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ELISA for Sulfonamides and Its Application for Screening in Water Contamination

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Two enzyme-linked immunosorbent assays (ELISAs) were tested for their suitability for detecting sulfonamides in wastewater from various stages in wastewater treatment plants (WWTPs), the river into which the wastewater is discharged, and two swine-rearing facilities. The sulfamethoxazole ELISA cross-reacts with several compounds, achieving detection limits of <0.04 μg/L for sulfamethoxazole (SMX), sulfamethoxypyridine, sulfachloropyridine, and sulfamethoxine, whereas the sulfamethazine (SMZ) ELISA is more compound specific, with a detection limit of <0.03 μg/L. Samples from various stages of wastewater purifications gave 0.6−3.1 μg/L by SMX-ELISA, whereas river samples were ∼10-fold lower, ranging from below detection to 0.09 μg/L. Swine wastewater samples analyzed by the SMX-ELISA were either at or near detectable limits from one facility, whereas the other facility had concentrations of ∼0.5 μg/L, although LC-MS/MS did not confirm the presence of SMX. Sulfamethazine ELISA detected no SMZ in either WWTP or river samples. In contrast, wastewater samples from swine facilities analyzed by SMZ-ELISA were found to contain ∼30 μg/L [piglet (50−100 lb) wastewater] and ∼7 μg/L (market-weight hog wastewater). Sulfamethazine ELISA analyses of wastewater from another swine facility found concentrations to be near or below detection limits. A solid phase extraction method was used to isolate and concentrate sulfonamides from water samples prior to LC-MS/MS multiresidue confirmatory analysis. The recoveries at 1 μg/L fortification ranged from 42 ± 4% for SMZ to 88 ± 4% for SMX ($n = 6$). The ELISA results in the WWTPs were confirmed by LC-MS/MS, as sulfonamide multiresidue confirmatory analysis identified SMX, sulfapyridine, and sulfasalazine to be present in the wastewater. Sulfamethazine presence at one swine-rearing facility was also confirmed by LC-MS/MS, demonstrating the usefulness of the ELISA technique as a rapid and high-throughput screening method.

KEYWORDS: Analysis; ELISA; LC-MS/MS; residue; sulfonamides

INTRODUCTION

Pharmaceuticals are emerging environmental contaminants causing great concern because of their ubiquitous occurrence in urban wastewater and their potential effect on humans as well as biota. In a 2002 report, the U.S. Geological Survey identified 95 emerging pollutants in U.S. waterways, among which were sulfachlorpyridazine, sulfadimethoxine, sulfamerazine, sulfamethizole, sulfamethoxazole (SMX), and sulfathiazole (*1*). Unlike many pollutants such as dioxins and polybrominated diphenyl ethers, the sulfonamide class of antibiotics is not highly lipophilic. Nevertheless, they are persistent and resistant to biodegradation, and although these compounds do show photochemical transformations (*2*), the environmental importance of these reactions is probably minimal. In fact, bacteria may transform biologically inactive conjugates (metabolites) back to the original biologically active compound. For example, Göbel et al. (*3*) found SMX exhibited increased concentrations in the wastewater treatment process that could be accounted for by transformation of the 4-*N*-acetyl metabolite back to the parent compound. In addition, several studies have found SMX was not completely removed (33−75%) by sewage treatments and, thus, is discharged into the environment by wastewater treatment plants (WWTPs) with effluent concentrations varying from 0.22 to 0.68 μg/L (*3, 4*). Similar results have been reported for sulfapyridine (*3*). Antibiotics, even in low concentration, may result in the development of resistant

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bacteria, posing a potential problem for both animal and human medicine.

Sulfonamides are used as veterinary pharmaceuticals for both prophylactic and therapeutic treatment, as well as in subtherapeutic amounts as growth promoters. These compounds can be excreted unchanged or as metabolites in either urine or feces. Sulfonamides show low soil sorption and can be present in surface water (*5*). Hence, the application of liquid manure from animals or municipal sewage sludge as fertilizer to produce sustainable nutrient recycling can increase the potential for environmental pollution. See Sarmah (*6*) and Sukul and Spiteller (*7*) for reviews of the potential environmental contamination from these and other veterinary chemicals.

Sulfonamides are common therapeutic agents used in human medicines primarily in the treatment of bacterial infections. They are estimated to be 16−21% of annual antibiotic usage, making them the most important group of antibiotics consumed by humans (*8*). Therefore, it is no surprise that the sulfonamide SMX is one of the most prevalent pharmaceuticals found in wastewater (*1*). Reduction of these chemicals in the environment requires knowledge of their concentration and their source (human or veterinary use), which entails monitoring by analytical methodology capable of high throughput.

