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Evaluation of Offline Tandem and Online Solid-Phase Extraction with Liquid Chromatography/Electrospray Ionization-Mass Spectrometry for Analysis of Antibiotics in Ambient Water and Comparison to an Independent Method

Scientific Investigations Report 2007–5021

**U.S. Department of the Interior
U.S. Geological Survey**

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By M.T. Meyer, E.A. Lee, G.M. Ferrell, J.E. Bumgarner, and Jerry Varns

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DIRK KEMPTHORNE, Secretary

U.S. Geological Survey
Mark D. Myers, Director

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Front cover: Photographs showing offline tandem solid-phase extraction (left) and online-solid-phase extraction (right) set up for water samples.

Back cover: Photographs showing two liquid chromatographs/mass spectrometers used to analyze antibiotics.

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Conversion Factors and Abbreviations

Multiply	By	To obtain
acre	4,047	square meter (m^2)
cubic centimeter (cm^3)	0.06102	cubic inch (in^3)
cubic feet per second (ft^3/s)	0.02832	cubic meter per second (m^3/s)
foot (ft)	0.3048	meter (m)
gram (g)	0.03527	ounce (oz)
inch (in.)	25.4	milliliter (mL)
kilopascal (kPa)	0.1450	pound-force per square inch (lb/in^2)
kilovolts (kV)	0.001	volts (V)
liter (L)	0.2642	gallon (gal)
liter per hour (L/h)	0.0063	barrels per hour (bbl/h)
metric ton	2,205	pound (lb)
microgram (µg)	3.527×10^{-8}	ounce (oz)
microliter (µL)	2.642×10^{-7}	gallon (gal)
micrometer (µm)	3.937×10^{-5}	inch (in.)
milligram (mg)	3.53×10^{-5}	ounce (oz)
millimeter (mm)	0.03937	inch (in.)
nanogram (ng)	3.527×10^{-11}	ounce (oz)
ounce, fluid (oz)	0.02957	liter (L)
pound (lb)	453.6	gram (g)
pound per square inch (lb/in^2)	6.895	kilopascal (kPa)

Temperature can be converted to degrees Celsius (˚C) or degrees Fahrenheit (˚F) by the equations:

$$˚C = 5/9\ (˚F - 32)$$

$$˚F = 9/5\ (˚C) + 32.$$

Abbreviated Water-Quality Units

microgram per liter (µg/L)

microgram per milliliter (µg/mL)

microsiemens per centimeter at 25 degrees Celsius (µS/cm)

milligram per liter (mg/L)

milligram per milliliter (mg/mL)

milliliter (mL)

milliliter per minute (mL/min)

millimolar (mM)

nanogram per microliter (ng/µL)

Other Abbreviations or Acronyms Used in this Report

ACE	automated cartridge exchange
ACN	acetonitrile
ACS	American Chemical Society
AFO	animal feeding operation
AR	antibiotic resistant
BLM	beta lactams-macrolides
C_{18}	carbon-18
CAFO	confined animal feeding operation
CAS	Chemical Abstract Service
CCV	continuing calibration verification
EDTA	ethylene diamine tetraacetic acid
ESI	electrospray ionization
HLB	hydrophilic-lipophilic balance cartridge
HPD	high-pressure dispenser
HPLC	high-performance liquid chromatography
kV	kilovolts
LC/ESI-MS	liquid chromatography/electrospray ionization-mass spectrometry
LC/MS/MS	liquid chromatography/mass spectrometry/mass spectrometry
LOQ	limit of quantitation
M3	Cahill and others (2004) pharmaceutical method
MCX	mixed-mode HLB cation exchange cartridge
min	minutes
OGRL	Organic Geochemistry Research Laboratory (Lawrence, Kansas)
R^2	correlation coefficient
RSD	relative standard deviation
SIM	selected-ion monitoring
SPE	solid-phase extraction
SQ	sufonamides-quinolines
TET	tetracyclines
USEPA	U.S. Environmental Protection Agency
USGS	U.S. Geological Survey
cc	cubic centimeter
n	number of samples
V	volts
w/V	weight to volts
±	plus or minus
>	greater than
%	percent

Evaluation of Offline Tandem and Online Solid-Phase Extraction with Liquid Chromatography/Electrospray Ionization-Mass Spectometry for Analysis of Antibiotics in Ambient Water and Comparison to an Independent Method

By M.T. Meyer[1], E.A. Lee[2], G.F. Ferrell[3], J.E. Bumgarner[4], and Jerry Varns[4]

Abstract

This report describes the performance of an offline tandem solid-phase extraction (SPE) method and an online SPE method that use liquid chromatography/mass spectrometry for the analysis of 23 and 35 antibiotics, respectively, as used in several water-quality surveys conducted since 1999. In the offline tandem SPE method, normalized concentrations for the quinolone, macrolide, and sulfonamide antibiotics in spiked environmental samples averaged from 81 to 139 percent of the expected spiked concentrations. A modified standard-addition technique was developed to improve the quantitation of the tetracycline antibiotics, which had "apparent" concentrations that ranged from 185 to 1,200 percent of their expected spiked concentrations in matrix-spiked samples. In the online SPE method, normalized concentrations for the quinolone, macrolide, sulfonamide, and tetracycline antibiotics in matrix-spiked samples averaged from 51 to 142 percent of their expected spiked concentrations, and the beta-lactam antibiotics in matrix-spiked samples averaged from 22 to 76 percent of their expected spiked concentration.

Comparison of 44 samples analyzed by both the offline tandem SPE and online SPE methods showed 50 to 100 percent agreement in sample detection for overlapping analytes and 68 to 100 percent agreement in a presence-absence comparison for all analytes. The offline tandem and online SPE methods were compared to an independent method that contains two overlapping antibiotic compounds, sulfamethoxazole and trimethoprim, for 96 and 44 environmental samples, respectively. The offline tandem SPE showed 86 and 92 percent agreement in sample detection and 96 and 98 percent

agreement in a presence-absence comparison for sulfamethoxazole and trimethoprim, respectively. The online SPE method showed 57 and 56 percent agreement in sample detection and 72 and 91 percent agreement in presence-absence comparison for sulfamethoxazole and trimethoprim, respectively. A linear regression with an R^2 of 0.91 was obtained for trimethoprim concentrations, and an R^2 of 0.35 was obtained for sulfamethoxazole concentrations determined from samples analyzed by the offline tandem SPE and online SPE methods.

Linear regressions of trimethoprim and sulfamethoxazole concentrations determined from samples analyzed by the offline tandem SPE method and the independent M3 pharmaceutical method yielded R^2 of 0.95 and 0.87, respectively. Regressed comparison of the offline tandem SPE method to the online SPE and M3 methods showed that the online SPE method gave higher concentrations for sulfamethoxazole and trimethoprim than were obtained from the offline tandem SPE or M3 methods.

Introduction

Antibiotics are used extensively in human health and agriculture. In agriculture, antibiotics are used primarily in large animal feeding operations (AFOs), but they also are used in aquaculture and to control fungi in fruit orchards (McManus and others, 2002). Although increased prevalence of antibiotic resistant (AR) infections has been attributed primarily to the overprescription of antibiotics and the unwillingness of a substantial number of patients to complete the prescription cycle (Tenover and Hughes, 1996; Cole and others, 2003), there is increasing concern that the routine use of subtherapeutic levels of antibiotics in confined animal feeding operations (CAFOs) could generate strains of pathogenic AR bacteria and AR-resistant human infections (Harrison and Lederberg, 1998; Molbak and others, 1999; Smith and others, 1999). There also is uncertainty as to whether the transport of antibiotics, along

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with other pharmaceuticals, in surface water and ground water from urban and agricultural sources has deleterious effects on water quality (Levy, 1998a).

Data on the annual amount of antibiotics produced in the United States and the percentage of the antibiotics associated with various human and agricultural uses are sparse. In general, annual production of antibiotics in the United States is about 23,000 metric tons (Levy, 1998b). Estimates of the percentage of antibiotics used for human treatment range from less than 50 to 65 percent, whereas estimates on the amount of antibiotics administered to livestock range from 10 to more than 50 percent (Mellon and others, 2001; Animal Health Institute, 2002). A substantial portion of the antibiotics administered to humans or animals is excreted in the active form (Elmund and others, 1971; Feinman and Matheson, 1978; Alcock and others, 1999), and abiotic processes also may revert a portion of the pharmaceutical metabolites back to the parent form (Langhammer and Buening-Pfaue, 1989). In this report, the term metabolite refers to direct and indirect biotic and abiotic degradation products of a parent antibiotic.

A wide range of pharmaceutical compounds are transported into surface and ground water from human and agricultural sources in Europe and the United States (Holm and others, 1995; Buser and others, 1998; Roloff, 1998; Ternes, 1998; Campagnolo and others, 2002; Kolpin and others, 2002). Because of the diversity of antibiotic usage, several sources can transport antibiotics to surface and ground water. These include discharge of treated wastewater, permitted and nonpermitted discharge of raw sewage, unsaturated zone transport and surface runoff from land-applied waste from human and agricultural sources, leakage and flooding from large waste-storage ponds and pits in CAFOs, and leakage from septic systems. Determination of whether a relation between AR bacterial populations, or human and ecosystem health, and the occurrence of antibiotics in surface and ground water are important areas of research (Meyer, 2004). The development of analytical methods capable of identifying and quantifying low concentrations of pharmaceutical compounds in surface and ground water is an essential component in addressing this problem.

Although many methods have been developed for the analysis of antibiotics in food, serum, and urine over the last two decades, only since the late 1990s have researchers begun to develop a range of sensitive methods to study pharmaceuticals, including antibiotics, in environmental samples. For example, Hirsch and others (1998) used lypholization and liquid chromatography/tandem mass spectrometry (LC/MS/MS) to analyze for compounds from the beta lactam, macrolide, sulfonamide, and tetracycline classes of antibiotics in surface water. They also tested a C_{18} solid-phase extraction (SPE) method for the analysis of multiple classes of antibiotics. Lindsey and others (2001) used the Waters (Milford,

Massachusetts) Oasis hydrophilic-lipophilic balance (HLB) SPE cartridge and an LC/MS method to analyze sulfonamide and tetracycline antibiotics in surface water. Other methods published since 2000 have used various SPE extraction methodologies and LC/MS/MS. Most of the methods for analysis of antibiotics in water by SPE and LC/MS/MS or LC/MS were developed by Meyer and others (2000), Bruno and others (2001), Golet and others (2001), Ternes and others (2001), Zhu and others (2001), Christian and others (2003), Grant and others (2003), Loffler and Ternes (2003), Reverte and others (2003), Snow and others (2003), Turiel and others (2003), and Cahill and others (2004).

This report describes and compares two independently developed antibiotic methods: (1) an offline tandem-SPE, LC/electrospray-ionization (ESI) MS method, and (2) an online single-pass SPE LC/ESI-MS method of which both were used to examine the occurrence, fate, and transport of human and agriculturally used antibiotics in surface and ground water. The offline tandem SPE method of analysis for antibiotics was used from 1999 to 2003 to analyze more than 1,000 surface- and ground-water samples from national stream-water reconnaissance studies, watershed and field-scale studies on antibiotic and antibiotic-resistant bacterial transport from urban and agricultural sources in multiple States, and wastewater outfall transport studies. The online SPE method of analysis for antibiotics was used from 2000 to 2003 to analyze more than 500 samples from similar studies. A suite of antibiotic compounds (table 1) used for human health, the health and management of agricultural livestock, and veterinary medicine was selected for study. The goals of developing these methods were: (1) to examine the occurrence of antibiotics in surface water downstream from large CAFOs (results in Meyer and others, 2000; Campagnolo and others, 2002); (2) to examine the occurrence of antibiotics in susceptible watersheds on a national scale (Kolpin and others, 2002); and (3) to assess whether selected antibiotic compounds can be useful discriminators of contaminant sources. Samples analyzed by these two methods also were compared with the same samples analyzed by an independently developed SPE LC/ESI-MS method that has two commonly detected antibiotic compounds that overlap with the two methods described in this report.

