Anal. Chem. **2001,** *73,* 4640−4646

Analysis of Trace Levels of Sulfonamide and Tetracycline Antimicrobials in Groundwater and Surface Water Using Solid-Phase Extraction and Liquid Chromatography/Mass Spectrometry

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A method has been developed for the trace analysis of two classes of antimicrobials consisting of six sulfonamides (SAs) and five tetracyclines (TCs), which commonly are used for veterinary purposes and agricultural feed additives and are suspected to leach into ground and surface water. The method used solid-phase extraction and liquid chromatography/mass spectrometry (LC/MS) with positive ion electrospray. The unique combination of a metal chelation agent (Na_2EDTA) with a macroporous copolymer resulted in quantitative recoveries by solid-phase extraction (mean recovery, 98 ± 12%) at submicrogram-per-liter concentrations. An ammonium formate/formic acid buffer with a methanol/water gradient was used to separate the antimicrobials and to optimize the signal intensity. Mass spectral fragmentation and ionization characteristics were determined for each class of compounds for unequivocal identification. For all SAs, a characteristic *m/z* 156 ion representing the sulfanilyl fragment was identified. TCs exhibited neutral losses of 17 amu resulting from the loss of ammonia and 35 amu from the subsequent loss of water. Unusual matrix effects were seen only for TCs in this first survey of groundwater and surface water samples from sites around the United States, requiring that TCs be quantitated using the method of standard additions.

Two commonly used classes of antimicrobials are sulfonamides (SAs) and tetracyclines (TCs). SAs, which are synthetic, are classified only as antimicrobials. TCs are naturally occurring or semisynthetic and, therefore, are considered to be antibiotics, a subclass of antimicrobials. SA and TC compounds rely on different mechanisms to defeat bacteria. SAs compete with *p*-aminobenzoic acid to prevent the synthesis of folic acid in bacteria[1]. TCs, however, bind to bacterial ribosomes to prevent tRNA access to the receptor sites[1]. Increasingly, swine, poultry, and other livestock are being raised in large confined animal-feeding operations (CAFOs) that require the use of SA and TC antimicrobials. The antimicrobials prevent epidemics and increase the animals' rate of weight gain when it is fed to them at milligram-per-kilogram concentrations. Thus, some antimicrobials may end up in CAFO wastewater and may be transported into groundwater and surface water.

As a result, concern is growing about antimicrobials affecting water quality because they can accelerate the evolution of antimicrobial-resistant bacteria.[2−4] In addition to increasing resistance among bacteria that have been exposed, if the genetic code for resistance is stored on the R-plasmids, resistance can be transferred to other bacteria.[4,5] Although the antimicrobials given to humans often are not the same as those given to animals, the structures can be similar enough that antimicrobials used for animals can cause resistance to those used for humans. For example, studies have shown that when streptococci and staphylococci bacteria developed resistance to tylosin, a common animal-feed additive, they also developed a resistance to erythromycin used by the human caretakers.[4]

These concerns have led to the need for a sensitive and reliable method of analysis for antimicrobials in ground and surface water in order to survey the U.S. water supplies. Early HPLC work using phosphate buffers has explored the chromatography of sulfonamides without concentration of the SAs prior to analysis.[6] Occasionally, antimicrobials will occur in water at levels that can be detected without preconcentration of the sample. Semiquantitative radioimmunoassay and LC/MS methods have been developed to analyze for these classes of compounds.[7] In most samples, however, the concentrations of the individual analytes are suspected to be < 2 μg/L.

A common method for extracting analytes from an aqueous matrix is solid-phase extraction (SPE). At least one method has been developed for the extraction of SA pesticides from water.[8] That method involves stacking an anion-exchange and an alumina SPE cartridge for the isolation of SAs. SPE methods also have been developed for SAs and TCs in blood, meat, and other biological matrixes.[9−17] Smallridge et al.[18] were among the first

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(1) Marzo, A.; Dal Bo, L. *J. Chromatog. A* **1998**, *812*, 17−34.
(2) Hirsh, R.; Ternes, T.; Haberer, K.; Kratz, K. *Sci. Total Environ.* **1999**, *225*, 109−118.
(3) Chef-Sandford, J. C.; Aminov, R. I.; Krapac, I.; Garrigues-Ifanifan, N.; Mackie, R. I. *App. Environ. Microbiol.* **2001**, *67*, 1494−1502.
(4) Khachatourians, G. G. *Can. Appl. Microbiol. J.* **1998**, *159* (9), 1129−1136.
(5) DuPont, H. L.; Steele, J. H. *Rev. Infect. Dis.* **1987**, *9* (3), 447−450.
(6) Mokry, M.; Klimes, J.; Zahradnicek, M. P*harmazie* **1994**, *49* (5), 333−335.
(7) Meyer, M. T.; Bumgarner, J. E.; Varns, J. L.; Daughtridge, J. V. Thurman, E. M.; Hostetler, K. A, *Sci. Total Environ.* **2000**, *248* (2−3), 181−188.
(8) Rodriguez, M.; Orescan, D. B. *Anal. Chem.* **1998**, *70* (13), 2710−2717.