A number of investigators have attempted to determine the risk of pharmaceuticals found in environmental water samples, but because of the use of different models and assumptions, there has been no agreement among the studies (*9–12*). Some studies suggest SMX could be a problem, whereas other studies suggest that it might not be.

Because several structurally similar compounds are included in the sulfonamide class, sophisticated multiresidue analysis methods such as LC/MS or LC-MS/MS have been developed and continue to be the focus of recent studies (*3, 13–17*). These methods not only allow the quantitative analysis of closely related compounds but also allow unequivocal confirmation of the chemical species. Unfortunately, the instrumentation is expensive and has high operating costs, making it unsuitable for routine monitoring analysis. Ion suppression due to matrix effects has frequently been a problem, leading to inaccurate quantitation. To counter this problem, different approaches such as extensive cleanup methods, use of different internal standards, or matched matrices for recovery adjustment have been reported. These adaptations make it difficult to compare different instrumental methods.

Immunoassays are particularly suitable for high-throughput analysis. In addition to offering portability, they provide simplicity of operation. Although immunoassays show some matrix effects, these are often easily corrected by dilution or by using a compensating medium for the construction of the standard curve. In contrast, the LC-MS-based methods often show ion suppression or enhancement, which is corrected for by using isotopic standards, which may or may not be available. Because of these advantages and because of the diverse structural differences in the sulfonamides class, many different immunoassays for sulfonamides have been developed throughout the past decade (*18–21*). Recently, the focus is on developing an ELISA using an antibody that detects a broad spectrum of sulfonamides, rather than a single member of the class (*22–24*).

In this paper we utilize two sulfonamide ELISAs to determine sulfonamide concentration in various stages of wastewater from two different WWTPs and in river water, as well as from swine-rearing facilities. The ELISA results were confirmed by LC-MS/MS for the presence of sulfonamide.

MATERIALS AND METHODS

Materials. SMX was purchased from US Pharmacopeia (Rockville, MD). Sulfacetamide, sulfaguanidine, sulfathiazole, sulfadiazine, sulfapyridine, sulfameter, sulfamethoxypyridine, sulfamerazine, sulfamethizole, sulfamethazine (SMZ), sulfachloropyridine, sulfadimethoxine, sulfabenzamide, sulfaquinoxaline, and sulfasalazine were obtained from Sigma-Aldrich (St. Louis, MO). Sulfamethoxazole-d_4 and sulfadiazine-d_4 were obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). Glass fiber filter G4 was obtained from Fisher Scientific (Pittsburgh, PA). Solid phase extraction Oasis HLB cartridges were purchased from Waters (Milford, MA). The production of the rabbit anti-SMX and rabbit anti-SMZ antibodies used in these ELISAs were published previously (*18, 24*), and these reagents were incorporated into assay kits by Abraxis LLC (Warminster, PA).

Sample Collection. Wastewater samples from various treatment stages were obtained from two different treatment plants along the Red River Valley (one trickling system, one activated sludge treatment) along the North Dakota and Minnesota border. River waters were collected from the Red River of the north, which flows north, in proximity to the adjacent cities of Fargo, ND/Moorhead, MN (center of town, "metro") as well as upstream (south, 12 km from metro) and downstream (north, 17 km from metro). Treatment plant release wastewater was collected between the metro and north sampling sites. Samples were transported on ice to the laboratory. Raw wastewater was collected from two swine-rearing facilities. Samples were stored at −20 °C in the dark prior to processing.

Sample Treatment for ELISA. Samples were centrifuged at 1000*g* for 10 min, and the supernatants were filtered through G4 glass fiber filters and stored at −20 °C until assayed.

Sample Treatment for LC-MS/MS. Sample preparation procedures were modified from Göbel et al. (*25*). Briefly, 20 mL of the water sample was spiked with 10 ng of SMX-d_4 and sulfadiazine-d_4 (100 μL of 100 ng/mL working solution in 50% MeOH/H_2O v/v) and 25 μL of 1 M H_2SO_4, resulting in a pH of approximately 4. The samples were passed through a preconditioned Oasis HLB cartridge (60 mg sorbent) at <5 mL/min. Conditioning consisted of 2 × 1.5 mL of 50% MeOH/ethyl acetate followed by 2 × 1.5 mL of 2.5% ammonia−water in MeOH. Once samples were loaded, cartridges were washed with 1.5 mL of 5% MeOH/H_2O, dried under vacuum for 1 h, and eluted with 2 × 1.5 mL of 50% MeOH/ethyl acetate and 2 × 1.5 mL of 2.5% ammonium water in MeOH. Solvent was removed with a stream of nitrogen and the sample reconstituted with 200 μL of 50% acetonitrile/H_2O containing 0.2% formic acid. After vortexing and centrifuging at 50*g* for 5 min, the mixture was transferred to glass vials and stored in the dark at −20 °C until analyzed.