Acknowledgments

Experimental Section

Materials

The antibiotic compounds analyzed by the offline tandem SPE and online SPE-LC/MS methods, their molecular weight, Chemical Abstract Service numbers, and general animal and human usage are shown in table 1. The analytical standards for amoxicillin, ampicillin, carbadox, cefotaxime, chlortetracycline, cloxacillin, demeclocycline, doxycycline, enrofloxacin, erythromycin, flumequine, lincomycin, lomefloxacin, minocycline, norfloxacin, ofloxacin, oxacillin, oxolinic acid, oxytetracycline, penicillin G, penicillin V, roxithromycin, sulfachlorpyridazine, sulfadiazine, sulfadimethoxine, sulfamerazine, sulfamethazine, sulfamethizole, sulfamethoxazole, sulfathiazole, tetracycline, trimethoprim, tylosin, and virginiamycin, and surrogate standards meclocycline, nalidixic acid, and oleandomycin were obtained from Sigma Aldrich (St. Louis, Missouri); ciprofloxacin from Research Diagnostics (Flanders, New Jersey) and Fluka (Switzerland); clinafloxacin from Axxora (San Diego, California); ormetoprim from Alpharma Inc. (Fort Lee, New Jersey); sarafloxacin from Abbott Laboratories (Shawnee Mission, Kansas); sulfamethazine-$^{13}C_6$ and erythromycin-$^{13}C_1$ (surrogate standards) from Cambridge Isotopes (Andover, Massachusetts); terbuthylazine (internal standard) from Supelco (Bellefonte, Pennsylvania); and simetone (internal standard) from the U.S. Environmental Protection Agency (USEPA) repository. The purity of all the standards was greater than 90 percent, except for chlortetracycline (75 percent) and tetracycline (80 percent). Because of the lack of stable isotope labeled antibiotic standards, unlabeled antibiotics that either were not registered for use or are low-use antibiotics in the United States were chosen for use as surrogate standards. Acetic acid, ammonium acetate, ammonium formate, formic acid, Na_2-EDTA, and Na-hydroxide were all American Chemical Society (ACS) certified. Acetonitrile and methanol were high-performance liquid chromatography (HPLC) grade.

Sample Collection

Water samples were collected using established U.S. Geological Survey (USGS) protocols (Shelton and Capel, 1994). All water samples were filtered in the field or in the laboratory using baked (450 ˚C for 8 hours), 0.7-µm glass-fiber filters and stored in 125-mL or 1-L amber, glass bottles. For the offline tandem SPE method, all samples were shipped overnight to the USGS, North Carolina Water Science Center, Raleigh, North Carolina (1998–2000), or the Florida Integrated Science Center, Ocala Water Quality and Research Laboratory, Ocala, Florida (2000–03). For the online SPE method, all samples were shipped overnight to the USGS, Kansas Water Science Center, Organic Geochemistry Research Laboratory (OGRL), Lawrence, Kansas. All samples were logged in and stored at 4 ˚C until they were prepared for

extraction. Samples generally were extracted within 14 days after they were received at the laboratory.

Offline Tandem Solid-Phase Extraction

Sample Preparation

For the offline tandem SPE method, individual 1-mg/mL stock solutions of each analyte were prepared in methanol or reagent-grade water with 20 mM ammonia acetate, pH 5.6 standard units and stored at -10 ˚C. An antibiotic standard mix solution containing 2.5 ng/µL of each analyte and a surrogate standard mix containing 2.5 ng/µL sulfamethazine-$^{13}C_6$, erythromycin-$^{13}C_1$, and meclocycline were prepared in 20 mM ammonia acetate, pH 5.6 standard units. A 10-ng/µL internal standard solution of terbuthylazine was prepared in methanol. All solutions were stored in amber glass bottles at –10 ˚C. Acetonitrile and methanol were HPLC grade from Fisher (Pittsburgh, Pennsylvania). Ammonia hydroxide and Na_2-EDTA were ACS certified. Erythromycin and erythromycin-$^{13}C_1$ were converted to erythromycin-H_2O and erythromycin-$^{13}C_1$-H_2O by acidifying the fortified standard solutions and samples to pH 3.0 standard units with (H_2SO_4) prior to extraction. Reagent-grade water was generated using a deionized water system with two mixed-bed resins and one activated carbon cartridge following treatment using a Barnstead or Millipore cartridge water treatment with ultraviolet disinfection and 0.2-µm filtration.

For the offline tandem SPE method, antibiotic compounds were extracted from water samples using a tandem SPE procedure. This SPE method used a Waters Oasis HLB and mixed-mode cation exchange (MCX) (6 cc, 200 mg) SPE cartridges conditioned with 2 mL reagent water, 2 mL methanol, 2 mL methanol with 5 percent ammonia hydroxide, 2 mL reagent water, and 2 mL pH 3.0 standard units (H_2SO_4) reagent water. Aliquots (500 mL) of each water sample were prepared in 500-mL baked, glass amber bottles for extraction by adding 5 mL of a 0.1-mg/mL solution of Na_2-EDTA and adjusting the pH to 3.0 using concentrated H_2SO_4. Each sample then was spiked with 50 µL of the 2.5-ng/µL surrogate standard mix. The MCX cartridges then were connected into the valves of a 24-port vacuum rack, and the HLB cartridges were attached to the top of the MCX cartridges using large-volume SPE adapters (fig. 1) with the tubing nuts bored out to 3/8 in. to accept the tip of the SPE cartridge. A large-volume sampling line was connected to the HLB cartridge, and the sample passed through the HLB and MCX cartridges with a vacuum of 20 mm Hg. The HLB and MCX cartridges for each sample then were rinsed individually with 2 mL reagent water. For the elution step of the method, the HLB and MCX SPE cartridges were stacked in reverse order with the MCX stacked on top of the HLB cartridge. First, the HLB cartridge was attached to a vacuum rack with disposable Teflon sleeves and loaded with 2 mL methanol. Then, the MCX cartridge was attached to the

Table 1. Antibiotics selected for study, their molecular weight, Chemical Abstract Service number, and general human and animal usage.

Compound	Molecular weight (grams)	Chemical Abstract Service number	General human and animal usage
Beta lactams			
Amoxicillin	365.4	26787–78–01	human, cats, cattle
Ampicillin	349.4	69–53–4	dogs, cats, cattle, human
Cefotaxime	477.4	64485–93–4	human
Cloxacillin	475.9	7081–44–9	human, cattle
Oxacillin	401.4	7204–38–2	cattle, human
Penicillin G	356.4	69–57–8	human, swine, cattle
Penicillin V	350.4	87–08–1	human
Macrolides			
Erythromycin	735.9	114–07–8	humans, poultry, swine
Erythromycin-H_2O	715.5	114–07–8	erythromycin degradate
Roxithromycin	837.1	80214–83–1	humans
Tylosin	916.1	1401–69–0	chickens, swine, cattle
Virginiamycin	525.6	21411–53–0	poultry, swine, cattle
Quinolones			
Ciprofloxacin	331.4	85721–33–1	human, swine, chickens
Clinafloxacin	365.8	105956–97–6	human
Enrofloxacin	359.4	93106–60–6	cattle, swine, poultry, dogs, cats
Flumequine	261.3	42835–25–6	cattle, swine, chickens, fish
Lomefloxacin	351.3	98079–51–7	human
Norfloxacin	319.3	70458–96–7	human, poultry
Ofloxacin	361.4	82419–36–1	poultry, human
Oxolinic acid	261.2	14698–29–4	cattle, swine, chickens, fish
Sarafloxacin	385.4	98105–99–8	poultry, fish
Sulfonamides			
Sulfachloropyrizadine	284.7	80–32–0	swine, calves, dogs
Sulfadiazine	250.3	68–35–9	horses, humans
Sulfadimethoxine	310.3	122–11–2	fish, poultry
Sulfamerazine	264.3	127–79–7	fish, poultry
Sulfamethazine	283.5	57–68–1	swine, cattle
Sulfamethizole	270.3	144–82–1	dogs, cats
Sulfamethoxazole	253.3	723–46–6	human
Sulfathiazole	255.3	72–14–0	swine
Tetracyclines			
Chlorotetracycline	515.3	57–62–5	swine, poultry, cattle, sheep, ducks
Demeclocycline	501.3	127–33–3	human, cattle
Doxycycline	480.9	564–25–0	human, dog
Minocycline	493.9	10118–90–8	human, cattle
Oxytetracycline	496.9	79–57–2	poultry, fish, swine, cattle, sheep, bees, lobsters
Tetracycline	480.9	60–54–8	humans, dogs, cattle
Others			
Carbadox	262.2	1789875	swine
Lincomycin	406.5	154–21–2	poultry, swine
Ormetoprim	274.3	6981–18–6	fish, poultry
Trimethoprim	290.3	738–70–5	human, dogs, horses

(A) Solid-phase extraction setup for water samples

Solid phase extraction adapter and sample tube

Oasis hydrolphilic-lipophilic balance solid-phase extraction cartridge (HLB)

SPE adapter

Mixed-mode cation exchange solid-phase extraction cartridge (MCX)
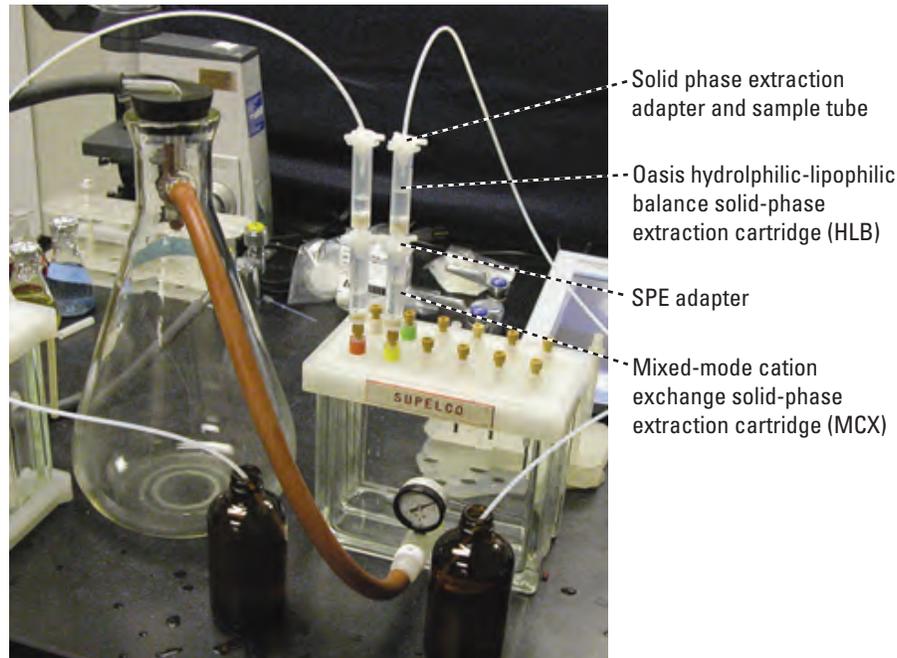
(B) Solid-phase elution setup for extracted water samples

Mixed-mode cation exchange solid-phase extraction cartridge (MCX)

SPE adapter

Oasis hydrolphilic-lipophilic balance solid-phase extraction cartridge (HLB)

Elution vacuum rack with disposable teflon sleeves

Elution test tube
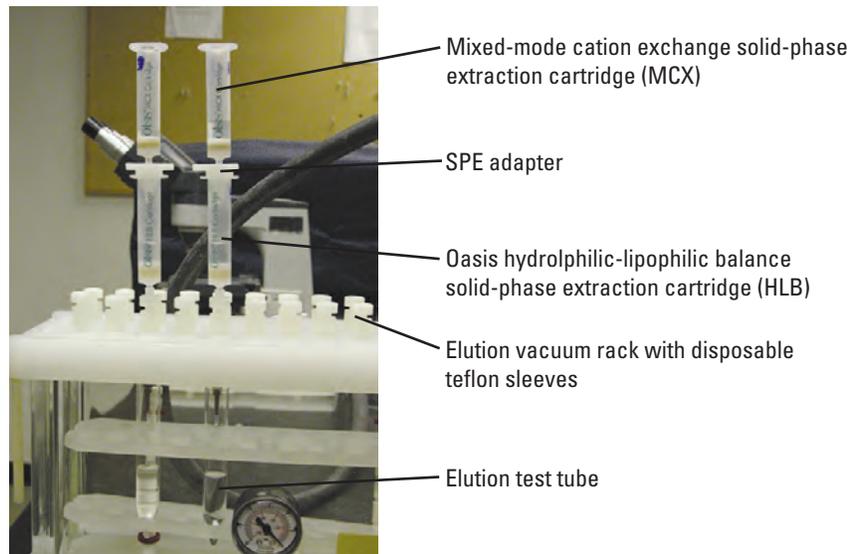
Figure 1. Solid-phase extraction apparatus and setup for extraction of antibiotics.

top of the HLB cartridge and also loaded with 2 mL methanol. The cartridges then were eluted into a 15-mL glass, conical-bottom test tube. Both cartridges were eluted with additional 2- x 3-mL aliquots of methanol followed by the elution of the MCX cartridge separately to prevent the complete elution of all the humic substances adsorbed to the HLB cartridge with 2 mL of a 5-percent ammonia hydroxide-methanol solution. The sample eluate then was spiked with 50 µL of the 10-ng/µL terbuthylazine internal standard solution. Sample eluates were vortexed and evaporated at 50 ˚C under a gentle stream of nitrogen (15 lb/in^2) to a volume of approximately 20 µL using a Zymark TurboVap (Hopkinton, Massachusetts). Three hundred microliters (300 µL) of 20 mM ammonia acetate reagent water (pH 5.6 standard units) then were added to the sample eluate, vortexed, and transferred to a glass chromatography vial. The sample eluates were stored at –10 ˚C until analysis.