10.1021/ac010514w
Published on Web 08/23/2001

to extract sulfamethazine and sulfathiazole from swine feed. Online chromatographic separation[19,20] and liquid−liquid extraction[20] also have been used to separate SAs from a complex matrix. With the exception of Carson et al.,[13] these reports concentrated on the analysis of fortified controls rather than field samples. Furthermore, the concentration detection limits in those methods were greater than 2 mg/L. Once these extraction techniques were applied to aqueous samples containing <5 μg/L tetracyclines, extraction efficiency decreased to less than 50% and, in some cases, approached zero.

Other methods, such as luminescent *Escherichia coli*, have been investigated for the detection of TCs in biological matrixes.[21] Again, the concentration detection limits are too high to be practical for analysis of water. Additionally, a method has been developed that uses lyophilization to extract TCs in combination with SPE to extract SAs from water.[2,3] In groundwater studies conducted in Germany, Hirsh et al. reported detection of SAs at concentrations >0.1 μg/L[2].

Many antimicrobials are nonvolatile with high molecular weights, and they respond well to positive electrospray ionization, which makes liquid chromatography/mass spectrometry (LC/MS) an excellent choice for separation and analysis. This paper presents an ultrasensitive method using solid-phase extraction with an EDTA addition to extract antimicrobials from water and to identify them unequivocally with LC/MS using positive ion electrospray with a limit of quantitation of 0.1 μg/L. This low limit of quantitation is environmentally meaningful in that concentrations of antimicrobials are commonly detected in this survey. Six SAs (sulfachloropyridazine, sulfadimethoxine, sulfamerazine, sulfamethazine, sulfamethoxazole, and sulfathiazole) in addition to five TCs (chlortetracycline, doxycycline, minocycline, oxytetracycline, and tetracycline) were analyzed using this method (Figure 1). This paper also discusses the chromatographic separation of TC epimers, the mass spectral characteristics for each of the classes of antimicrobials, and a first report of unusual mass spectra enhancements of the TCs in a nonvolatile matrix.

EXPERIMENTAL SECTION

Sample Preparation. Groundwater and surface water samples were collected from sites throughout the U.S. All samples were filtered through 0.7-μm glass-fiber filters (Whatman or equivalent) into 1-L amber glass bottles and stored at 4 °C until they were extracted, typically within two weeks. Aqueous samples were prepared for extraction by adding 75 μL of 40% H_2SO_4, 100 μL of 1.23 mg/L $^{13}C_6$-sulfamethazine as a surrogate, 200 μL of 1.23 mg/L meclocycline as a surrogate, and a 1-mL scoop of disodium ethylenediaminetetraacetate (Na_2EDTA) to a bottle containing 123 mL of water. For controls, appropriate amounts of MeOH solution containing 1.23 mg/L of each of the analytes were added. The bottles were placed on an orbital shaker for 60 min at 100 rpm to dissolve the Na_2EDTA. To test the behavior of SAs and TCs spiked into a matrix, water was collected from Poison Creek in Idaho. Before it was used as a matrix, the water was analyzed using the method developed here, and no SAs or TCs were found.

Solid-Phase Extraction. Analytes were extracted using the 60-mg hydrophilic-lipophilic balance (HLB) cartridge from Waters (Millford, MA). Cartridges were preconditioned with 3 mL of MeOH, 3 mL of 0.5 N HCl, and 3 mL of distilled water. Water samples then were passed through the cartridges at 10 mL/min. After isolation, cartridges were rinsed with 1 mL of distilled water to remove excess Na_2EDTA. The analytes were eluted using 5 mL of MeOH into a test tube containing 100 μL of the internal standard, 0.123 mg/L simatone. Simatone was chosen as an internal standard, because it eluted within the same chromatographic time window as the analytes, responded well in ESI(+), and did not exhibit any noticeable matrix effect. The extracts then were concentrated under a flow of N_2 to an approximate volume of 50 μL. To this, 75 μL of mobile phase A was added. The resulting solutions were transferred to 0.3-mL amber autosampler vials. Amber vials were used to prevent photodegradation of TCs.

LC/MS Conditions. Antimicrobials were separated using a 100- × 4.6-mm Luna C8(2) column with a 3-μm pore size (Phenomenex, Torrance, CA). A binary gradient with a flow rate of 0.60 mL/min was used. Mobile phase A contained 10 mM ammonium formate in 90/10 water/methanol with 0.3% formic acid. Mobile phase B contained 10 mM ammonium formate with 0.5% formic acid in MeOH. The gradient was as follows: B = 9% for the first 5 min, increased to 42% by 15 min, and increased to 100% by 20 min. All of the compounds eluted within 25 min. A 5-min post time allowed reequilibration of the column. Mass spectra were acquired in positive ion electrospray (ESI(+)) on an HP 1100 LC coupled to a 1946B LC/MS (Agilent, Palo Alto, CA). The drying gas was operated at a flow rate of 10 mL/min at 400 °C. The nebulizer pressure was 30 psig, the capillary was set at 4000 V, and the fragmentor was set at 100 V. For each compound (with the exception of simetone), the protonated molecular ion, $[M + H]^+$, and at least one confirming ion were acquired (Table 1). Quantitation was based on the ratio of the base peak ion (protonated adduct of the molecular ion) of the analyte to the base peak ion of the internal standard.