ELISA Procedure. Two ELISAs, one designed for SMX and another one for SMZ, were used to determine the sulfonamide levels. Both ELISAs showed some cross-reactivity to structurally related compounds as shown in **Table 1**. The SMX-ELISA utilized a 96-well plate format. Briefly, 75 μL/well of standards (0, 0.025, 0.05, 0.1, 0.25, and 1 μg/L) in water or samples were added to the plate followed by the addition of 50 μL/well of primary antibodies and incubated at room temperature for 20 min followed by the addition of SMX−horseradish peroxidase (HRP) (50 μL/well) and further incubated for 40 min at room temperature. Plates were washed with 250 μL/well of phosphate-buffered saline containing 0.05% Tween 20 (PBST) three times. A solution of hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine (TMB) (150 μL/well) was added to the plate, and the color reaction was developed at room temperature for 30 min, terminated by the addition of 100 μL/well of 1 M H_2SO_4. The absorbance was read at 450 nm using a Bio-Rad model 550 ELISA plate reader (Bio-Rad Laboratories, Hercules, CA). The unknown concentration was computed from the standard curve. For each assay, a positive control was included (0.2 μg/L solution of the appropriate sulfonamide).

The SMZ-ELISA uses a magnetic particle immunoassay format rather than the original 96-well plate format (*18*) to improve assay kinetics. A mixture of 250 μL/tube of either SMZ standard (0, 0.05, 0.5, and 5 μg/L) or samples, 250 μL/tube of SMZ-HRP, and 500 μL/tube of SMZ antibody conjugated paramagnetic particles was incubated (room temperature, 30 min) followed by 1 mL PBST (1 mL/tube) washes. A solution of hydrogen peroxide and TMB (500 μL/tube) was

Table 1. Performance Parameters of the Sulfamethoxazole and Sulfamethazine ELISAs[a]

	sulfamethoxazole ELISA			sulfamethazine ELISA		
compound	90% B/B_0[b] (μg/L)	50% B/B_0[b] (μg/L)	% cross-reactivity[c]	90% B/B_0[b] (μg/L)	50% B/B_0[b] (μg/L)	% cross-reactivity
sulfamethoxazole (SMX)	**0.030**	**0.255**	**100**	250	4300	<0.1
sulfamethoxypyridazine	0.033	0.146	175	20	>1000	<0.1
sulfachloropyridazine	0.034	0.18	142	100	880	0.1
sulfadimethoxine	0.036	0.42	61	10	150	0.6
sulfamethizole	0.25	2.5	10	700	>1000	<0.1
sulfasalazine	0.94	7.9	3.2	70	>1000	<0.1
sulfapyridine	0.73	7.6	3.4	64	>1000	<0.1
sulfameter	0.17	12	2.1	100	>1000	<0.1
sulfaquinoxaline	0.13	26.5	1	30	290	0.3
sulfadiazine	14	120	0.2	12	130	0.7
sulfacetamide	52	250	0.1	1000	>1000	<0.1
sulfamerazine	41	580	<0.1	0.08	3.9	23
sulfaquanidine	110	1010	<0.1	NT	NT	<0.1
sulfabenzamide	160	1750	<0.1	1	>1000	<0.1
sulfamethazine (SMZ)	375	7600	<0.1	**0.03**	**0.88**	**100**

[a] Determined in ELISA buffer. NT, not tested. [b] B/B_0 was defined as absorbance value with competitor/absorbance value without competitor. Limit of detection (μg/L) is defined as 90% B/B_0, whereas assay sensitivity is defined as 50% B/B_0. [c] % Cross-reactivity = (50% B/B_0) tested compound/(50% B/B_0) SMX or SMZ × 100.

added to the plate and the color reaction developed (room temperature, 20 min). The color development was stopped by the addition of 500 μL/tube of 1 M H_2SO_4, and the absorbance read at 450 nm (Photometric Analyzer, Abraxis, Warminster, PA). Initially, filtered water samples were assayed without any dilutions, and if the sulfonamides concentrations exceed the highest concentration of the calibration curve, then samples were diluted with assay buffer and reanalyzed.