Sample sets consisted of 10 water samples, a matrix-spiked sample, a duplicate water sample, two fortified reagent-water samples, and two reagent-water blanks.

Sample Analysis

Samples were analyzed using liquid chromatography/electrospray-mass spectrometry (LC/ESI-MS) in positive-ion mode using selected-ion monitoring (SIM). Samples were analyzed for the macrolides and sulfonamides using either a Hewlett Packard (now Agilent Technologies, Inc., Palo Alto, California) 1100 series-1 LC/MS or Waters Corporation (Milford, Massachusetts) 2695 LC/ZQ MS. Quinolone and tetracycline antibiotics were analyzed using the Waters LC/MS system. The macrolide and sulfonamide classes of antibiotics along with carbadox, lincomycin, and trimethoprim were analyzed using the Hewlett Packard system utilizing a

Table 2. Mobile-phase composition and gradient for sulfonamide-macrolide and quinolone-tetracycline liquid chromatography/mass spectrometry analysis.

[mM, millimolar; ACN, acetonitrile]

Time (minute)	Mobile-phase A (percent)	Mobile-phase B (percent)
Sulfonamide-macrolide analysis		
0	95	5
2	95	5
7	90	10
16	64	36
20	48	52
23	0	100
26	0	100
28	95	5
38	95	5

Mobile-phase A: 90/10 (20 mM ammonia acetate pH 5.6/ACN)
Mobile-phase B: 20/80 (mobile phase ACN)

Time (minute)	Mobile-phase A (percent)	Mobile-phase B (percent)
Quinolone-tetracycline analysis		
0	91	9
5	91	9
15	58	42
20	0	100
23	0	100
25	91	9
35	91	9

Mobile-phase A: 90/10 (20 mM NH_4-formate, 0.3-percent formic acid/methanol)
Mobile-phase B: (methanol with 20 mM NH_4-formate, 0.5-percent formic acid)

gradient separation (table 2) modified from Hirsch and others (1998) with a Luna 3.0- x 150-mm, 3.5-μm phenylhexyl column (Phenomenex, Torrance, California). The quinolone and tetracycline compounds were analyzed using a gradient separation similar to Lindsey and others (2001; table 2B) with a Phenomenex Luna 3.0- x 150-mm, 3.0-μm C_8(2), or Waters 3.0- x 150-mm, 3.5-μm C18 MS Xterra column.

The LC analysis conditions were as follows: sample injection volume, 20 μL; flow rate, 0.3 mL/min; autosampler temperature (Waters, Milford, Massachusetts, only), 20 ˚C; and column heater temperature, 50 ˚C. The MS conditions were as follows: Waters ZQ, drying gas, 500 L/h; cone gas, 50 L/h; capillary voltage, 3.0 kV; extractor voltage, 0; RF lens voltage, 0.3; source temperature, 100 ˚C; desolvation temperature, 220 ˚C; low and high mass resolution, 15.0; and ion energy, 0.3. For the Hewlett Packard 1100 MS: capillary voltage, 3.0 kV; drying gas, 45 lb/in^2; and nebulizer, 15 lb/in^2.

Compounds were identified using the retention time (± 0.1 min) relative to the nearest surrogate or internal standard and the ratio (± 25 percent) of the integrated areas of the quantitation ion to each of the confirming ions (table 3). One to two confirming ions were used for each compound. Structural information on the confirming ions is provided in table 3. The structures were identified from existing literature (Kagan and Grostic, 1972; Niessen, 1998; Lindberg and others, 2004) and structural elucidation using ChemDraw software (Cambridge, Massachusetts).

Samples were quantified using a linear-regressed duplicate multiple-point standard curve. The standard curve was constructed by fortifying duplicate 500-mL reagent-grade water samples with the antibiotic standard mix at 0.0, 0.01, 0.02, 0.05, 0.10, 0.20, 0.50, and 1.0 μg/L. The solutions then were prepared and extracted using the same SPE procedure as the water samples. The correlation coefficients exceeded 0.99 for all of the analytes. For high-response compounds, the quinolones, lincomycin, sulfadimethoxine, and trimethoprim standard curves were constructed using the 0.01- through 0.50-μg/L standards. In cases where the y-intercept was greater than the lowest quantifying standard, a separate low-level standard curve was constructed using the 0.01- through 0.05-μg/L standards. For the other compounds, standard curves were constructed using the 0.02- or 0.05- through 1.0-μg/L solutions. Concentrations of detected analytes were calculated using the ratio of the integrated areas of the quantifying ion of the analyte to the quantifying ion of the internal standard (terbuthylazine). The limit of quantitation (LOQ), or reporting level, for each analyte ranged from 0.03 to 0.10 μg/L for the Hewlett Packard LC/MS analyses and from 0.01 to 0.05 μg/L for the Waters LC/MS (table 3). Quantitation was performed using Target 4.0 (Thru-Put Systems, Boca Raton, Florida) and MassLynx 3.5 (Waters Corp., Milford, Massachusetts) for the Hewlett Packard and Waters LC/MS data, respectively.

Table 3. List of base peak and confirming ions for antibiotic mass spectral analysis for offline tandem solid-phase extraction method.

[LC/MS, liquid chromatography/mass spectrometry; M+H, molecular plus hydrogen; µg/L, micrograms per liter; --, not determined]

Compound	Hewlett Packard LC/MS reporting level (µg/L)	Waters LC/MS reporting level (µg/L)	Quantitation ion	Quantitation ion fragment	Confirming ion 1	Confirming ion 1 fragment, reference	Confirming ion 2	Confirming ion 2 fragment, reference
Macrolides								
Erythromycin-H$_2$O	0.05	0.02	716.5	M+H	558.5	M-desosamine-H$_2$O+H,[1]	158.1	desosamine+H,[1]
Roxithromycin	.05	.02	837.7	M+H	679.4	M-desosamine+H,[1]	158.1	desosamine+H,[1]
Tylosin	.05	.02	916.6	M+H	772.4	--	174.1	M-4,5-Dimethoxy-2-methyl-tetrahydro-pyran-3-ol-H
Quinolones								
Ciprofloxacin	--	.01	332.1	M+H	288.2	M-CO$_2$+H,[2]	231.1	M-H$_2$O-N-methylpyrrolidine,[3]
Enrofloxacin	--	.01	360.3	M+H	316.1	M-CO$_2$+H,[2]	342.1	M-H$_2$O-N-methylpyrrolidine,[3]
Norfloxacin	--	.01	320.1	M+H	276.1	M-CO$_2$+H,[2]	231.1	M-H$_2$O-N-methylpyrrolidine,[3]
Sarafloxacin	--	.01	386.1	M+H	342.1	M-CO$_2$+H,[2]	299.3	M-H$_2$O-N-methylpyrrolidine,[3]
Sulfonamides								
Sulfachloropyrizadine	.10	.05	285.1	M+H	156.0	NH$_2$C$_6$H$_5$SO$_2$,[3]	287.1	Chlorine 37 isotope
Sulfadimethoxine	.05	.02	311.1	M+H	156.0	NH$_2$C$_6$H$_5$SO$_2$,[3]	245.1	M-H$_2$SO$_2$
Sulfamerazine	.05	.02	265.1	M+H	156.0	NH$_2$C$_6$H$_5$SO$_2$,[3]	108.1	aminophenyl,[3]
Sulfamethazine	.05	.02	279.1	M+H	156.0	NH$_2$C$_6$H$_5$SO$_2$,[3]	186.1	CH$_3$OC$_4$N$_2$NH$_2$SO$_2$,[3]
Sulfamethizole	.10	.05	271.0	M+H	156.0	NH$_2$C$_6$H$_5$SO$_2$,[3]	108.1	aminophenyl,[3]
Sulfamethoxazole	.10	.05	254.1	M+H	156.0	NH$_2$C$_6$H$_5$SO$_2$,[3]	108.1	aminophenyl,[3]
Sulfathiazole	.10	.05	256.0	M+H	156.0	NH$_2$C$_6$H$_5$SO$_2$,[3]	108.1	aminophenyl,[3]
Tetracyclines								
Chlorotetracycline	--	.05	479.2	M+H	481.2	Chlorine 37 isotope	462.2	Chlorine 37 isotope
Demeclocycline	--	.05	465.2	M+H	448.1	M-NH$_3$+H,[2]	430.0	M-H$_2$O-NH$_3$+H,[1]
Doxycycline	--	.05	445.2	M+H	428.2	M-NH$_3$+H,[2]	--	--
Minocycline	--	.05	458.2	M+H	441.1	M-NH$_3$+H,[2]	352.1	--
Oxytetracycline	--	.05	461.2	M+H	426.2	M-H$_2$O-NH$_3$+H,[1]	443.3	M-NH$_3$+H,[2]
Tetracycline	--	.05	445.1	M+H	428.2	M-NH$_3$+H,[3]	410.1	M-H$_2$O-NH$_3$+H,[1]
Others								
Carbadox	.10	.05	231.1	M-OCH$_2$	263.0	M+H	--	--
Lincomycin	.05	.02	407.3	M+H	126.2	1-Methyl-3-propyl-pyrrolidin,[4]	--	--
Trimethoprim	.03	.02	291.1	M+H	230.2	M-2(CH$_3$O)+H,[2]	--	M-trimethoxyphenyl,[1]
Surrogate and internal standards								
Sulfamethazine-^{13}C$_6$	--	--	285.0	M+H	186.1	--	284.1	--
Erythromycin-^{13}C$_1$-H$_2$O	--	--	717.5	M+H	559.5	--	159.1	--
Meclocycline	--	--	477.2	M+H	479.2	--	460.2	--
Terbuthylazine	--	--	230.0	M+H	232.0	--	174.0	--

[1] Hirsch and others (1998).
[2] Lindberg and others (2004).
[3] Niessen (1998).
[4] Kagan and Grostic (1972).

Online Solid-Phase Extraction

Sample Preparation

For the online SPE method, 1 mg/mL stock solutions of each analyte were prepared in methanol or 50/50 methanol with 0.1 M Na-hydroxide for the quinolones. Antibiotics were analyzed in three groups—the beta lactams-macrolides (BLM) along with carbadox, lincomycin, ormetoprim, and trimethoprim; quinolones-sulfonamides (SQ); and tetracyclines (TET) (table 4). A separate 1.0-ng/μL standard mix and surrogate standard mix containing the appropriate analytes for each analysis group were used. The surrogate standard for the BLM group was oleandomycin, for the SQ group it was $^{13}C_6$-sulfamethazine and nalidixic acid, and for the TET group the surrogate standard was meclocycline. A 1-ng/mL working standard mix for each antibiotic group was made by diluting 123 μL of the standard mix for the appropriate group into 123 mL of reagent-grade water in a 125-mL baked, amber glass bottle. A working surrogate standard mix for each antibiotic group was prepared by diluting 500 μL of the surrogate standard mix for the appropriate group into 75 g of reagent-grade water for the BLM and SQ groups and 65 g of reagent-grade water plus 10 mL of a 5-percent EDTA solution for the TET group in a 125-mL baked, amber glass bottle. The working internal standard mix (simetone) was prepared by diluting the 1.23-ng/μL internal standard mix 20:1 for the BLM and SQ groups and 40:1 for the TET groups to a final volume of 2 mL in reagent-grade water. A matrix-spike solution was prepared for each antibiotic group by diluting the 1.0-ng/μL standard mix 10:1 to a final volume of 1 mL in reagent-grade water in a 2-mL glass chromatography vial.

Water samples were prepared by pipetting 10-mL sample aliquots into 10-mL glass vials (Chromocol/SunSri, Wilmington, North Carolina). A duplicate sample and matrix-spike sample also were prepared for each set of 10 samples. Blanks solutions were prepared by adding 10 mL of reagent-grade water into 10-mL glass vials. Matrix-spiked samples were prepared by adding 50 μL of the matrix-spike solution to each of the designated samples. Standard solutions were prepared at concentrations of 0.05, 0.10, 0.20, 0.50, and 1.0 μg/L using the volumes of reagent-grade water and working antibiotic working standard mix listed in table 5. Reagent-grade water continuing calibration verification (CCV) samples were prepared at 1.0 μg/L. Working surrogate standard mix for the appropriate group (750 μL) was added to all the samples, standards, and blanks. The 10-mL vials then were capped, mixed, and stored at 2-4 ˚C until analysis.