RESULTS AND DISCUSSION

Recovery of Analytes by SPE. A variety of cartridges were tested to find the most efficient extraction method for both SAs and TCs. The cartridges included a 500-mg C-18 from Waters, a 150-mg Environmental+ (ENV+) (Jones Chromatography, Lakewood, CO), a 60-mg HLB cartridge from Waters, and a 500-mg HLB large particle cartridge, also from Waters (Table 2). The C-18 was tested because of its ability to effectively retain a large number of compounds[22] and its ease of use. The ENV+ and HLB

(9) Blanchflower, W. J.; McCracken, R. J.; Haggen, A. S.; Kennedy, D. G. J. *Chromatogr. B* **1997**, *692*, 351−360.
(10) Kennedy, D. G.; McCracken, R. J.; Cannavan, A.; Hewitt, S. A. J. *Chromatogr. A* **1998**, *812*, 77−98.
(11) Weimann, A.; Bojesen, G. *Anal. Lett.* **1998**, *31* (2), 2053−2066.
(12) Schenck, F. J.; Callery, P. S. *J. Chromatogr. A* **1998**, *812*, 99−109.
(13) Carson, M. C.; Ngoh, M. A.; Hadley, S. W. *J. Chromatogr. B.* **1998**, *712*, 113−138.
(14) Oka, H.; Ito, Y.; Kagami, T.; Harada, K. *J. Chromatogr. A.* **1998**, *812*, 309−319.
(15) Fedeniuk, R. W.; Shand, P. J. *J. Chromatogr. A.* **1998**, *812*, 3−15.
(16) Pouliquen, H.; Gouelo, D.; Larhantec, M.; Pilet, N.; Pinault, L. *J. Chromatogr. B.* **1997**, *702*, 157−162.
(17) Goicoechea, H. C.; Oliveri, A. C. *Anal. Chem.* **1999**, *71* (19), 4361−4368.
(18) Smallridge, R. L.; Kentzer, E. J.; Stringham, K. R.; Kim, E. H.; Lehe, C.; Stringham, R. W.; Mundell, E. C. *J. Assoc. Off. Anal. Chem.* **1998**, *71* (4), 710−717.
(19) Klimes, J.; Mokry, M. *Pharamzie* **1997**, *52* (6), 448−450.
(20) Kristiansen, G. K.; Brock, R.; Bojesen, G. *Anal. Chem.* **1994**, *66*, 3253−3258.
(21) Kurittu, J.; Lönnberg, S.; Virta, M.; Karp, M. *J. Agric. Food Chem.* **2000**, *48*, 3372−3377.