LC-MS/MS Analysis. The LC-MS/MS setup consisted of a Waters Alliance 2695 pump equipped with a Waters PDA detector 1996 utilizing a Q-TOF API-US mass spectrometer using MassLynx 4.1 to acquire and process data. Initially each sulfonamide (sulfacetamide, sulfaguanidine, sulfathiazole, sulfabenzamide, sulfadiazine, sulfapyridine, sulfamethizole, sulfaquinoxaline, sulfamerazine, SMZ, SMX, sulfasalazine, sulfachloropyridine, sulfadimethoxine, sulfameter, and sulfamethoxypyridazine) to be analyzed was directly infused using electrospray ionization in positive mode to identify the precursor ion, product ions, and optimum cone voltage used for the multiresidue analysis. For each sulfonamide, the sum of the precursor ion and the two most abundant product ions were used for confirmation as summarized in Supporting Information (Table SI 1). With the exception of sulfasalazine, all other sulfonamides have m/z 156 $[M - RNH_2]^+$ as a base peak, and therefore m/z 156 is included for all sulfonamides except sulfasalazine. For the multiresidue analysis the LC system was equipped with an Atlantis C18 column (3 μm, 2.1 × 100 mm; Waters) with a flow rate of 0.2 mL/min. The HPLC column was maintained at 30 °C and the autosampler at 4 °C. The binary gradient system was as follows: solvent A, H_2O with 0.2% formic acid, solvent B, 95% acetonitrile/H_2O with 0.2% formic acid, and at time 0, 75% A; 15 min 30% A; 18 min 0% A; 23.1 min 75% A; run ends at 35 min. Standard curves of each sulfonamide consisted of 0.5, 1, 2, 5, 10, 20, 100, 200, and 500 μg/L, with 20 μL injections. Sulfamethoxazole and sulfadiazine were corrected for internal standard recovery, whereas the remaining sulfonamides were quantitated using only external standard curves. A quadratic function was fit to each curve, and in general the R^2 values were >0.99. In general, the use of a quadratic function was superior to a linear function, because the response decreased at higher concentrations and the linear function did not extrapolate well to low concentrations.

RESULTS AND DISCUSSION

The sensitivity (50% B/B_0) and limit of detection (90% B/B_0) along with the cross-reactivity relative to the standard analyte [(50% B/B_0) tested compound/(50% B/B_0) SMX or SMZ] are given in **Table 1**. The SMX antibody recognized several structurally related sulfonamides (**Figure 1**), showing high cross-reactivity with sulfamethoxypyridazine (175%), sulfachloropyridazine (142%), and sulfadimethoxine (61%). The high reactivity of the first two could be explained by similarity in their structure, but the cross-reactivity of sulfadimethoxine, which shows little structural similarity to SMX, is rather surprising. Because SMX use far exceeds the use of other sulfonamides, their cross-reactivity is not likely to be of practical significance. Samples that screened positive by SMX-ELISA should be analyzed using secondary methods to identify specific sulfonamides present. The SMZ antibody showed cross-reactivity (23%) with only sulfamerazine, presumably because of their high structural similarity. The two ELISAs both have within- and between-day variations of <16% (spike levels for sulfamethoxazole were 0.05, 0.25, and 0.5 μg/L; spike levels for sulfamethazine were 0.25, 0.5, 1.0, 2.5 μg/L). In separate experiments, the recoveries for spike levels of 0.1, 0.25, and 0.5 μg/L were 92, 99, and 100% for SMX-ELISA and 106, 108, and 116% for SMZ-ELISA, respectively (data not shown).

The results for the use of SMX-ELISA for wastewater and river water measurements are shown in **Table 2**. The results show significant levels of sulfonamides (expressed as SMX equivalents) for all stages of wastewater treatment in both WWTPs. Confirmatory analysis with LC-MS demonstrated SMX was present. The ELISA results showed levels of SMX decreased ~50% for WWTP1 in March and April at different stages of the WWTP treatment process (**Table 2**), although the concentrations showed variations within the treatment process as well as at the different sample times. These concentration results were similar to the findings of other workers using LC-MS methods. Göbel et al. (*3*) found variable elimination of SMX in wastewater from two WWTPs using tertiary treatment in Switzerland, with much of the variation purportedly due to conversion of metabolites back to parent during the processing. The levels of the *N*-acetyl metabolite consistently decreased with processing at both plants, and the total (SMX + metabolite) decreased by about 50% for two of the three sampling periods at one plant. Influx levels were 0.85−1.6 μg/L for the *N*-acetyl-SMX and 0.23−0.57 μg/L for SMX. Batt et al. (*4*) found SMX levels from 0.21 to 2.8 μg/L in four different WWTPs in New York, including those using different tertiary treatments, with concentrations decreasing by 33−75% from the influent to the effluent. The river samples in our study all showed at least 10-fold lower sulfonamide concentrations by SMX-ELISA compared to effluent of the WWTPs, with no evidence of correlation between concentration and proximity to WWTP release location. In general, the concentration trend obtained by SMX-ELISA was also observed by LC-MS/MS (**Table 2** and **Figure 2**).

