the first injection. This loss would not have been corrected for, and would have led to inaccurate concentrations if quantification was based on an external calibration curve. In performing the standard addition technique, standards are directly spiked into the sample solution immediately after preparation (within 5 min apart), therefore the analyte and the standard added have nearly equal exposure time for sorption. Therefore, loss in the spiked standard due to sorption mimics the loss of the analyte in the sample, therefore minimizing the discrepancy created from sorption to glassware.

Water Cleanup and Solid Phase Extraction Development. During sample extraction with HLB, large amounts of matrix components, such as humic substances, are also retained. Therefore, either analytes need to be selectively eluted from the cartridge or the matrix needs to be selectively washed off of the sorbent prior to analyte elution, to avoid matrix interferences in the LC−MS/MS. The initial SPE rinse with water/methanol (95/5, v/v) ensured all salts and weakly retained matrix components were washed off the cartridge. Then, the first elution solvent using 10 mL of ethyl acetate/methanol (9/1, v/v) mixture permitted the effective elution of free estrogens, sulfonamides, and tetracyclines. The high composition of a nonpolar solvent such as ethyl acetate allowed for minimal coelution of

Table 2. MDL and MQL Levels Determined in 0.5 L of Surface Water or 5 g of Soil

analyte	water MDL (ng/L)	water MQL (ng/L)	water MQL (n = 3) % RSD	soil MDL (ng/g)	soil MQL (ng/g)	soil MQL (n = 3) % RSD
			Sulfonamides			
SPD	0.4	1.2	6	0.01	0.06	8
SMT	0.3	1.0	15	0.01	0.06	19
SMX	0.3	1.0	6	0.01	0.06	21
SMR	0.3	1.0	10	0.01	0.06	13
STZ	0.3	1.0	8	0.01	0.06	11
SMI	0.3	1.0	10	0.01	0.06	24
SCP	0.3	1.0	4	0.01	0.06	22
SDM	0.3	1.0	4	0.01	0.06	6
SMZ	0.3	1.0	4	0.01	0.06	7
			Tetracyclines			
OTC	0.9	3.0	5	0.1	0.6	10
ATC	0.9	3.0	5	0.1	0.6	11
ETC	0.9	3.2	8	0.1	0.6	8
TC	0.9	3.0	7	0.1	0.6	4
ECTC	0.9	3.1	3	0.1	0.6	8
CTC	0.9	2.9	6	0.1	0.6	8
ACTC	1.0	3.3	13	0.1	0.7	12
			Estrogens			
E2-3G	0.3	0.9	8	0.04	0.2	4
E1-3G	0.3	1.0	4	0.04	0.2	11
EE2-3G	0.3	1.0	9	0.04	0.2	19
E2-17S	0.3	1.1	4	0.04	0.2	8
17β-E2-3S	0.3	0.9	4	0.04	0.2	10
17α-E2-3S	0.3	1.0	8	0.04	0.2	7
E1-3S	0.3	1.0	8	0.04	0.2	4
E3	0.4	1.2	14	0.08	0.5	11
17β-E2	0.4	1.2	8	0.08	0.3	5
17α-E2	0.3	1.0	3	0.08	0.5	8
EE2	0.4	1.2	7	0.08	0.3	22
E1	0.3	1.0	8	0.04	0.2	16

matrix components. The chemical nature of humic substances, which contain functional groups such as carboxylic or phenolic acids, renders the humic substances to be more polar and acidic, and less likely to be eluted from the SPE sorbent using nonpolar solvents.[32] The observed colorless nature of the initial eluate and the dark brown (matrix) color of the HLB sorbent after elution provide visual evidence that much of the humic substances in the matrix was retained in the SPE cartridge.

The subsequent washings with 10 mL of acid-wash (containing 5% methanol and 2% acetic acid, by volume) followed by a 10 mL basic-wash (containing 5% methanol and 2% ammonium hydroxide, by volume) to rinse the SPE sorbent allowed for the removal of basic and acidic matrix interferences, respectively, with negligible losses of the analytes. The remaining estrogen conjugates in the HLB sorbent were eluted using a 2% ammonium hydroxide in methanol solution and collected in a separate vial.