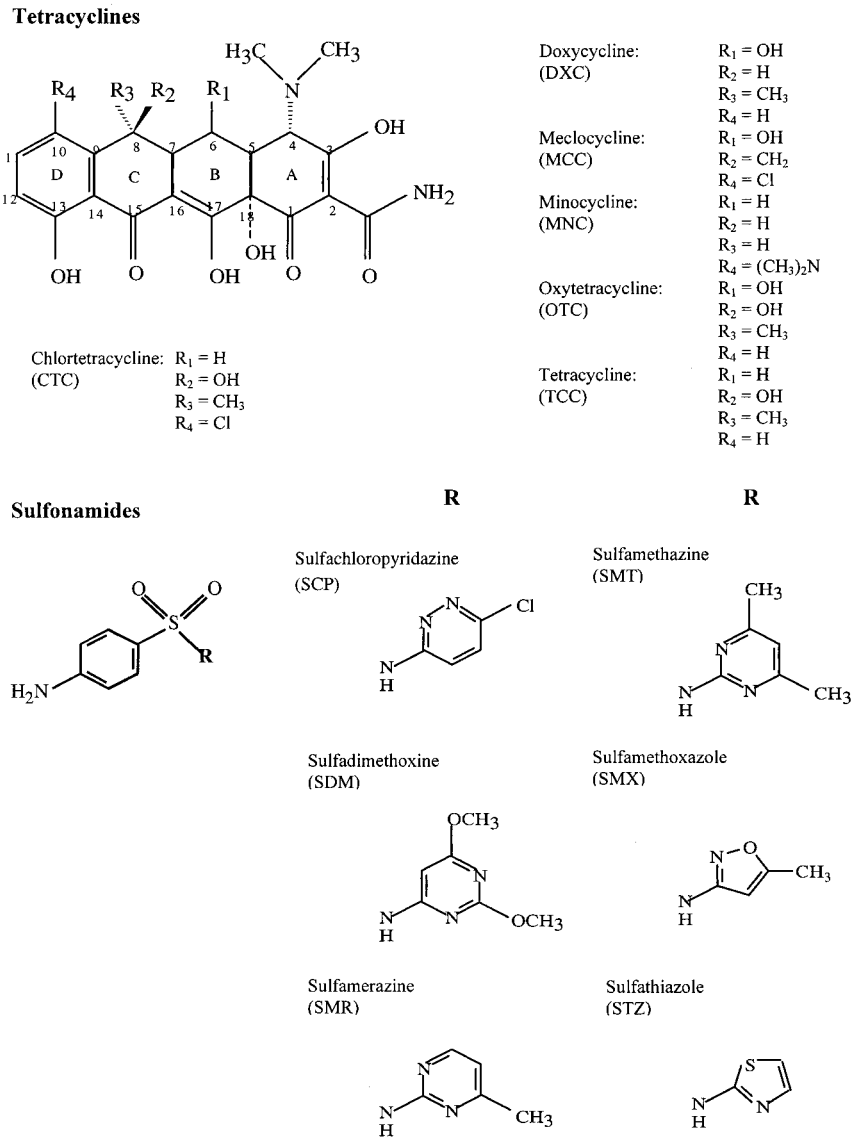

Figure 1. Structures of tetracyclines and sulfonamides.

cartridges were tested because they are designed to retain both hydrophilic and hydrophobic compounds with high capacity[22] and because neither cartridge contains silanols, which have been found to bind irreversibly to TCs.[12] Furthermore, sodium EDTA was added to the sample prior to extraction to chelate metals that may be present in solution or sorbed on the surface of the sorbent. These metals have been found to chelate TCs[9,23] and to prevent their effective recovery from biological matrixes.

Because SAs are negatively charged at neutral pH, and have p*K*1 values ranging from 5.4 to 7.5 and p*K*2 values around 2.5, they are highly water soluble. Therefore, recovery experiments were carried out at pH 2.5 (Table 2). At both neutral pH and pH < 3.0, SAs were extracted by all of the cartridges tested and could be eluted by methanol quantitatively; recoveries ranged from 84 to 130%. Thus, all of the cartridges tested were effective for the isolation of the SAs, and no additional treatments were required.

Table 1. Molecular Ion Adduct and Confirming Ions for the Tetracycline and Sulfonamide Antimicrobials

	MW	$\{M + H\}^+$	confirming ion 2	confirming ion 3
	tetracyclines			
minocycline	457.48	458	441	
tetracycline	444.44	445	428	410
oxytetracycline	460.44	461	444	426
chlortetacycline	478.89	479	481	462
simetone-ISTD	197.24	198		
doxycycline-H2O	462.46	446	428	
meclocycline-ISTD	476.87	477	460	
	sulfonamides			
sulfathiazole	255.32	256	156	
sulfamerazine	264.31	265	156	172
C13−6 sulfamethazine-ISTD	284.33	285	162	
sulfamethazine	278.33	279	156	
sulfachloropyridazine	284.73	285	156	
sulfamethoxazole	253.28	254	156	
sulfadimethoxane	310.33	311	156	

Of the four cartridges tested, the 60-mg HLB was chosen as the best overall sorbent with a mean recovery of SAs of 97.7 ± 12.2%.

(22) Thurman, E. M.; Mills, M. S. *Solid-Phase Extraction Principles and Practice*; Wiley and Sons: New York, 1998; pp 232−233.
(23) Varatarian, V. H.; Goolsby, B.; Brodbelt, J. S. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1089−1098.

Table 2. Recoveries of Sulfonamides and Tetracyclines from Various SPE Cartridges with Duplicate Samples[a]

	500-mg C-18	150-mg ENV+	60-mg HLB	500-mg HLB
sulfachloropyridazine	92%	79%	88.2%	87.1%
sulfadimethoxine	88	79	95.5	80.0
sulfamethazine	115	102	130	75.8
sulfamerazine	125	113	83.8	74.2
sulfathiazole	144/NR	80	108	87.5
chlortetracycline	108/NR	0	108	89.8/NR
oxytetracycline	144/NR	0	109	112/NR
tetracycline	139/NR	0	107	146/NR

[a] Calculations are based on the internal standard simatone, which was added after extraction. NR = not reproducible within +20% of the mean.

Table 3. Recoveries of Sulfonamides and Tetracyclines from 123 mL of Distilled Water[a]

antimicrobial	recovery, %	σ
sulfachloropyridazine	88	14
sulfadimethoxine	96	12
sulfamerazine	84	9.0
sulfamethazine	130	17
sulfamethoxazole	91	13
sulfathiazole	98	9.9
chlortetracycline	89	13
doxycycline	101	7.5
oxytetracycline	100	14
tetracycline	98	13

[a] Recoveries are the average of duplicates of 0.20, 1.0, and 2.0 g/L. No concentration dependence was seen for the recovery of any of the analytes.

The most efficient recoveries of individual SAs are listed in Table 3.

Because TCs have been shown to chelate metals[9,23] and to bind to silanol surfaces, special techniques have been used in past studies to improve recovery. For example, EDTA has been used in the extraction of higher concentrations of TCs[3,13] to improve recovery by chelating metal ions. TCs may bind residual metals on SPE cartridges, thereby irreversibly binding to the cartridge and lowering recovery.