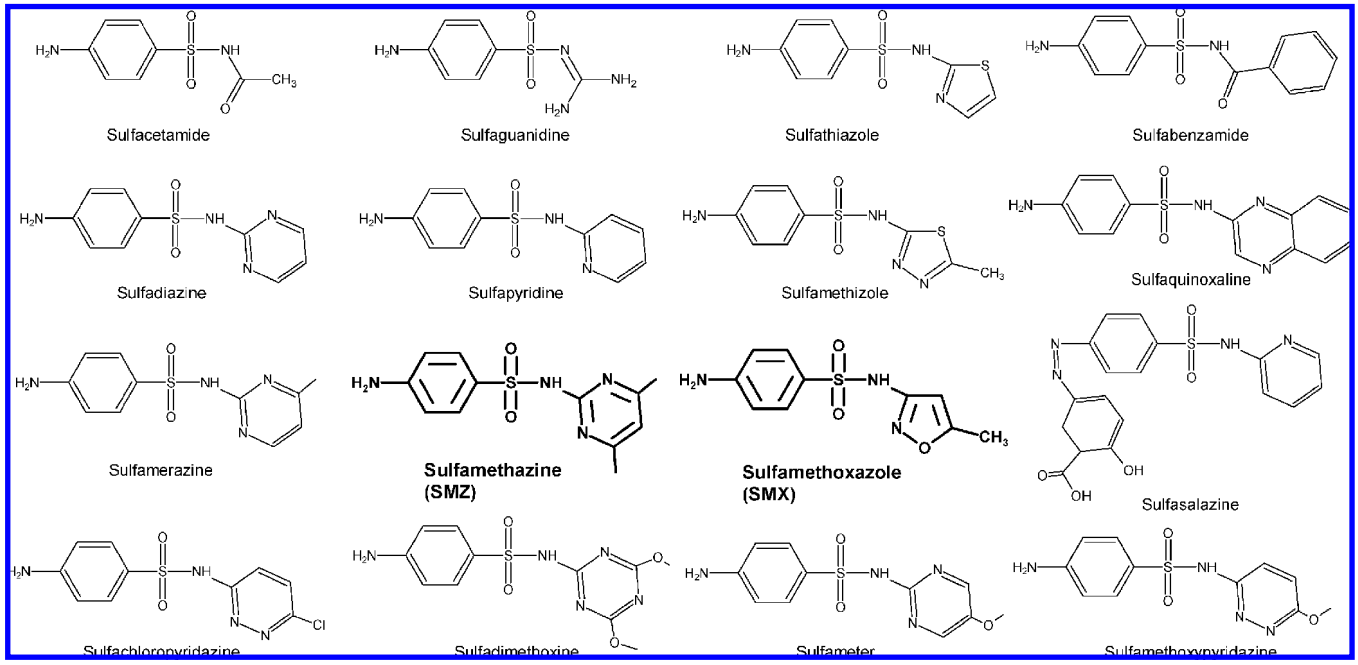

Figure 1. Structures of sulfonamides analyzed by ELISAs or LC-MS/MS for the current study.

Table 2. Sulfamethoxazole Analysis of Wastewater Treatment Plant and Red River Sample by ELISA[a] and LC-MS/MS[b] (μg/L)

	WWTP 1[c]				WWTP 2[c]				river[d]		
description	March 2007	April 2007	June 2007	description	March 2007	April 2007	June 2007	description	March 2007	April 2007	June 2007
raw influent				return activated sludge				north			
ELISA	2.2	2.5	1.4	ELISA	3.1	2.8	1.3	ELISA	0.08	0.05	ND[e]
LC-MS/MS	0.7	2.4	0.8	LC-MS/MS	1.9	1.3	0.9	LC-MS/MS	ND	ND	ND
BOD trickling				final clarifier				metro			
ELISA	2.1	2.0	1.8	ELISA	0.8	1.0	0.6	ELISA	0.07	0.08	0.01
LC-MS/MS	0.6	1.0	1.1	LC-MS/MS	0.4	1.6	0.2	LC-MS/MS	ND	ND	ND
intermediate clarifier				NH_3 basin influent				south			
ELISA	2.2	2.5	2.0	ELISA	1.0	1.4	0.6	ELISA	0.09	0.07	ND
LC-MS/MS	0.7	1.7	1.4	LC-MS/MS	5.4	2.0	0.8	LC-MS/MS	ND	ND	ND
NH_3 trickling				NH_3 basin effluent							
ELISA	1.1	1.4	1.3	ELISA	1.4	1.6	0.9				
LC-MS/MS	0.4	0.6	1.1	LC-MS/MS	1.9	1.2	0.2				
final effluent				final effluent							
ELISA	1.2	1.1	1.7	ELISA	1.7	3.0[f]	1.2				
LC-MS/MS	1.4	0.8	1.2	LC-MS/MS	2.2	1.2	0.5				