A full sample run consisted of a five-point standard curve, five reagent-water blanks, two 1.0-μg/L CCVs, four duplicate samples, four 1.0-μg/L matrix-spike samples, and 40 environmental samples. A duplicate sample, a matrix spike, and a blank were placed after every 10 environmental samples, and in addition, a 1.0-μg/L CCV sample was placed after every 20 environmental samples. Just prior to analysis the sample vials were loaded onto the online SPE instrument (Spark-Holland, Netherlands). The prepared samples in the 10-mL vials were placed in 31 of the 32 sample positions in the autosampler tray. Ten milliliters (10 mL) of the appropriate modifier solution (table 6) were added to a 10-mL glass vial and placed in the 32nd position of the autosampler. Table 6 also shows the solutions used for the high-pressure dispenser (HPD) of the online SPE instrument for each of the three antibiotic groups. HLB Prospekt SPE cartridges (Waters, Milford, Massachusetts) were used for the BLM and SQ analyses; the glyphosate, mixed-mode SPE cartridge (proprietary, Spark-Holland, Netherlands) was used for the TET analysis. The NaOH-EDTA solution (table 6) was used to rinse and deactivate the sample lines on the HPD. The SPE cartridges were prepared in the loading clamp on the automated cartridge exchange (ACE) unit by rinsing with 1 mL of acetonitrile (ACN) and 1 mL of reagent-grade water. Just prior to sample loading the autosampler added 250 μL of the modifier solution to the sample vial and then mixed the sample by aspirating and dispensing 5 mL of the sample two times. Then 10 mL of the prepared sample were pumped from the sample vial and pushed through the SPE cartridge by the HPD. The cartridge then was transferred to the elution clamp of the ACE.

Sample Analysis

Sample analyses were performed using LC/ESI-MS in positive-ion mode using selected-ion monitoring on the Agilent 1100 LC and Model D LC/MSD Mass Spectrometer. After the cartridge was placed in the elution clamp, the compounds were eluted by the HPD with 750 μL of acetonitrile as the LC binary pump, mobile-phase mixture was routed through the SPE cartridge. An isocratic pump was used to add aqueous solution to dilute out the organic solvent from the binary mobile phase to focus the compounds on the head of the LC column. The flow rates and mobile-phase composition for each of the antibiotic groups are shown in table 7. The LC autosampler injected 20 μL of the working internal standard solution as soon as the SPE cartridge elution was started. The compounds for all three antibiotic groups were separated on a 150- X 3.0-mm Luna C_{18} (2) LC column with 30-μm stationary phase at a temperature of 50 °C. The mass spectrometer conditions were as follows for the Hewlett Packard 1100 MS: capillary voltage, 3.0 kV; drying gas, 45 lb/in²; and nebulizer, 15 lb/in².

Compounds were identified using the retention time (±0.1 min) relative to the nearest surrogate or internal standard and the ratio (± 25 percent) of the integrated areas of the quantitation ion to each of the confirming ions (table 4). One to two confirming ions were used for each compound. Structural information on the confirming ions is provided in table 4. The structures were identified from existing literature (Kagan and Grostic, 1972; Niessen, 1998; Lindberg and others, 2004) and structural elucidation using ChemDraw software (Cambridge, Massachusetts).

Table 4. List of base peak and confirming ions for antibiotic mass spectral analysis for online solid-phase extraction method.

[LC/MS, liquid chromatography/mass spectrometry; M+H, molecular plus hydrogen; μg/L, micrograms per liter; --, not determined]

Compounds	Hewlett Packard LC/MS reporting level (μg/L)	Quantitation ion	Quantitaton ion fragment	Confirming ion 1	Confirming ion 1 fragment, reference	Confirming ion 2	Confirming ion 2 fragment, reference
Beta lactams							
Amoxicillin	0.20	366	M+H	349	--	--	--
Ampicillin	.10	350	M+H	106	--	160	--
Cefotaxime	.10	456	M+H	396	--	--	--
Cloxacillin	.10	436	M+H	438	--	--	--
Oxacillin	.10	402	M+H	160	--	--	--
Penicillin G	.10	335	M+H	160	--	--	--
Penicillin V	.10	351	M+H	160	--	--	--
Macrolides							
Erythromycin-H_2O	.05	716	M+H	558	--	158	--
Erythromycin	.05	734	M+H	576	--	158	--
Roxithromycin	.05	837	M+H	679	--	158	--
Tylosin	.05	916	M+H	174	--	--	--
Virginiamycin	.05	526	M+H	508	--	548	--
Quinolines							
Ciprofloxacin	.05	332	M+H	314	M+H-18	--	--
Clinafloxacin	.05	366	M+H	368	chlorine 37 isotope	--	--
Flumequine	.05	262	M+H	244	M+H-18	--	--
Lomefloxacin	.05	352	M+H	334	M+H-18	--	--
Norfloxacin	.05	320	M+H	302	M+H-18	--	--
Ofloxacin	.05	362	M+H	318	M+H-18	--	--
Oxolinic Acid	.05	262	M+H	244	M+H-18	--	--
Sarafloxacin	.05	386	M+H	368	M+H-18	--	--
Sulfonamides							
Sulfachloropyridazine	.05	285	M+H	156	$NH_2C_6H_5SO_2$,[1]	--	--
Sulfadiazine	.05	251	M+H	156	$NH_2C_6H_5SO_2$,[1]	--	--
Sulfadimethoxine	.05	311	M+H	156	$NH_2C_6H_5SO_2$,[1]	--	--
Sulfamerazine	.05	265	M+H	156	$NH_2C_6H_5SO_2$,[1]	--	--
Sulfamethazine	.05	279	M+H	156	$NH_2C_6H_5SO_2$,[1]	--	--
Sulfamethoxazole	.05	254	M+H	156	$NH_2C_6H_5SO_2$,[1]	--	--
Sulfathiazole	.05	256	M+H	156	$NH_2C_6H_5SO_2$,[1]	--	--
Tetracyclines							
Anhydro-chlorotetracycline	.10	461	M+H	444	--	--	--
Anhydro-tetracycline	.10	427	M+H	410	--	--	--
Chlorotetracycline	.10	479	M+H	481	chlorine 37 isotope	--	--
Demeclocycline	.10	465	M+H	448	M-NH_3+H,[2]	--	--
Doxycycline	.10	445	M+H	428	M-NH_3+H,[2]	--	--
Epi-chlorotetracycline	.10	479	M+H	481	chlorine 37 isotope	--	--

Table 4. List of base peak and confirming ions for antibiotic mass spectral analysis for online solid-phase extraction method.—Continued

[LC/MS, liquid chromatography/mass spectrometry; M+H, molecular plus hydrogen; μg/L, micrograms per liter; --, not determined]

Compounds	Hewlett Packard LC/MS reporting level (μg/L)	Quantitation ion	Quantitaton ion fragment	Confirming ion 1	Confirming ion 1 fragment, reference	Confirming ion 2	Confirming ion 2 fragment, reference
Tetracyclines—Continued							
Epi-iso-chlorotetracycline	0.10	479	M+H	481	chlorine 37 isotope	--	--
Epi-oxytetracycline	.10	461	M+H	444	--	--	--
Epi-tetracycline	.10	445	M+H	428	--	--	--
Iso-chlorotetracycline	.10	479	M+H	481	chlorine 37 isotope	--	--
Minocycline	.10	458	M+H	441	M-NH$_3$+H,[2]	--	--
Oxytetracycline	.10	461	M+H	444	M-NH$_3$+H,[2]	--	--
Tetracycline	.10	445	M+H	428	M-NH$_3$+H,[1]	--	--
Other							
Carbadox	.05	263	M+H	231	--	--	--
Lincomycin	.05	407	M+H	126	--	359	--
Ormetoprim	.05	275	M+H	259	--	--	--
Trimethoprim	.05	291	M+H	261	--	230	--
Surrogates and internal standards							
Sulfamethazine-^{13}C$_6$	--	285	M+H	162	--	--	--
Meclocycline	--	477	M+H	460	--	--	--
Naxidilic acid	--	233	M+H	215	--	--	--
Oleandomycin	--	688	M+H	544	--	--	--
Simetone	--	198	M+H	--	--	--	--

[1] Niessen (1998).

[2] Lindberg and others (2004).

Samples were quantified using a five-point standard curve with a quadratic fit. Concentrations of detected analytes were calculated using the ratio of the integrated areas of the quantifying ion of the analyte to the quantifying ion of the internal standard (terbuthylazine). The limit of quantitation (LOQ) or reporting level for each analyte ranged from 0.05 to 0.10 μg/L. Quantitation was performed using Target 4.0 software.

Evaluation of Analytical Methods for Analysis of Antibiotics

Offline Tandem Solid-Phase Extraction

Figure 2 shows SIM chromatograms of an extracted sample spiked at 0.20 μg/L for the offline tandem SPE method for analysis of sulfonamide-macrolide and quinolone-tetracycline antibiotics. These data show that the chromatography and mass-spectral signatures adequately identify the antibiotic compounds reported using this method. During methods development, erythromycin was not detected in any of the acidified, spiked reagent-water, or environmental samples. Thus, it was assumed that the erythromycin was quantitatively converted to the dehydrated degradation product (erythromycin-H$_2$O).

Table 8 shows the general performance data for this method, which show that, except for lincomycin and to a lesser extent doxycycline, the HLB cartridge retained more than 95 percent of the analytes in spiked distilled water samples. The MCX cartridge was used for two reasons: (1) the substantial breakthrough of lincomycin, which is an extensively used antibiotic in agriculture, using the HLB cartridge and the high retention of lincomycin on the MCX cartridge; and (2) to provide extra ion-exchange and sorption capacity for varying matrices that potentially could affect the retention of the more basic compounds on the HLB cartridge. The recovery of the 23 antibiotic compounds and the long-term (1999–2003) variation of fortified reagent-water samples and spiked surface- and

Table 5. Proportions of blank solution to working standard mix used to prepare standard solutions.

[μg/L, micrograms per liter; mL, milliliter]

Concentration (μg/L)	Volume (mL)	
	Blank solution	Working standard mix (1.0 μg/L)
0	10	--
.05	9.5	0.50
.10	9.0	1.0
.20	8.0	2.0
.50	5.0	5.0
1.0	0	10

ground-water samples from their theoretical spiked concentrations also are shown in table 8.

The recovery of each antibiotic compound was determined for the offline tandem SPE method by regressing 20 extracted spiked reagent-water samples against a duplicate external multipoint standard curve. The standard solutions were fortified, in duplicate, at 0.05, 0.1, and 1.0 μg/L; seven samples were spiked at 0.20 μg/L, and seven others were spiked at 0.50 μg/L. The recoveries were calculated by regressing the data for each analyte from the extracted samples against the external standard curve. Matrix effects of pharmaceuticals in different solvent systems have been reported previously (Vargo and Whichman, 2003). To minimize potential matrix effects that may confuse recovery calculations, the external standards were made by adding the same solvents and volumes used to elute the SPE cartridges to test tubes. The solutions were spiked with same amount of antibiotic-, surrogate-, and internal-standard solutions as were used to generate the extracted standard curves. The spiked solutions then were evaporated to approximately 20 μL and brought to a

final volume of 320 μL using the same procedure as described for the offline tandem SPE extracted samples.

The mean recovery of each compound ranged from 86 to 116 percent (table 8), and the relative standard deviation ranged from 4.6 to 16.7 percent. Variation between the slopes of the internal and external standard curves for each compound was similar to the recovery data in table 8. The average difference between the slopes of the internal and external curves for each antibiotic was 4.9 ± 11.6 percent. These data show that the recoveries are suitable for quantitative analyses. The recoveries for the classes of antibiotics reported for this method are similar to recoveries of other SPE-LC/MS methods (Hirsch and others, 1998; Lindsey and others, 2001) used for antibiotic analysis.

Other compounds that were initially incorporated into the offline tandem SPE method in 1998 included ivermectin, roxarsone, spectinomycin, vancomycin, and virginiamycin. They are not reported herein because the recoveries were less than 30 percent and the LOQs generally were greater than 0.20 μg/L. Of these five compounds, only virginiamycin was detected in any environmental sample collected between 1998 and 2000. Ivermectin, roxarsone, spectinomycin, and vancomycin were removed from the method in 2000.