We prevented chelation of metals by the TCs in two ways. The first was to wash metals off the cartridge using a solution of 0.5 N HCl. The second way was to add a strong chelator to the sample that would outcompete the TCs for metal ions. Na_2EDTA was chosen because it is an excellent metal chelator that is sufficiently soluble in water and does not interfere with the extraction of the SAs. The HLB gave reproducible recoveries for the 60-mg cartridge (Tables 2 and 3), and somewhat less reproducible recoveries for the 500-mg cartridge (Table 2). However, the TCs were not eluted from the C-18 and ENV+ cartridges and had no recovery or nonreproducible recovery. When extractions were performed without rinsing the cartridge with HCl or adding Na_2-EDTA to the sample, TC recoveries decreased by at least 50% (data not shown).

Irreversible binding is also due to the silanols of C-18 cartridge interacting with the TCs, except under extreme pH conditions, which could damage both the silanol and the TCs[1,2,9,11,16]. It is common to use either a phosphoric acid (H_3PO_4) solution[1,2,16] or an oxalic acid solution[9,11] when chromatographing TCs using a C-18 LC column, but these are not appropriate for elution of TCs from an SPE cartridge. Phosphoric acid could not be used to elute TCs from an SPE cartridge, because H_3PO_4 concentrated during evaporation of the eluant and degraded the analytes. Oxalic acid was not used to elute the analytes, because it cannot be volatilized by electrospray ionization.

The extraction of spiked 1.0 μg/L TCs from deionized water using a 500-mg C-18 cartridge resulted in approximately the same recovery for the TCs when compared to the recovery using a 60-mg HLB cartridge (Table 2). However, large variations in concentrations were observed, and signal enhancement was apparent for the TCs. The recovery decreased if either Na_2EDTA or acid was omitted from the procedure. To avoid the problem of TCs interacting too strongly with the silanols of an SPE cartridge, the 60-mg HLB cartridge, which does not contain a silanol backbone, was used. ENV+ cartridges also were tested, but although SAs exhibited excellent recoveries, no TCs were recovered using the ENV+ cartridge (Table 2). The 500-mg, large-pore HLB cartridge recovered analytes as well as the smaller cartridge, although because the 60-mg cartridge resulted in more reproducible recoveries than the 500-mg cartridge, the smaller cartridge was chosen. Because of the consistently reproducible recoveries of TCs, it was assumed that they did not exhibit matrix effects in distilled water.

Three concentrations of chlortetracycline, doxycline, oxytetracycline, and tetracycline were extracted using the 60-mg HLB cartridge, and recoveries ranged from 89 to 101%. The average recovery was 92.2 ± 13.6% (Table 3). No concentration dependence was observed for TC recoveries in the concentration range of 0.1−2.0 μg/L. The recovery of minocycline was not included in these calculations. The average recovery for minocycline was 17.4 ± 3.8%, which is probably due to the two amino groups that are present that enhance cation exchange at the pH of isolation. The addition of H_2SO_4, HCl, or Na_2EDTA did not affect the extraction efficiency of SAs (data not shown). After the effects of chelation and silanol binding were eliminated, a decrease in pH to <3.0 was required to increase the hydrophobicity of TCs to allow sorption to the SPE cartridges (Table 3).

Several solvents were tested for efficient elution of SAs and TCs. Acetone, acetonitrile, ethyl acetate, 2-propanol, and methanol, were tested. Each of the solvents recovered at least 60% of the SAs from both distilled water and the water from Poison Creek. Recovery of each of the TCs was at least 80% in distilled water, but recovery from the Poison Creek water was dependent on the solvent that was used. Overall recovery efficiency of TCs from Poison Creek water using each of the solvents was as follows: methanol > acetonitrile ≃ 2-propanol > acetone > ethyl acetate. The recovery efficiency corresponds to the solubility of humic and fulvic acid (natural dissolved organic matter) in the solvents. These findings indicate that the TCs associate with humic material, possibly through hydrogen bonding of the carboxylic acid groups of the humic materials to the keto-enol moieties of the TCs, and cannot be easily eluted separately.

In conclusion, sulfonamides and tetracyclines interact with SPE cartridges by different mechanisms. SAs rely primarily on hydrophobic interactions, whereas TCs rely on several mechanisms,