Soil Extraction and Sample Cleanup. Soil samples present an added challenge in analysis because more matrix interferences are associated with soils. More importantly, analytes of interest may be sorbed strongly onto soil, requiring harsh extraction conditions that may lead to undesirable coextraction of matrix components. The tetracyclines are particularly notorious as reported in many studies showing greater than 96% sorption of TC and CTC[33] in soil, and variable extraction recoveries from soil depending on soil properties.[34] Therefore, isolating analytes of interest and reducing the amount of coextracted matrix were the main goals in this study. The extraction solvent used in ASE is a solution of water/methanol/acetone (50/25/25, v/v/v) with 25 mM EDTA, 0.6 M sodium chloride, and 2% ammonium hydroxide. The water and methanol are sufficiently polar solvents to extract the metabolites of interest. The acetone allows for the extraction of relatively nonpolar free estrogens. The 2% ammonium hydroxide minimizes electrostatic interactions between antibiotics and soil due to cation-exchange mechanisms that are favored under acidic conditions.[34−36] The EDTA complexes with metals that would otherwise interact with tetracyclines, and reduce extraction recoveries. After collection of the extraction solution from the ASE, a dilution to less than 5% organic solvent is needed to prevent early breakthrough of analytes from the HLB.

LC−MS/MS Method Development. The analysis of sulfonamides and tetracyclines required +ESI, which was achieved during the first 10.5 min of the LC−MS/MS run. The ionization

Table 3. Low, Medium, and High SPE Percent Recovery ± Relative Standard Deviation (n = 3) of Spiked Analytes in Surface Water and Soil

analyte	water % recovery MQL	water % recovery 30 ng/L	water % recovery 100 ng/L	soil % recovery MQL	soil % recovery 30 ng/g	soil % recovery 100 ng/g
			Sulfonamides			
SPD	88 ± 3	86 ± 5	100 ± 3	59 ± 11	68 ± 16	79 ± 21
SMT	95 ± 12	72 ± 12	93 ± 4	63 ± 12	90 ± 15	95 ± 14
SMX	104 ± 6	76 ± 16	97 ± 5	84 ± 13	85 ± 11	83 ± 15
SMR	74 ± 14	85 ± 7	102 ± 9	61 ± 5	83 ± 16	83 ± 10
STZ	116 ± 3	83 ± 4	81 ± 5	93 ± 6	77 ± 19	69 ± 1
SMI	87 ± 7	86 ± 9	79 ± 5	59 ± 12	70 ± 4	73 ± 16
SCP	100 ± 3	94 ± 18	88 ± 4	68 ± 7	107 ± 14	99 ± 18
SDM	84 ± 3	83 ± 13	86 ± 5	54 ± 9	62 ± 14	53 ± 9
SMZ	82 ± 1	83 ± 8	85 ± 1	60 ± 8	66 ± 12	66 ± 13
			Tetracyclines			
OTC	111 ± 14	65 ± 5	62 ± 3	115 ± 9	53 ± 11	56 ± 9
ATC	44 ± 11	68 ± 5	60 ± 5	30 ± 6	41 ± 14	40 ± 18
ETC	42 ± 16	48 ± 2	52 ± 10	33 ± 12	32 ± 3	23 ± 4
TC	85 ± 8	91 ± 6	67 ± 5	57 ± 11	54 ± 13	46 ± 15
ECTC	49 ± 5	66 ± 9	47 ± 6	24 ± 15	26 ± 11	25 ± 19
CTC	92 ± 7	68 ± 16	72 ± 10	45 ± 9	33 ± 19	40 ± 14
ACTC	48 ± 5	65 ± 19	36 ± 7	22 ± 10	21 ± 9	28 ± 16
			Estrogens			
E2-3G	99 ± 11	101 ± 11	86 ± 2	84 ± 6	70 ± 15	64 ± 4
E1-3G	107 ± 7	105 ± 6	89 ± 4	83 ± 4	75 ± 15	66 ± 9
EE2-3G	107 ± 6	96 ± 7	86 ± 4	60 ± 7	69 ± 12	68 ± 5
E2-17S	86 ± 6	106 ± 4	99 ± 1	81 ± 7	67 ± 13	71 ± 7
17β-E2-3S	77 ± 6	88 ± 11	92 ± 6	71 ± 1	65 ± 8	69 ± 12
17α-E2-3S	72 ± 2	87 ± 6	99 ± 1	64 ± 4	70 ± 13	71 ± 6
E1-3S	94 ± 8	87 ± 4	102 ± 2	65 ± 5	74 ± 14	74 ± 3
E3	123 ± 13	114 ± 7	88 ± 6	70 ± 13	67 ± 5	65 ± 6
17β-E2	98 ± 11	101 ± 3	85 ± 3	58 ± 24	55 ± 9	62 ± 12
17α-E2	89 ± 8	108 ± 6	88 ± 12	54 ± 3	56 ± 8	56 ± 18
EE2	107 ± 10	93 ± 18	85 ± 14	105 ± 23	63 ± 8	67 ± 16
E1	88 ± 14	92 ± 12	85 ± 5	59 ± 13	60 ± 16	61 ± 18