[a] Sulfamethoxazole ELISA values are means of two assays performed on two different days. Samples were analyzed without cleanup. The average difference between the duplicate samples was 17.7% in wastewater. [b] LC-MS/MS values are confirmatory, only semiquantitative. Samples were subjected to SPE prior to analysis. On-column detection limit was 62 pg, and LOQ was 100 pg. [c] Sulfapyridine and sulfsalazine were detected in all WWTP samples. Cross-reactivity of these compounds with the sulfmethoxazole ELISA was <4%. See Supporting Information for values. [d] Location relative to central metropolitan location. [e] ND, not detected. [f] Chlorination and dechlorination steps were added.

Statistical analysis using a paired t test for 30 wastewater samples analyzed by both methods demonstrated there were no differences ($P = 0.07$). However, deletion of the point that showed an unusually high LC-MS/MS value caused the differences to become significant ($P < 0.05$). The narrow range of values, the high scatter, makes it difficult to interpret the results. The tendency of the ELISA to give slightly higher results is due to the intercept as shown in Supporting Information Figure SI 1. The slope is approximately 1. Although there are 38 data points (one outlier was omitted), the river water samples (9 data points) indicate both analyses give essentially zero when the analyte is present in only low concentration, indicating the intercept is significant only in the wastewater samples. One possible explanation would be the presence of the SMX metabolites, which could cross-react with the SMX antibody. In the river water neither the metabolites nor the parent analyte is present in high enough amount to give a significant response by SMX-ELISA. In addition to SMX, the LC-MS/MS confirmed the presence of sulfapyridine and sulfasalazine, but only in very low levels (in some cases, below the limit of quantitation, see Supporting Information Table SI 2). These compounds should have made little contribution to the SMX value obtained, as their cross-reactivity was <4% in the ELISA utilized. All municipal wastewater and river samples were below the limit of detection in the analysis using the SMZ-ELISA, which is expected given the primarily veterinary use of this sulfonamide.

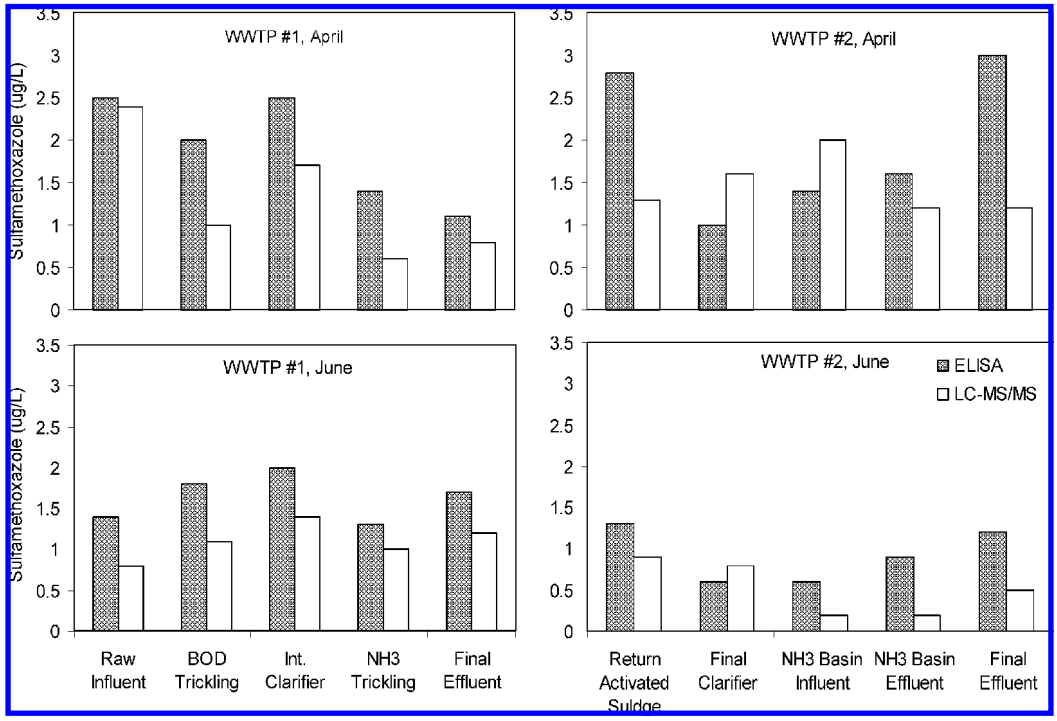

Figure 2. Comparison of ELISA and LC-MS/MS determination of sulfamethoxazole from different stages of two wastewater treatment plants in April and June 2007. Detection limits were 2 pg per well for SMX-ELISA and 62 pg on-column for LC-MS/MS.