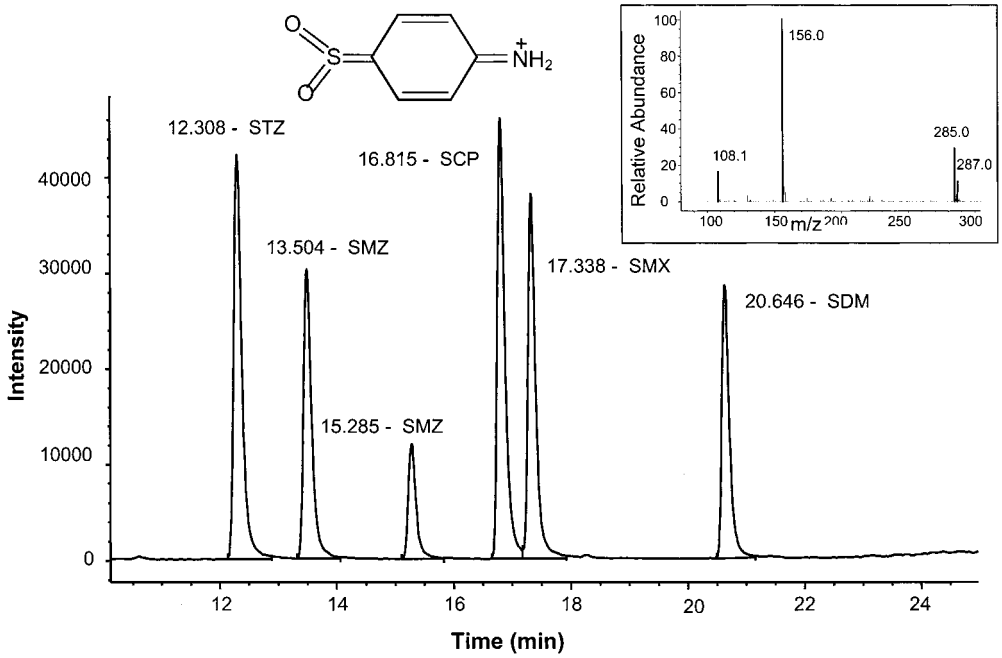

Figure 2. Extracted ion chromatogram for *m*/*z* 156 for each of the sulfonamides. A scan of sulfachloropyridazine is shown in inset. The fragmentor was set at 100 V.

including hydrophobic interactions, hydrogen bonding, chelation and cation exchange.

Common Fragmentation. All 11 antimicrobials in this study were tested by multiple LC/MS ionization methods. SAs were sensitively detected using ESI(+) and ESI(−) as well as APCI-(+). TCs were sensitively detected by ESI(+), APCI(+), and APCI-(−). Under optimized conditions for both classes of compounds, both ESI(+) and APCI(+) will work well. ESI(+) was chosen for this method, because it was the most sensitive toward chlorotetracycline, which was the TC that was suspected to be most important in this survey of antimicrobials.

Each class of antimicrobial compounds exhibited characteristic fragmentation in positive electrospray ionization. Figure 2 shows the mass spectrum of sulfachloropyridazine (SCP) with fragmentation to the 156 ion, which is characteristic of the class of SAs. This fragment represents the sulfanilyl ring that defines the class and was the only common ion seen under these conditions. Figure 2 also shows the extracted ion chromatogram for the class of SAs using the characteristic 156 ion. This fragment was also seen by Pleasance et al. using collision-induced dissociation[24] and by Kristiansen et al. using thermospray tandem mass spectrometry.[20] Thus, this ion was used for confirmation of the SA antimicrobials with the protonated adduct of the molecular ion as the quantitation ion.

For TCs, neutral losses of 17 and 35 amu were seen (Figure 3). The loss of 17 amu corresponded to the loss of NH_3. The loss of 35 amu corresponded to the loss of NH_3 with the subsequent loss of H_2O. Both of these losses agree with the findings of other research groups[9−11,13,14,23]. All of the compounds exhibited a loss of 17 amu. Peaks representing 35-amu losses for doxycycline and minocycline were not seen, indicating that the hydroxyl groups at position 6, ring B are lost as H_2O. This is in contrast to the findings of Hirsh et al.[3], who found that electrospray MS/MS analysis resulted in the appearance of $[M + H - NH_3 - H_2O]$ for doxycycline but did not report seeing $[M + H - NH_3]$ for oxytetracycline. These results indicate that although the general ions for a class of molecules remain the same, specific ions for each analyte can vary according to the MS method that is used. We did see the loss of 18 amu due to the loss of H_2O without the loss of NH_3, indicating that under the spray-chamber conditions described herein for collision induced dissociation, the loss of NH_3 initiated the loss of H_2O in a two-step process. Additionally, identical losses also were seen using APCI(+) and APCI(−).

Matrix Effects. Spiked recoveries of TCs and SAs were tested in distilled water, groundwater, and surface water. SA recoveries from all three matrixes were approximately 100% and did not show matrix effects. In addition, the recoveries of TCs from distilled water were ∼100%, with little deviation; therefore, it was assumed that matrix effects for the TCs were not seen in distilled water. However, when various groundwater and surface water samples spiked with 2.0 μg/L TCs were extracted and analyzed, the calculated concentrations varied from 0 to 30 μg/L, on the basis of distilled water. This result was indicative of matrix interference that made quantitation unreliable. Most matrixes resulted in an increase in signal intensity; however, some resulted in total suppression of the MS signal. Replicate spiked samples from the same source gave reproducible matrix effects. When TC samples were analyzed using APCI(+), signal enhancement also was seen. APCI(−), the least-sensitive ionization mode for TCs, also resulted in signal enhancement, although not to the magnitude of the other two ionization methods. For fragment ions within each compound, signal enhancement was equal. Matrix effects were seen for all of the TCs.