preference for all sulfonamides and tetracyclines would be in the positive mode due to lower detection limits.[37] The method for ionization of sulfonamides and tetracyclines is considered as wrong-way-round ionization because the mobile phase is highly basic (pH 10.4) and provides adequate protonation of the antibiotics. In this method, wrong-way-round ionization is defined as the ability to observe $[M + H]^+$ from strongly basic solution and was previously described in detail by Tso et al.[22,38] After all the sulfonamides and tetracyclines have eluted, the polarity was switched to −ESI to allow analysis of estrogens and their conjugates. The switching to −ESI was necessary because estrogens have higher ionization efficiencies in negative mode LC−MS/MS[18] providing lower detection limits. The development of an LC−MS/MS method using −ESI at a high pH to facilitate deprotonation of conjugated metabolites and free estrogens was also reported previously by Tso et al.[19] For the simultaneous analysis of estrogens, sulfonamides, and tetracyclines, it was critical to design the mobile phase gradient so that the estrogens and their conjugates elute after 10.5 min, after all sulfonamides and tetracyclines have eluted.

The retention times of sulfonamides were highly sensitive to changes in the mobile phase pH. The effect of pH on sulfonamides is demonstrated in Figure 2. Starting at a high pH of 10.4 (Figure 2A), it can be seen that a slight change to pH 9.5 mobile phase (Figure 2B) results in dramatic changes in the capacity factors (k') of sulfonamides. Similarly, addition of acetic acid to adjust the mobile phase to pH 4.5 (Figure 2C) resulted in large changes in k' of sulfonamides. On the contrary, these changes in pH resulted in minimal changes in the k' for tetracyclines and estrogens (data not shown) that would effect polarity switching. The sensitivity of the k' for sulfonamides with pH changes has important implications in the LC−MS/MS method because it was critical to have sufficient retention and separation of sulfonamides in the basic mobile phase that is necessary to ionize estrogens. On the other hand, it was necessary to have the sulfonamides elute before 10.5 min to allow the instrument to switch from +ESI to −ESI. This critical balance was achieved using a mobile phase at pH of 9.5, which provided sufficient k' to retain sulfonamides in the column. The last eluting sulfonamide (SMZ) eluted at approximately 1 min before the first estrogen, allowing sufficient time for switching polarities from +ESI to −ESI. However, it was

Table 4. Analysis of Litter/Soil and Runoff Water and Recovery of Spiked Surrogate Standards in Soil and Runoff Water