The long-term performance of the offline tandem SPE method was evaluated by normalizing the analyzed concentration of spiked reagent-water samples analyzed between 1999 and 2003 to their theoretical spiked concentrations (table 8). The reagent-grade water samples for the offline tandem SPE method were spiked at concentrations between 0.02 and 1.0 μg/L. For the offline tandem SPE method, the average concentration of each antibiotic varied between 95 and 106 percent of the theoretical spiked concentration, and the relative standard deviations varied from 8.2 to 20.1 percent. The reagent-grade water spike data show that the performance of the offline tandem SPE method was relatively stable over several years. In addition, more than 200 reagent-water blanks

Table 6. Solutions for online solid-phase extraction instrument and high-performance liquid chromatograph.

[EDTA, ethylene diamine tetraacetic acid; M, molar; Na, sodium; w/V, weight to volts; %, percent; --, not applicable]

Solution	Valve position	Beta lactam-macrolide	Sulfonamide-quinolone	Tetracycline
Sample modifier solutions	--	10% NaCl/0.5% EDTA in reagent water (w/V)	0.25 mL formic acid to 9.75 mL reagent water	0.25 mL formic acid to 9.75 mL reagent water
Online solid-phase extraction	--			
Triathalon autosampler	1	reagent water	reagent water	reagent water
	2	2% formic acid	2% formic acid	2% formic acid
High-pressure dispenser	1	reagent water	reagent water	reagent water
	2	acetonitrile	acetonitrile	acetonitrile
	3	1 M NaOH with 0.02% (5% Na$_2$-EDTA)	--	--
	4	5 mM NH$_4$-acetate	0.3% formic acid	0.3% formic acid

Table 7. Flow rates and mobile-phase composition for analysis by online solid-phase extraction method.

[mL/min, milliliter per minute; %, percent; mM, millimolar; NH$_4$, ammonium]

Minutes	Isocratic pump flow rate (mL/min)	Quaternary pump flow rate (mL/min)	Mobile-phase composition			
			Mobile-phase A (%)	Mobile-phase B (%)	Mobile-phase C (%)	Mobile-phase D (%)
Beta lactam-macrolide						
			5 mM NH$_4$-acetate	acetonitrile	reagent water	methanol
0	0	0.35	90	10	0	0
19	0	.35	0	100	0	0
22	0	.35	0	100	0	0
Sulfonamide-quinolone						
	0.3 % formic acid		0.3 % formic acid	acetonitrile	reagent water	methanol
0	.102	.252	95	5	0	0
20	.102	.252	0	100	0	0
20.01	0	.36	0	100	0	0
Tetracyclines						
	0.3 % formic acid		0.3 % formic acid	1% formic acid	reagent water	methanol
0	0	.36	95	0	0	5
.50	0	.36	95	0	0	5
.51	.27	.135	60	0	0	40
5.50	.27	.135	60	0	0	40
5.51	0	.36	60	0	0	40
12.99	0	.36	28	0	0	72
13	0	.36	0	28	0	72
20	0	.36	0	0	0	100

were analyzed between 1999 and 2003, and none of the method analytes were detected in any of these blank samples.

To evaluate the performance of the offline tandem SPE method in different matrices, 29 surface-water samples from 10 States and 16 ground-water samples from five States were spiked from 0.02 to 1.0 μg/L and analyzed between 2000 and 2003. The data from the offline tandem SPE method show that the concentrations of the quinolone, macrolide, and sulfon-amide antibiotics varied from an average of 81 to 139 percent of the theoretical spiked concentrations with relative standard deviations that ranged from 16.8 to 39.8 percent (table 8). Table 8 also shows that the percentage variation for the theoretical concentration of the surrogates was less and more variable in the spiked surface- and ground-water samples than in the spiked reagent-water samples. These data indicate that varying matrices affect either the recovery of the compounds on the SPE cartridges or affect the ionization of the com-pounds in the ESI source chamber of the MS.

The data in table 8 also show that, although the results of analysis of surface- and ground-water samples were more variable than the results of analysis of reagent-water samples, the results generally averaged within ± 20 percent of the

expected concentration with the exception of carbadox and the macrolides, which tended to have higher average concentra-tions than the expected concentration and standard deviations greater than 30 percent. Interestingly, the erythromycin-^{13}C$_1$-H$_2$O surrogate standard did not display the same enhance-ment characteristics as the unlabeled erythromycin-H$_2$O. The area counts of carbadox, erythromycin-H$_2$O, and tylosin in many cases were slightly higher than in the equivalent spiked reagent-water samples but not enough to account for the average 30-percent increase over the expected concentration. The overestimation is caused by the combination of a slight enhancement in area counts of these antibiotics and a decrease in the response of the internal standard for some samples. Slight enhancement of the macrolide antibiotics also was observed in the online SPE method (Ed Lee, USGS, oral com-mun., October 2004).

Table 8 shows that for the offline tandem SPE method the matrix-spike concentrations of the tetracycline antibiotics, including the surrogate standard meclocycline, varied from an average of 185 to 1,200 percent of the theoretical spiked concentrations with large standard deviations. The area counts of the tetracycline antibiotics in the surface- and ground-water

(A) Sulfonamide and macrolide antibiotics
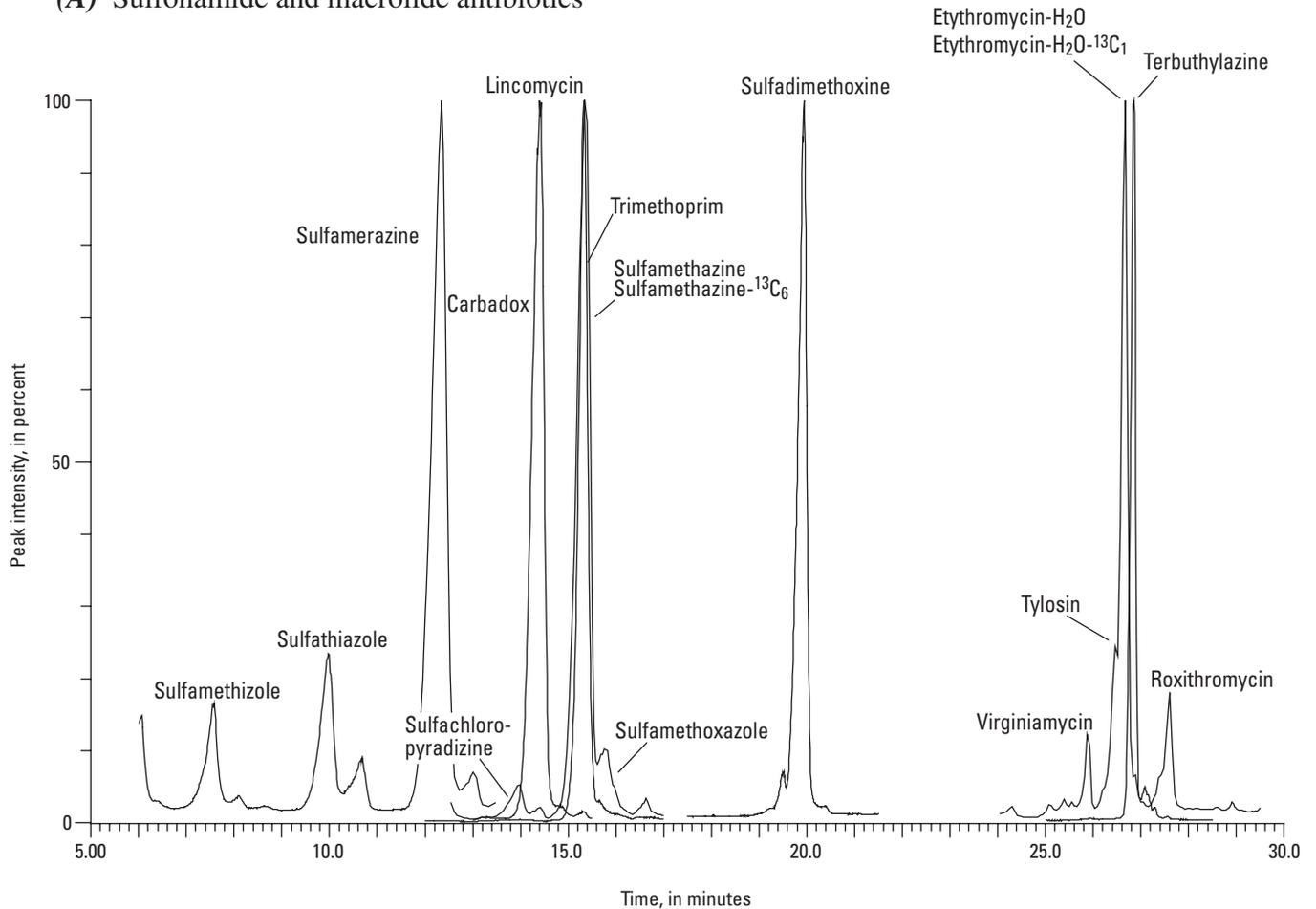
Figure 2. Selected ion-monitoring chromatograms of an extracted sample spiked at 0.20 microgram per liter for offline tandem solid-phase extraction method used in analysis of *(A)* sulfonamide-macrolide and *(B)* quinolone-tetracycline antibiotics.

samples were, in general, substantially higher than in equivalently spiked reagent-grade water samples. These data indicate that the ionization of the tetracycline antibiotics can be enhanced substantially in this particular method. Lindsey and others (2001) also observed enhanced ionization for the tetracycline class of antibiotics. The large standard deviations also demonstrate that the degree to which these compounds were affected was matrix dependent. Because the range of variability for the individual compounds was different, they could not be normalized to the surrogate standard for quantitation.

Tetracycline antibiotics readily form chelate complexes with metal ions and also sorb to proteins and silanol groups (Zia and Price, 1976; Ishino and others, 1981; Lee and Everett, Jr., 1981; VanBogert and Kroon, 1981). EDTA is a strong chelating agent that is added to the water samples to prevent tetracyclines from binding to divalent metals and the functional groups on the SPE cartridge during extraction. Because of their complexing characteristics, tetracyclines were detected rarely in surface or ground water, and therefore, it would have been an inefficient use of time and resources to perform standard addition on every sample. Thus, a modified standard-

addition technique was tested for tetracyclines on 10 of the spiked sample extracts. Two 100-μL aliquots of the sample extract were pipetted into 2-mL chromatography vials with 200-μL glass inserts. Ten microliters (10 μL) of a 10-ng/μL standard mix in 20 mM NH_4-acetate (pH 5.6 standard units) were pipetted into one vial, and 10 μL of 20 mM NH_4-acetate (pH 5.6) were pipetted into the other vial to keep the extract volumes and sample matrix the same. The samples then were reanalyzed, and the tetracycline antibiotics quantified using standard addition:

$$C_{ws} = ((R_{us}/(R_s - R_{us})) \times C_s)^*(V_{se}/V_{ws}),\qquad(1)$$

where | C_{ws} | is the concentration of the analyte in the extracted water sample in nanograms per milliliter;
| R (response) | is the area of the analyte divided by the area of the internal standard;
| R_{us} | is the response of the unspiked sample analyte;
| R_s | is the response of the spiked analyte;
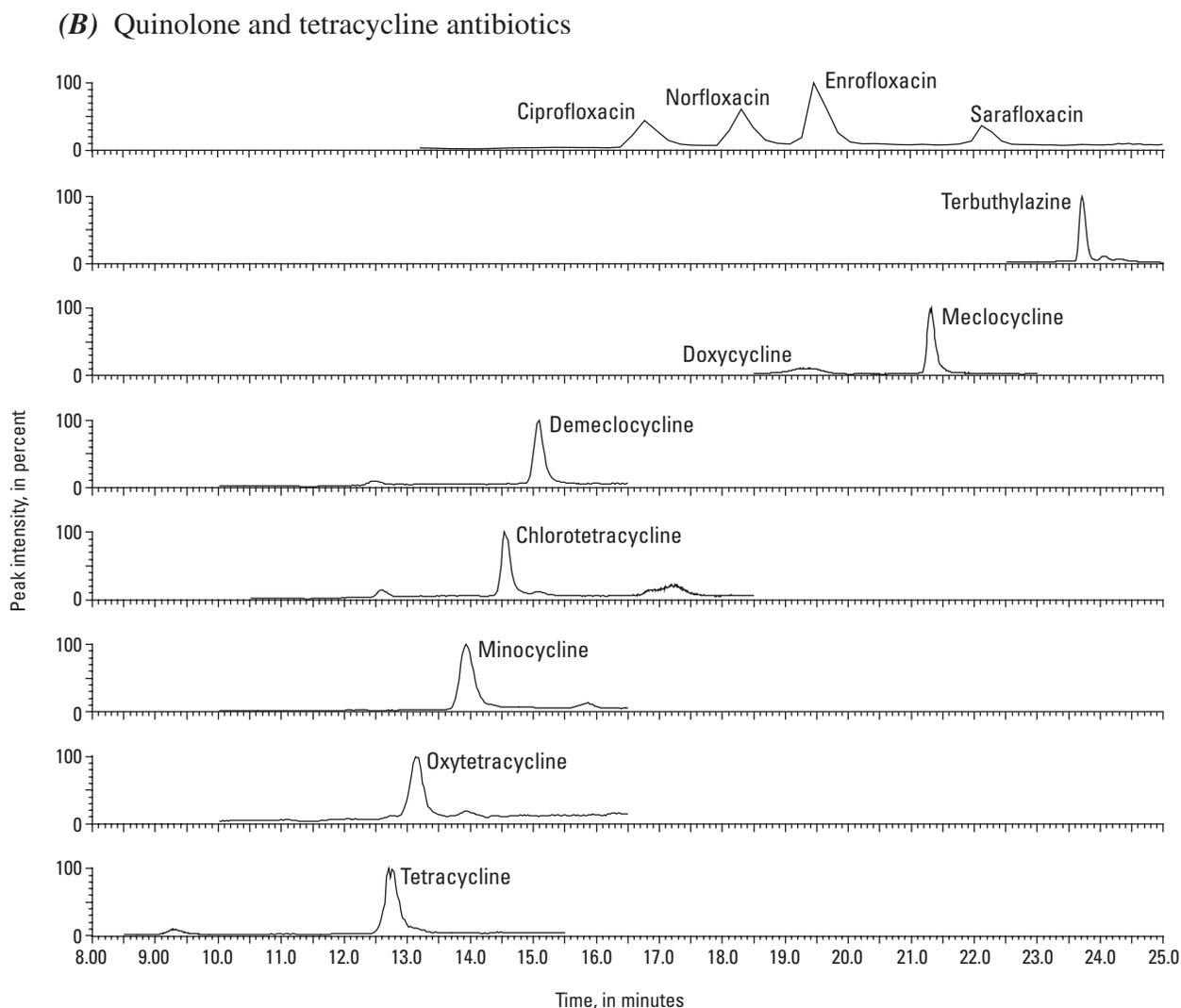
(B) Quinolone and tetracycline antibiotics

Figure 2. Selected ion-monitoring chromatograms of an extracted sample spiked at 0.20 microgram per liter for offline tandem solid-phase extraction method used in analysis of *(A)* sulfonamide-macrolide and *(B)* quinolone-tetracycline antibiotics.—Continued

C_s is the concentration of the spiked analyte in the sample extract in nanograms per microliter;

V_{se} is the volume of the sample extract in microliters; and

V_{ws} is the volume of the water sample in milliliters.

Table 8 shows that the modified standard addition brought the analyzed concentrations within an average of 84 to 95 percent of theoretical spiked concentrations and that the standard deviations ranged from 12.4 to 19.2 percent. This technique allowed for samples to be analyzed without extracting an extra spiked sample for quantitation. Furthermore, the sample extract only needed to be divided into aliquots, spiked, and rerun if one of the problematic analytes was detected. The data in table 8 show that the offline tandem SPE method is relatively robust in a wide variety of surface-water and ground-

water matrices. These data also indicate that, to the extent that compounds are available, stable, isotope-labeled compounds would be beneficial to provide more robust quantitation when using the ESI source.

Online Solid-Phase Extraction

Figures 3 through 5 show SIM chromatograms of an extracted sample spiked at 1.0 µg/L for the online SPE method used for the analysis of beta lactam-macrolide, sulfonamides-quinolone, and tetracycline antibiotics. These data show that the chromatography and mass-spectral signatures adequately identify the antibiotic compounds reported using this method.

A summary of the performance of the online SPE method from 1999 to 2003 for spiked reagent-water and spiked environmental samples is shown in table 9. The normalized percentage variation of the concentration of all the analytes

Table 8. Summary of recovery of antibiotics from fortified reagent-water solutions and average percentage variation of analyzed concentration of reagent-water and matrix spikes to their theoretical spiked concentrations for offline tandem solid-phase extraction method.

[RSD, relative standard deviation; MCX, mixed-mode cation exchange; SS, surrogate standard; IS, internal standard; n, number of samples; ±, plus or minus; --, no data]

Compounds	Mean percentage recovery[1] (n=20)	Reagent-water spikes[2] (n=100)	Matrix spikes[2] (n=40)	Standard addition (n=10)	Average±RSD, in percent breakthrough to MCX (n=3)
Sulfonamide-macrolide analysis					
sulfachloropyradizine	99±6.59	101±11.6	95±30.2	--	2.3±0.359
sulfadimethoxine	91±4.60	101±13.7	81±16.8	--	0.457±0.154
sulfamerazine	111±7.87	105±16.0	95±22.6	--	0.67±0.192
sulfamethazine	86±4.86	106±14.2	83±26.2	--	0.431±0.092
sulfamethizole	92±6.7	104±15.7	88±24.8	--	--
sulfamethoxazole	102±5.59	98±14.0	101±24.3	--	0.62±0.168
sulfathiazole	95±7.44	103±12.6	86±18.4	--	--
carbadox	100±9.35	95±13.0	134±36.4	--	2.9±0.260
lincomycin	110±15.1	98±11.0	98±32.7	--	91±2.28
erythromycin-H_2O	103±11.2	105±12.5	139±31.2	--	2.9±0.118
roxithromycin	115±12.7	99±11.3	120±39.8	--	1.7±0.271
trimethoprim	92±6.7	99±13.8	102±35.7	--	0.601±0.164
tylosin	116±13.1	95±11.3	111±36.9	--	1.4±0.534
erythromycin-^{13}C-H_2O (SS)	94±9.01	99±10.4	91±26.7	--	--
sulfamethazine-$^{13}C_6$ (SS)	86±10.5	99±12.2	82±30.3	--	--
terbuthylazine (IS)	--	100±8.2	84±30.1	--	--
Quinolone-tetracyline analysis					
ciprofloxacin	104±8.03	101±15.5	92±28.8	--	2.3±0.181
enrofloxacin	109±13.7	104±17.2	107±33.3	--	3.5±0.258
norfloxacin	102±11.8	103±16.2	106±33.7	--	2.2±0.166
sarafloxacin	105±10.4	102±16.5	88±29.6	--	1.1±0.054
chlorotetracycline	96±16.7	100±15.0	185±94	89±17.6	--
demeclocycline	86±12.1	103±16.6	670±450	84±19.2	4.6±0.054
doxycycline	97±14.4	100±20.1	440±260	86±12.4	14±1.69
minocycline	98±6.39	102±17.2	260±155	94±16.7	3.2±0.214
oxytetracycline	89±11.0	101±17.5	1200±740	91±13.8	3.2±0.051
tetracycline	92±10.5	103±16.2	730±420	95±14.4	2.9±0.255
meclocycline (SS)	92±6.70	106±18.3	220±104	--	--
terbuthylazine (IS)	--	102±10.9	82±28.6	--	--

[1] Percentage recovery based on variation of 20 extracted standard solutions between 0.05 and 1.0 microgram per liter regressed against a duplicate multipoint external standard curve.

[2] Average percentage variation of spiked sample concentrations normalized to theoretical spiked concentration ± relative percentage standard deviation.

(A) Beta-lactam antibiotics
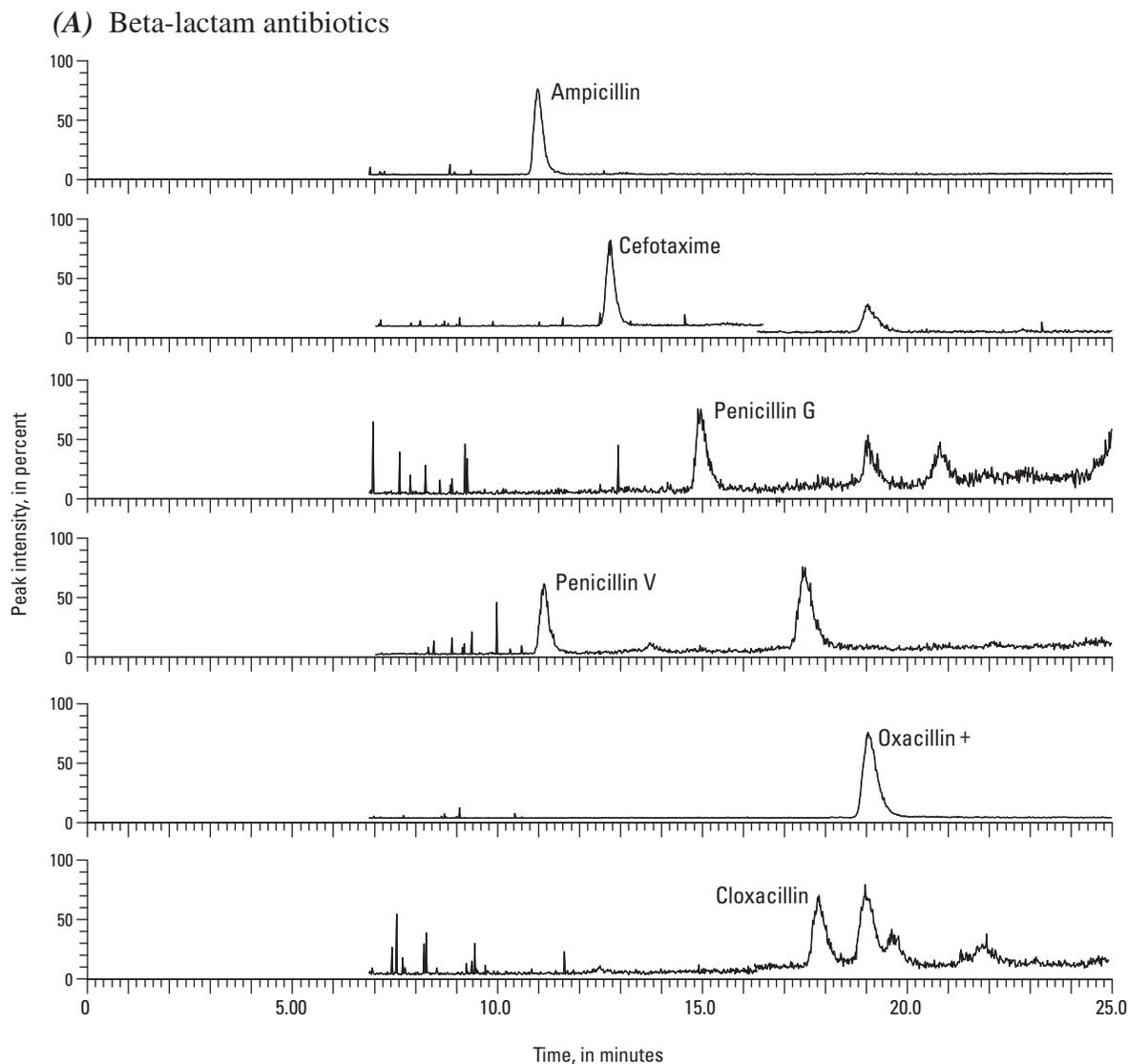
Figure 3. Selected ion-monitoring chromatograms of an extracted sample spiked at 1.0 microgram per liter for online solid-phase extraction method used in analysis of *(A)* beta-lactam and *(B)* macrolide antibiotics.

in 1.0-µg/L spiked reagent-water samples ranged from 71 to 112 percent of the expected spiked concentrations with relative percentage standard deviations ranging from 6 to 87 percent for all 39 analytes. Relative percentage standard deviations were less than 35 percent for 35 of the analytes. Amoxicillin and cefotaxime had the highest percentage of relative standard deviations at 87 and 72 percent, respectively. The normalized percentage concentrations of the surrogate standards for the three antibiotic classes analyzed ranged from 101 to 112 percent with relative percentage standard deviations ranging from 12 to 28 percent (table 9). The long-term reagent-water spiked data show that the method was stable during its use from 1999 to 2003.