Table 3. Sulfamethazine Concentrations in Swine Wastewater As Measured by SMZ-ELISA (μg/L)[a,b]

dilution factor[c,d]	piglet	market weight
1:2	exceed	7.8 ± 0.8
1:10	31.1 ± 2.2	7.2 ± 0.9
1:20	29.8[e]	NT[f]

[a] Final concentration of initial sample, corrected for dilution factors. Presence confirmed by LC-MS/MS. [b] Concentrations were <0.135 μg/L for SMZ in wastewater samples from second swine facility. [c] Standard error represents three separate measurements on different days. [d] Samples diluted with assay buffer, no extraction. [e] Mean of two different measurements on two different days. [f] Not tested.

The SMX-ELISA detected no SMX in samples from one swine facility, whereas wastewater from the second facility gave low values (0.4 and 0.7 μg/L, data not shown). LC-MS/MS data were not able to confirm the presence of SMX in these samples. The method discrepancy could be due to matrix effects in the ELISA and/or low signal-to-noise ratio resulting from low concentrations and high organic content in LC-MS/MS samples. Given that SMX is not approved for animal usage by the U.S. FDA (*26*), it was most likely that the SMX-ELISA results were false positives.

The SMZ-ELISA, however, detected sulfamethazine in these same swine wastewater samples, whereas wastewater from the other facility had none to below limit of quantitation concentrations. The findings of SMZ-positive samples in rearing facilities could be expected as SMZ is approved for use in swine as a growth promoter and in the treatment of infections. Sulfamethazine-positive samples had concentrations (~33 and 7 μg/L for wastewater from piglet and market weight swine, **Table 3**). The SMZ-ELISA results were similar to those reported by Choi et al. (*27*) for municipal and agricultural wastewater influent (4 and 97.2 μg/L, respectively) using LC-MS diode array analysis. These scientists found SMZ was not detectable in effluents, whether from municipal or agricultural WWTPs. They found five of eight river samples contained 0.1−0.3 μg/L of SMZ, indicative of nonpoint source contamination. Kandimalla et al. (*28*) from the Czech Republic reported ~10-fold higher concentrations of SMZ (570 μg/kg) in raw swine manure using the same polyclonal antibody that our SMZ-ELISA assay uses.

Table 4. Sulfonamide Recoveries in Nanopure versus Tap Water by LC-MS/MS[a]

	1 μg/L		2.5 μg/L	
compound	nanopure H_2O	tap H_2O	nanopure H_2O	tap H_2O
sulfathiazole	45 ± 5	50 ± 6	57 ± 7	56 ± 8
sulfadiazine	75 ± 9	79 ± 8	79 ± 11	70 ± 9
sulfapyridine	54 ± 6	48 ± 6	64 ± 8	63 ± 10
sulfamerazine	62 ± 6	52 ± 5	70 ± 3	64 ± 8
sulfamethizole	66 ± 4	72 ± 6	63 ± 2	67 ± 8
sulfamethazine (SMZ)	42 ± 4	37 ± 5	52 ± 3	46 ± 6
sulfachloropyridazine	51 ± 4	47 ± 8	56 ± 5	54 ± 6
sulfamethoxazole (SMX)	88 ± 4	91 ± 3	89 ± 5	89 ± 6
sulfadimethoxine	56 ± 3	42 ± 5	60 ± 3	49 ± 5
sulfabenzamide	55 ± 3	53 ± 5	57 ± 4	56 ± 4
sulfaquinoxaline	53 ± 3	47 ± 6	56 ± 4	50 ± 5
sulfasalazine	62 ± 3	68 ± 5	59 ± 5	71 ± 2

[a] Recoveries as percent ± standard error (n = 6).