(A) Average ± Standard Deviation for Analysis of Litter/Soil (ng/g) and Runoff (ng/L) Water (n = 2)

sample type	OTC	ATC	ETC	TC	ECTC	CTC	SMZ	E1
litter	65 ± 2	ND[a]	ND	ND	ND	ND	0.4 ± 0.2	41 ± 10
field soil A	9 ± 2	<MQL[b]	4 ± 4	10 ± 8	ND	ND	ND	2 ± 1
field soil B	ND	7 ± 5	ND	ND	ND	ND	ND	12 ± 2
field soil C	9 ± 4	ND	ND	ND	ND	ND	ND	2 ± 0
runoff A	ND	ND	ND	ND	1 ± 1	6 ± 0	ND	<MQL
runoff B	ND	ND	ND	ND	<MQL	ND	ND	58 ± 32
runoff C[c]	ND	ND	ND	ND	ND	ND	ND	13

sample type	E2-17S	17β-E2-3S	17α-E2-3S	E1-3S	E3	17β-E2	17α-E2
litter	75 ± 24	3 ± 3	4 ± 2	27 ± 11	9 ± 1	6 ± 1	3 ± 1
field soil A	3 ± 0	<MQL	0.6 ± 0.1	<MQL	0.7 ± 0	<MQL	<MQL
field soil B	26 ± 8	ND	ND	<MQL	ND	ND	ND
field soil C	ND	ND	ND	0.2 ± 0	ND	ND	ND
runoff A	214 ± 142	1.08 ± 0.98	<MQL	<MQL	ND	ND	ND
runoff B	ND	16 ± 8	17 ± 9	23 ± 10	ND	ND	12 ± 6
runoff C[c]	28	1	1	2	ND	2	1

(B) Recovery of Spiked Surrogate Standards (n = 2) in Soil at 5 ng/g and Runoff Water at 50 ng/L

sample type	SMX-d_4	DMC	E2-3G-d_3	E2-3S-d_4	E1-3S-d_4	E2-d_3	E1-d_4
litter	63 ± 3	49 ± 4	62 ± 1	58 ± 8	57 ± 2	48 ± 7	46 ± 5
field soil A	67 ± 4	47 ± 6	76 ± 7	67 ± 6	64 ± 5	42 ± 1	42 ± 11
field soil B	60 ± 14	41 ± 14	71 ± 19	68 ± 22	55 ± 16	40 ± 14	44 ± 19
field soil C	62 ± 4	34 ± 1	71 ± 3	74 ± 2	52 ± 0	55 ± 9	49 ± 3
runoff A	61 ± 15	67 ± 4	89 ± 18	86 ± 15	81 ± 8	99 ± 15	96 ± 10
runoff B	82 ± 8	62 ± 4	97 ± 7	80 ± 10	78 ± 10	64 ± 19	56 ± 12
runoff C[c]	57	46	86	74	62	59	67

[a] Not detected. [b] <MQL = less than method quantification limit. [c] Only 1 sample was available.

discovered that any addition of an acidic modifier would reduce the signal intensities of the estrogens. This suppression resulted in a decrease in signal intensities, from 10- to 100-fold for the estrogens, compared to mobile phases containing no acid. The addition of acidic modifiers like acetic or formic acid is known to cause extreme signal suppression effects by affecting the conductivity, surface-active electrolytes, and ion pairing of cations and anions for estrogens.[39−41]

Therefore, to adequately retain and separate the sulfonamides a mobile phase with no acidic modifier was used (pH 10.4) and a sample solution of water/acetonitrile (95/5, v/v) with 0.1% acetic acid was used instead. The acidic condition in the sample solution allows sufficient retention of the sulfonamides upon injection onto the column while the stronger organic solvent, acetonitrile, allowed for better separation than methanol. This mismatch between sample solutions and initial mobile phase composition allowed for sufficient retention of the most polar sulfonamide (SPD) beyond the void time, while still eluting all sulfonamides and tetracyclines before 10.5 min (Figure 3A,B) prior to the polarity switching to −ESI used for the estrogens (Figure 3C,D). The signal suppression effects observed in the mobile phase when acetic acid was used were not a significant source of suppression when included into the sample solution. This is most likely because only 10 μL of a 0.1% solution is introduced into the system.