Several matrix experiments have been completed in which the natural organic matter in the sample was removed by sorption onto C-18 prior to the spiking and analysis of the TA antimicrobi-

(24) Pleasance, S.; Blay, P.; Quilliam, M. A.; O'Hara, G. *J. Chromatogr.* **1991**, *558*, 155.

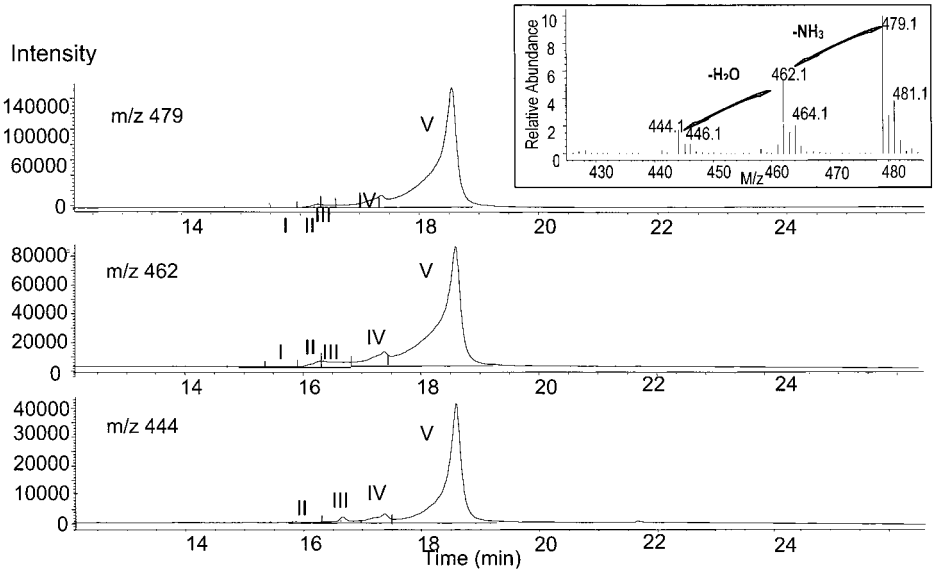

Figure 3. Chromatogram of $[M + H]^+$, $[M + H - NH_3]^+$, and $[M + H - NH_3 - H_2O]^+$ for chlortetracycline, indicating the five discernible epimers. Scan of CTC is shown in inset. The fragmentor was set at 100 V.

als. The TA signal enhancement was not seen after the removal of the natural organic matter. This result suggests that the natural organic matter is responsible for the matrix effects. The natural organic matter has surfactant properties that could enhance the electrospray signal by promoting ionization in electrospray positive. Others have reported matrix signal enhancement; for example, enhancement of the LC/MS signal caused by surface and groundwater matrixes was observed by Furlong et al. for the analysis of sulfonylurea herbicides,[25] but no hypothesis was presented. More experiments are underway in our laboratory to further investigate the source of the matrix effects associated with the natural organic matter present in the sample (i.e., humic and fulvic acids).

LC/MS Quantitation of Analytes. Concentrations for the SAs were calculated reproducibly by using either the internal standard simatone or the surrogate standard $^{13}C_6$-sulfamethazine using a conventional standard curve that was extracted through SPE, and no standard addition was required. A linear curve for extracted SAs, which is based on an internal standard, was calculated for concentrations from 0.05 to 5.0 μg/L. The quantitation ion was the proton adduct of the molecular ion. However, because of matrix effects, calculations of TC concentrations were based on the method of standard addition. The change of volume by the addition of 50 μL of spiking solution to a 123-mL sample was assumed to be negligible; therefore, we used the following equation to calculate concentration of TA antimicrobials,

$$\frac{[X]}{[S] + [X]} = \frac{I_x}{I_{s+x}}$$

where $[X]$ = the concentration of the unknown, $[S]$ = the concentration of the added standard, in this case 0.5 μg/L, and I = ratio of the intensity of the analyte signal to the intensity of the internal standard signal. The accuracy of these calculations at low concentrations was checked with a solution of $X = 0.05$ μg/L (one-half of the limit of quantitation based on extracted standards of 0.1 μg/L and a signal-to-noise ratio of 10:1). The sample of 0.05 μg/L was spiked with a standard addition of $S = 0.15$, 0.45, 0.95, and 1.95 μg/L. The calculations for X ranged from 0.043 to 0.051 μg/L, with an average of 0.047 ± 0.0034 μg/L. These data assured that the concentration was not varying on the basis of the amount of standard that was added in the standard addition. Therefore, an amount of 0.5 μg/L was used for the spiking solution, $[S]$.

A further challenge in quantitation of the TC was the formation of epimers as a function of the pH of the sample.[26] Chlortetracycline, doxycycline, minocycline, oxytetracycline, and tetracycline exhibited multiple epimers. The five partially separated chlortetracycline epimers for the $[M + H]^+$, $[M + H - NH_3]^+$, and $[M + H - NH_3 - H_2O]^+$ ions are illustrated in Figure 3. Because the epimers are not always clearly defined, particularly in samples with high background noise, only epimer V was used to quantitate chlortetracycline.