Matrix-spiked samples also were used to assess the long-term variation of the online SPE method in different matrices. The long-term matrix-spike data show greater average

variation from the expected spiked concentrations and greater percentage standard deviations than was observed with the reagent-water spikes. Between 78 and 95 matrix spikes were analyzed for each analyte except erythromycin-H_2O and carbadox. The matrix samples were spiked at 1.0 µg/L and analyzed between 1999 and 2003. The matrix-spiked samples consisted of 92 surface- and 31 ground-water samples. The surface-water samples were collected from 54 different streams in 18 States, and the ground-water samples were collected from 19 wells in 6 States. The data show that the average percentage of normalized concentration of the matrix-spiked samples varied from 22 to 142 percent of the theoretical spiked concentrations with relative standard deviations that ranged from 17 to 308 percent (table 9). The results from the online SPE method also show that, although the results of the surface- and ground-water analyses are more variable than the results from
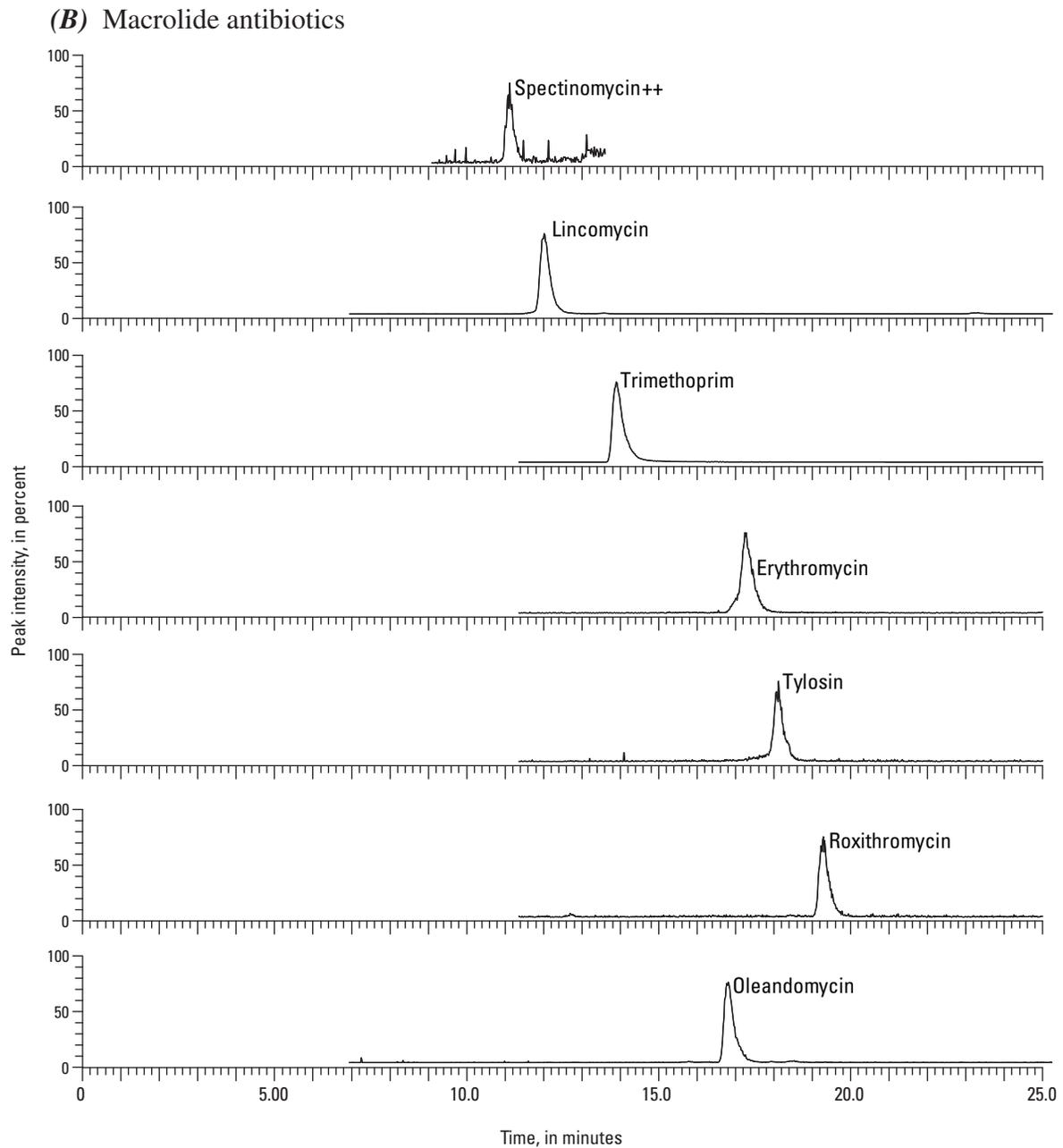
(B) Macrolide antibiotics

Figure 3. Selected ion-monitoring chromatograms of an extracted sample spiked at 1.0 microgram per liter for online solid-phase extraction method used in analysis of *(A)* beta-lactam and *(B)* macrolide antibiotics.—Continued

the reagent-water analyses, most of the compounds averaged within ±30 percent of the expected concentration with the exceptions of amoxicillin, ampicillin, cefotaxime, penicillin G, roxithromycin, tylosin, and all the tetracycline compounds except anhydro-tetracycline. Roxithromycin and tylosin had the highest percentage of normalized concentrations at 139 and 142 percent, respectively, and amoxicillin, ampicillin, cefotaxime, and anhydro-chlortetracycline had the lowest at 22, 42, 38, and 51 percent, respectively. Amoxicillin, minocycline, and tetracycline had the highest relative percentage standard deviations at 308, 111, and 130 percent, respectively. Erythromycin-H_2O was not evaluated in these samples as it

was added to the method in 2003, just before the method was transferred to the LC/triple quadrupole MS/MS. The percentage of normalized concentrations of the surrogate standards in water samples ranged from 94 to 130 percent, and the relative percentage standard deviations ranged from 34 to 40 percent. Also, none of the method analytes were detected in the 125 reagent-water blanks analyzed using the online SPE method.

The online SPE antibiotic data for the matrix-spiked surface- and ground-water samples were more variable than the data for the spiked reagent-water samples (table 9). This also was observed for the offline tandem SPE method data. These data indicate that varying matrices affect either the
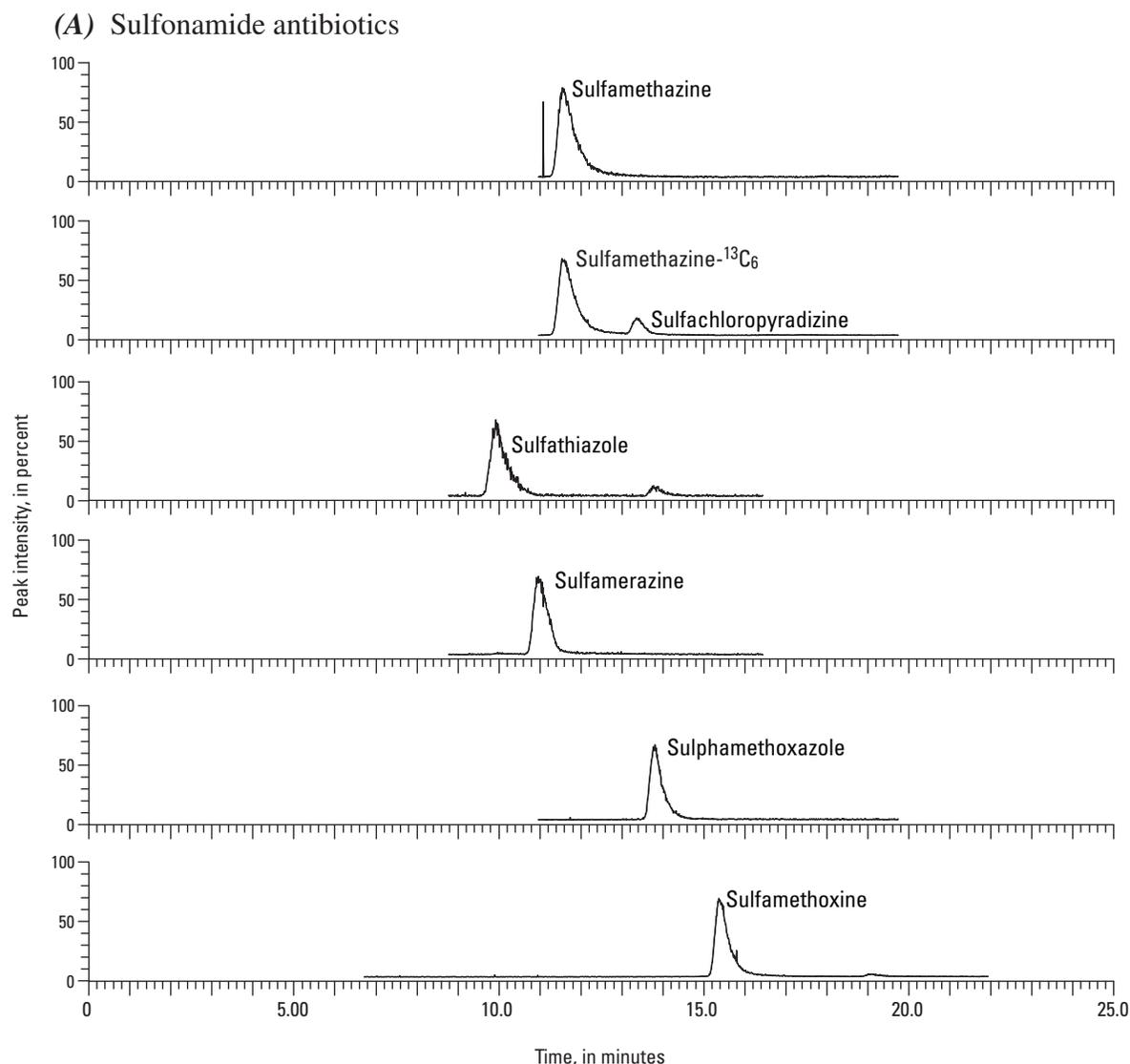
(A) Sulfonamide antibiotics

Figure 4. Selected ion-monitoring chromatograms of an extracted sample spiked at 1.0 microgram per liter for online solid-phase extraction method used in analysis of *(A)* sulfonamide and *(B)* quinolone antibiotics.

recovery of the compounds on the SPE cartridges or affect the ionization of the compounds in the ESI source chamber of the MS. Several researchers have reported signal suppression and less frequently signal enhancement from matrix effects in electrospray ionization (Buhrman and others, 1996; Barnes and others, 1997; Choi and others, 1999, 2001a,b; Dijkman and others, 2001; Pascoe and others, 2001; Reemtsma, 2001, 2002; Zrostlikova and others, 2002; Hajslova and Zrostlikova, 2003). Thus, it is likely that much of the increased variation in the matrix-spiked samples was due to "matrix" effects. Varying matrices concentrated during the SPE process can affect the efficiency with which an individual compound is ionized in the electrospray source. Ionization suppression or enhancement does not usually affect each compound equally. In addition, apparent enhancements can occur when an internal standard is more suppressed than the analyte to which

it is used to normalize. However, these data do not exclude the possibility that the recovery of the analytes in varying matrices can be responsible, at least to some degree, for some of the increased variation in the matrix-spiked samples. For the beta-lactam antibiotics, some of the increased variation in the average percentage of normalized concentrations also may be due to degradation. The beta-lactams have labile structures, and amoxicillin can readily degrade even under laboratory conditions. For example, Cahill and others (2004) observed poor recoveries for amoxicillin and speculated that it was due to degradation.