All sulfonamides, tetracyclines, and estrogens were analyzed by LC−MS/MS using two product ion transitions, which were used as identification points along with the retention time reproducibility criteria of ±1%. Furthermore, the increase in peak height after standard addition provided additional confirmation of analyte identities for quality control. The highest product ion signals were used for quantification. The relative standard deviation (RSD) of all quality control standards throughout each sequence analysis was less than 20%.

Method Performance Parameters. The MDL and MQL for each target analyte in water and soil samples are reported in Table 2. The reproducibility (n = 3) at MQL was determined by percent relative standard deviation (% RSD) for water (3−14%) and soil (4−24%), which are also reported in Table 2. In Table 3, the SPE recoveries of spiked analytes in water and soil samples at low (MQL), medium (30 ng/L), and high (100 ng/L) levels along with relative standard deviation are presented. The recoveries in water samples for sulfonamides and estrogens were all higher than 70%, while the tetracyclines ranged from 36 to 111%. The recoveries of the parent tetracyclines (OTC, TC, and CTC) were generally above 60%. In general, tetracyclines are known to undergo transformations in various environments, resulting in poor recoveries.[34] The poor recoveries can be attributed to the ability of tetracyclines to interconvert into their epimers based on pH conditions, interaction with free metals and natural organic matter in the matrix, and sorption onto glassware, which can all contribute to the low recoveries in the sample extraction.[42] The recoveries in spiked soil samples were lower relative to that in water, and can be attributed to the additional steps required in ASE and the increased matrix interferences from soil. Sulfonamides and estrogens have recoveries higher than 50%, while tetracyclines have recoveries ranging from 22 to 115%. In general, %

RSD for recovery ($n = 3$) in water was less than 18%, while % RSD for recovery in soil was less than 24%. An interday study (1 day) with all analytes spiked in surface water at 30 ng/L resulted in percent differences of less than 14%.

Application of the LC−MS/MS Method in the Analysis of Field Runoff and Soil Samples. The developed and validated method was used to analyze soil and field runoff water samples from an agricultural watershed that previously received poultry litter as fertilizer. Results from this analysis (Table 4A,B) demonstrate the ability for multiple analytes to be detected and quantified simultaneously in a single LC−MS/MS run. This is the first time that this single run analysis has been performed on these 28 compounds, which are typically performed in at least two separate LC−MS/MS methods. The surrogate standards were spiked in all samples, and recoveries are shown in Table 4B. Samples were not corrected for surrogate recoveries because all compounds were not available in deuterated forms, instead surrogates were used to monitor trends in the recoveries. The actual field samples showed that sulfonamides were generally not detected, except for trace levels of SMZ in the raw poultry litter. The tetracyclines were mostly found in soil samples rather than in water samples, which is consistent with the reported high soil sorption coefficients for tetracyclines ($K_d > 10^4$).[33] Of the free estrogens, E1 was the most abundant and frequently detected, along with 17β-E2, 17α-E2, and E3. These findings were also expected because a majority of the free estrogens can be oxidized into E1.[12,43] The highest concentrations of the free and conjugated estrogens were observed in the raw poultry litter. This was also expected because estrogens are naturally produced in the livestock and have been reported to be excreted in livestock waste.[44] In this study, the type of antibiotics given to the animals were not disclosed by the source of the poultry litter.

The ability to monitor multiple classes of compounds in a single analysis has several advantages for throughput and reducing overall cost when compared to separate analyses. This method used for free and conjugated estrogens, sulfonamides, and tetracyclines and sulfonamides by wrong-way-round ionization using a high pH mobile phase allows for sufficient detection limits and quantification for the screening of environmental samples.

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Funding Sources

This material is based upon the work supported by the United Sates Department of Agriculture (USDA) under Grant No. 2009-02424.

ACKNOWLEDGMENT

Any opinions, conclusion, or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the USDA.

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