The ratios of chlortetracycline epimers changed with fragmentation ion, although epimer V was always the largest. For example, the ion $[M + H - NH_3 - H_2O]^+$ did not exhibit epimer I. Under these conditions, both doxycycline and minocycline exhibited at least three epimers. Only one for each compound was discernible at concentrations less than 0.5 μg/L without matrix enhancement. The last doxycycline epimer and the first minocycline epimer were used for quantitation. Oxytetracycline exhibits two epimers, and the only the second one to elute was used for quantitation. Tetracycline exhibited three epimers, although without signal enhancement from the matrix, only the second one to elute could be seen at concentrations < 0.5 μg/L. This epimer was used to quantitate tetracycline. For all of the TCs, the ratio of epimers for each ion changed very little from sample to sample, which was probably due to the fact that all of the samples were adjusted to pH 2.5 for solid-phase extraction. The surrogate meclocycline did not exhibit epimers.

(25) Furlong, E. T.; Burkhardt, M. R.; Gates, P. M.; Werner, S. L.; Battaglin, W. A. *Sci. Total Environ.* **2000**, *248*, 135−146.

(26) Kennedy, D. G.; McCracken, R. J.; Carey, M. P.; Blanchflower, W. J.; Hewitt, S. A. *J. Chromatogr.* **1998**, *812*, 327−337.

Table 4. Antimicrobials Found in Groundwater and Surface Water Samples Collected from throughout the U.S.[a]

site	CTC	OTC	TCC	SDM	SMT	SMX	STZ
			Groundwater Sample				
groundwater, WA						0.22	
			Surface Water Samples				
Snake Creek, GA	0.15		0.11				
Cuyahoga River, Steele, OH						1.02	
North Dry Creek, Kearny, NE				0.06	0.22		
Suwannee River, GA		0.34					
four surface water samples, KS		0.07−1.34		0.24−15			0.08

[a] The first three samples listed are from a groundwater source, and the remaining samples are from surface water sources. Concentrations are in micrograms/liter.

Antimicrobials in Groundwater and Surface Water. Because SAs are water soluble and demonstrate little chelating ability and have a low binding constant for soil,[27] they have the potential to enter groundwater and surface water rapidly.[27,28] TCs, however, have been shown to be strong chelators[9,23] and sorb to soil strongly.[27] Furthermore, Stuer-Lauidsen et al.[28] found that despite a low octanol−water partitioning coefficient of 0.026 for oxytetracycline, the soil distribution coefficient in sludge was 1990. Therefore, it was hypothesized that although TCs might be detected in surface water, they may not occur in groundwater.

A summary of the antimicrobials detected in 144 water samples collected from April, 1999, through April, 2001, is illustrated in Table 4. Six of the samples collected were from groundwater or spring water sources, and the remainder of the samples were from surface water. TCs and SAs were detected in samples from 9 sites (6% detections) in concentrations ranging from 0.07 to >15 μg/L (Table 4). The majority of the detections were from surface water sites. According to our hypothesis, TCs were not detected in groundwater sites, and only one site contained a detection in groundwater. This sample was a groundwater site from Washington and contained the SA sulfamethoxazole.

Overall, 7 of the 144 groundwater and surface water sites were found to contain SAs, and six sites were found to contain TCs, reflecting the greater mobility of SAs. Chlortetracycline was detected at 0.15 μg/L in one surface water sample, and the most commonly detected TC was oxytetracycline. The concentrations of TCs versus SAs are similar. Data on the amount of each class used in the U.S. are not available. Several collection sites contained both SAs and TCs.

CONCLUSIONS

The analytical method described in this paper provides a means of extracting and analyzing submicrogram-per-liter concentrations of two classes of antimicrobials from water. Sulfonamides (SAs) can be quantitated easily from any water source using a variety of extraction methods, but the unusual behavior of tetracyclines (TCs) requires multiple sample-preparation steps. In addition, because of matrix enhancement, the method of standard addition is required for accurate determination of TC concentrations. Although the specific cause of the matrix enhancement of TCs was not determined, it has been shown to be dependent on tetracycline structure, matrix source, and the presence of natural dissolved organic matter. Work is continuing on this problem.

ACKNOWLEDGMENT

The authors thank Dana Kolpin of the U.S. Geological Survey for sample collection and the Toxic Substances Hydrology Program of the U.S. Geological Survey for supporting this work. The use of brand, firm, or trade names in this paper is for identification purposes only and does not constitute endorsement by the USGS.

Received for review May 7, 2001. Accepted July 19, 2001.

AC010514W

(27) Thurman, E. M.; Lindsey, M. E. Transport of Antibiotics in Soild and Their Potential for Groundwater Contamination. Presented at the 3rd SETAC World Congress, Brighton, U.K., May 22−25, 2000.

(28) Stuer-Lauridsen, F.; Birkved, M.; Hansen, L. P.; Holten Lützhøft, H.-C.; Halling-Sørensen, B. *Chemosphere* **2000**, *40*, 783−793.