Because the LC-MS/MS requires rather stringent purification of wastewater samples, the recovery from the purification process was determined to assess loss (**Table 4**). Apparent recoveries varied from 37 to 91% at 1 μg/L level and from 46 to 89% at the 2.5 μg/L level. Sulfamethoxazole and sulfadiazine showed the highest recoveries, in part due to the ability to correct with internal deuterated standards (~90 and 70−80%, respectively). Generally, a trend for higher recoveries was observed at the higher concentration (2.5 μg/L higher than 1.0 μg/L) with the exception of sulfamethizole, and those utilizing internal standard—sulfadiazine and SMX. The recovery differences between the nanopure water and tap water were generally small. Typically, a known amount of isotopically labeled standard was added to compensate for sample loss or ion suppression/enhancement of a given class of compounds. Application of this approach to the sulfonamide multiresidue analysis poses problems, as recoveries and ion suppression/enhancement may

differ among the analytes due to widely differing physicochemical properties of the sulfonamides. Some deuterated standards are unavailable, and inclusion of all potential deuterated sulfonamides is cost-prohibitive. In addition, whereas the LC-MS/MS method described herein is sound for confirmatory purposes, quantitation is somewhat limited due to the similar retention times for several sulfonamides. Once specific compounds of interest have been identified in a sample (LC-MS/MS screen), MS/MS acquisition modes could be modified to optimize for all compounds identified in the sample.

As mentioned above, in the municipal wastewater samples analyzed by LC-MS/MS, the sulfonamides that were present at highest frequency were SMX, sulfapyridine, and sulfasalazine (**Tables 4** and SI 2 of the Supporting Information). All three compounds are used for human medicine: SMX for urinary tract infections, sulfapyridine as a secondary agent for dermatitis, and sulfasalazine for ulcerative colitis, Crohn's disease, and rheumatoid arthritis. Because sulfapyridine is the breakdown product of sulfasalazine, it is impossible to identify the original source of sulfapyridine. Miao and co-workers (*29*) investigated antimicrobials in eight WWTPs in Canada and found SMX and sulfapyridine in all eight effluents. Other sulfonamides were found less frequently, including sulfadiazine and SMZ, which were found in only one of the eight effluents. Göbel (*25*) and co-workers studied the effluents from the primary, secondary, and tertiary stages of two WWTPs in Switzerland and found sulfapyridine and SMX at both plants in all three effluents. Sulfapyridine and SMX were poorly removed, although the *N*-acetylsulfamethoxazole decreased to approximately 10% in the effluent from the primary stage. SMZ was detected in the effluent at only one plant at low concentrations. Our results are in substantial agreement with these investigators.

In contrast to previous results, the only sulfonamide present in our swine wastewater samples was SMZ. The LC-MS/MS confirms the presence of SMZ (precursor and product ions), but the concentration is much lower (3.2 and 1.5 μg/L, for wastewater from piglet and market-weight swine) in comparison to the ELISA results. This could be due to the presence of SMZ metabolites that were not measured by the LC-MS/MS method or ion suppression. The SMZ-ELISA serial dilution results (**Table 3**) showed the concentrations were reasonably consistent, indicating minimal matrix effects after dilution for the pig wastewaters. Choi et al. (*27*) reported sulfathiazole at 10 times the SMZ concentration. The presence of sulfathiazole in their agricultural wastewater may be a reflection of excrement from different livestock species (potentially chicken and cattle in addition to swine, personal communication) and/or a different veterinary antibiotic usage in Korea.

In conclusion, this work demonstrated that two user-friendly ELISAs were able to identify the existence of SMX in municipal wastewater and SMZ in wastewater samples from swine-rearing facilities at the micrograms per liter level. A LC-MS/MS multiresidue method confirmed the existence of SMX in waters from WWTPs, and trace amounts of additional sulfonamides were found. The two methods compliment each other in that the ELISAs quickly identify samples requiring further study. The ELISAs represent an easy to use and effective method of analyzing antibacterial sulfonamides in wastewater, with the broad cross-reactivity of the SMX-ELISA and the selectivity of the SMZ-ELISA making them useful screening partners. These ELISAs would be effective for evaluation of modifications in wastewater processing. Future method development should include evaluation of these ELISAs in different matrices including other environmental samples and food, as well as development of a sample concentration step to lower detection/quantitation limits important for surface water and groundwater evaluation.

ABBREVIATIONS USED

ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; LC-MS/MS, liquid chromatography−mass spectrometry/mass spectrometry; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 0.05% Tween 20; SMX, sulfamethoxazole; SMZ, sulfamethazine; WWTP, wastewater treatment plant.

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Supporting Information Available: Tables showing sulfonamides' precursor ion and product ions for multiresidue analysis, positive ion electrospray, and sulfapyridine and sulfsalazine determination by LC-MS/MS (μg/L) of wastewater treatment plant samples; figure showing comparison of ELISA and LC-MS/MS analysis for sulfamethoxazole (μg/L), ELISA value = (0.95 ± 0.14) * LC-MS/MS value + (0.46 ± 0.16), $R^2 = 0.53$. This material is available free of charge via the Internet at http://pubs.acs.org.

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