The reagent-water spikes from the offline tandem SPE and online SPE methods show that they had acceptable performance during the time periods that they were used. Although the matrix-spiked sample data from both methods showed increased variability relative to the reagent-water spikes, the
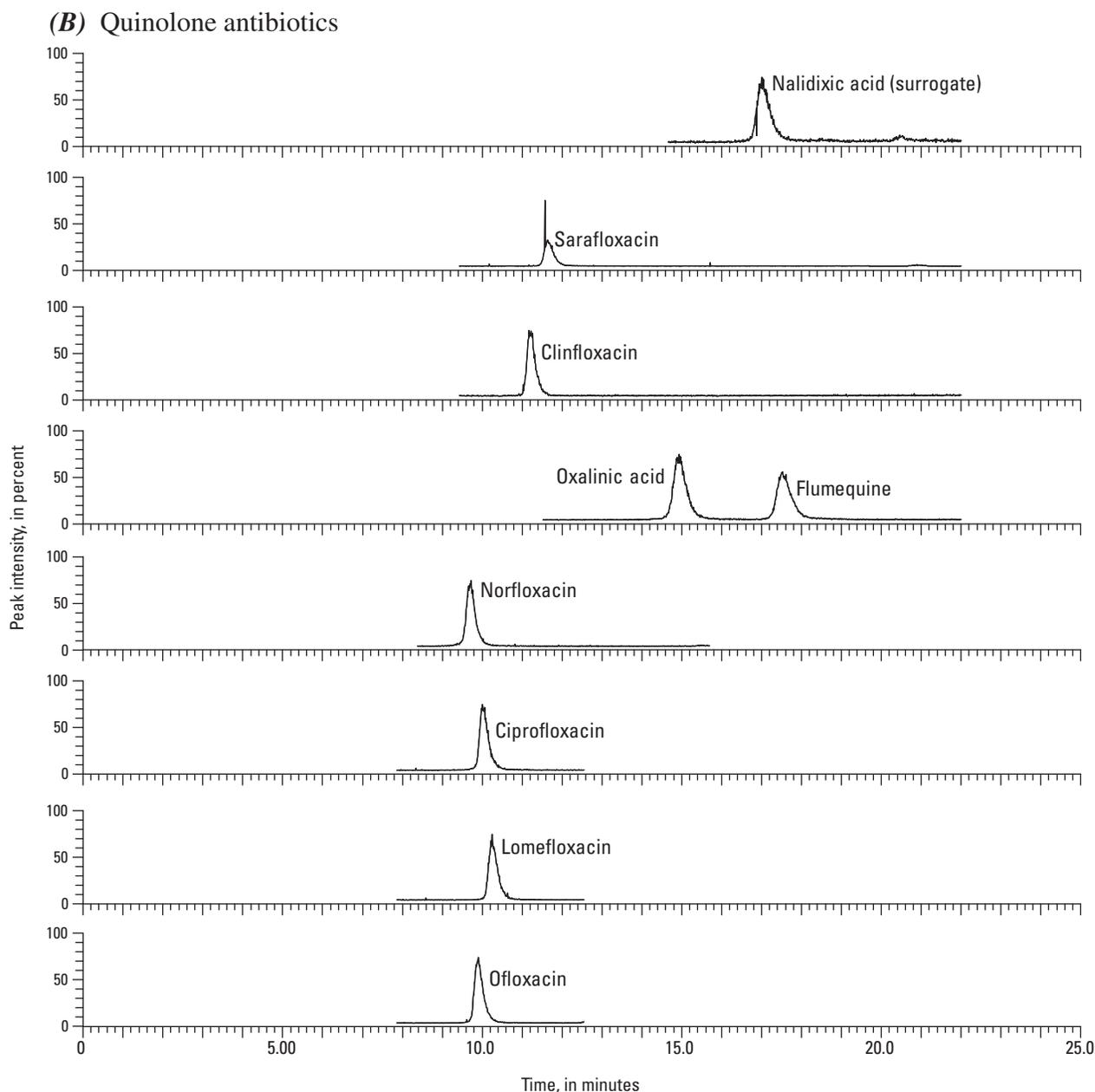
(B) Quinolone antibiotics

Figure 4. Selected ion-monitoring chromatograms of an extracted sample spiked at 1.0 microgram per liter for online solid-phase extraction method used in analysis of *(A)* sulfonamide and *(B)* quinolone antibiotics.—Continued

data also indicate that, for most of the analytes, the methods performed adequately for a wide variety of environmental samples. These data also show that matrix effects can be a substantial issue with LC/MS applications and is an issue that has not been adequately addressed in the literature. The matrix-spike data also suggest that, for specific studies in which concentration differences between samples are important, it may be preferable to use matrix-matched standard curves or multiple-point standard addition to increase the precision and accuracy of the method for that particular study.

Comparison of Offline Tandem and Online Solid-Phase Extraction and an Independent Method

The two independently developed single quadrupole LC/MS methods also were assessed for comparability in overlapping data sets and compared with a third, independently developed solid-phase extraction single quadrupole LC/MS method that contained two environmentally important antibiotics that overlapped with the offline tandem SPE and online
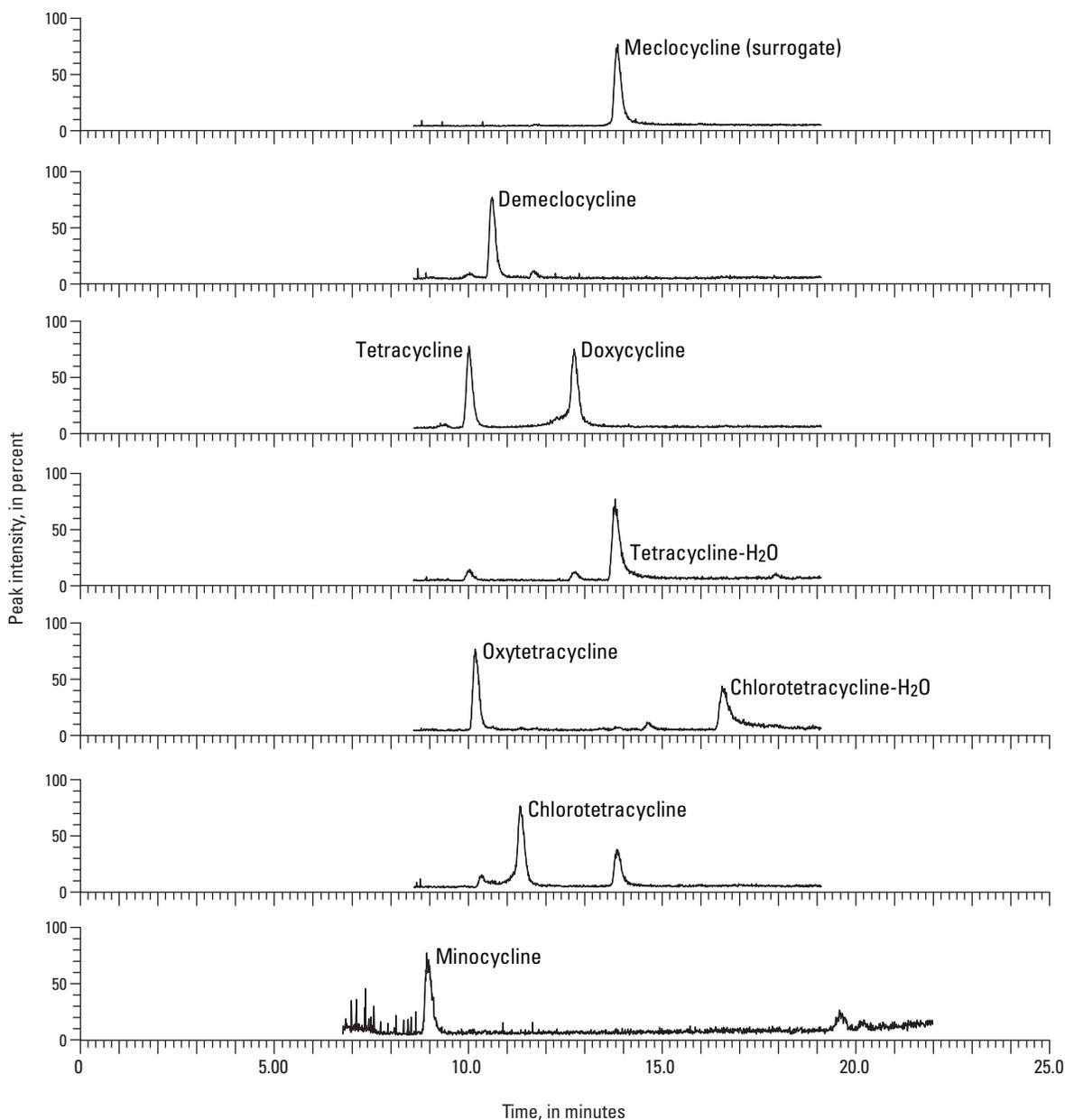
Figure 5. Selected ion-monitoring chromatograms of an extracted sample spiked at 1.0 microgram per liter for online solid-phase extraction method used in analysis of tetracycline antibiotics.

SPE methods. Ninety-six samples from three studies (Barnes and others, 2004; Stackelberg and others, 2004; Glassmeyer and others, 2005) were analyzed using the offline tandem SPE method and an independent SPE-LC/MS method for pharmaceuticals (Cahill and others, 2004; M3 method) developed at the USGS Methods Development Program, National Water-Quality Laboratory in Lakewood, Colorado. Forty-four samples from one of the studies also were analyzed using the offline tandem SPE and online SPE antibiotic methods as well as the M3 method. The three studies used in the comparisons included a ground-water reconnaissance of a landfill for five monitoring wells completed in a leachate plume from a municipal landfill (Barnes and others, 2004, five samples), a

study of the occurrence of pharmaceuticals from multiple samplings through a drinking-water plant in New Jersey including raw, settled, filtered, and finished water (Stackelberg and others, 2004, 47 samples), and a USEPA-USGS study (Glassmeyer and others, 2005) that sampled at one site upstream and three sites downstream from wastewater-treatments plants on 11 streams (44 samples) to assess persistence of wastewater contaminants in surface water.

The M3 method had two overlapping compounds (sulfamethoxazole and trimethoprim) with the offline tandem SPE and online SPE methods. Sulfamethoxazole and trimethoprim were the only antibiotics detected in any of the samples from the three studies using all three methods.

Table 9. Summary of average percentage of normalized concentration of spiked reagent-water and matrix samples for online solid-phase extraction method.

[μg/L, micrograms per liter; %, percent; ±, plus or minus; RSD, relative standard deviation; --, no data; SS, surrogate standard; IS, internal standard]

Compounds	Reagent-water spiked samples at 1.0 μg/L[1] (average%±%RSD)	Number of samples	Matrix-spiked samples at 1.0 μg/L[1] (average%±%RSD)	Number of samples
Sulfonamide antibiotics				
Sulfachloropyradradizine	102 ± 19.9	44	99.5 ± 45.4	95
Sulfadiazine	102 ± 17.8	44	85.3 ± 34.5	95
Sulfadimethoxine	103 ± 24.2	44	110.3 ± 33.5	95
Sulfamerazine	101 ± 17.2	44	91.9 ± 34.4	95
Sulfamethazine	103 ± 13.7	44	113.7 ± 41.6	95
Sulfamethoxazole	94.9 ± 14.4	44	90.0 ± 33.9	95
Sulfathiazole	105 ± 19.4	44	106.1 ± 42.1	95
Ciprofloxacin	102 ± 32.0	44	87.8 ± 41.7	95
Clinafloxacin	104 ± 23.3	44	95.2 ± 35.8	95
Quinolone antibiotics				
Flumequine	103 ± 20.6	44	125.2 ± 29.3	95
Lomefloxacin	102 ± 24.9	44	84.0 ± 32.8	95
Norfloxacin	100 ± 31.3	44	88.5 ± 44.2	95
Ofloxacin	98.0 ± 30.8	44	85.4 ± 41.6	94
Oxolinic acid	106 ± 17.7	44	127.0 ± 27.1	95
Sarafloxacin	95.1 ± 30.2	44	83.8 ± 38.7	94
Carbadox	102 ± 5.7	7	104.1 ± 16.7	10
Naxidilic acid (SS)	112 ± 27.7	44	130.3 ± 38.4	95
Sulfamethazine-$^{13}C_6$ (SS)	102 ± 11.7	44	106.1 ± 34.7	87
Beta lactam antibiotics				
Amoxicillin	71.3 ± 86.6	44	21.8 ± 308	86
Ampicillin	81.3 ± 35.2	47	41.9 ± 63.1	86
Cefotaxime	98.7 ± 72.2	47	37.6 ± 76.3	86
Cloxacillin	94.6 ± 21.5	47	76.3 ± 50.4	86
Oxacillin	93.1 ± 25.6	47	71.3 ± 69.6	86
Penicillin G	89.2 ± 21.9	47	67.9 ± 66.9	86
Penicillin V	90.7 ± 19.9	47	73.0 ± 81.1	86
Macrolide antibiotics				
Erythromycin	97.1 ± 30.3	47	103 ± 49.5	85
Erythromycin-H_2O	102 ± 34.5	6	--	--
Roxithromycin	87.0 ± 38.5	47	139 ± 59.2	85
Tylosin	83.2 ± 44.3	47	142 ± 69.5	85
Virginamycin	95.5 ± 20.9	47	102 ± 51.0	85
Lincomycin	97.9 ± 23.2	47	76.1 ± 30.9	85
Ormetopriim	97.4 ± 21.8	45	95.8 ± 28.7	81
Trimethoprim	98.6 ± 21.6	47	85.0 ± 34.4	86
Oleandomycin (SS)	102 ± 18.6	47	109 ± 40.0	85
Tetracycline antibiotics				
Chlorotetracycline	92.0 ± 20.7	41	87.1 ± 35.9	80
Anhydro-chlorotetracycline	77.3 ± 38.8	40	51.4 ± 64.4	81
Demeclocycline	92.4 ± 18.4	41	83.6 ± 40.0	81
Doxycycline	97.5 ± 17.4	40	95.1 ± 38.2	81
Minocycline	89.0 ± 38.2	41	73.8 ± 111	81
Oxytetracycline	95.9 ± 16.7	41	89.0 ± 37.4	81
Tetracycline	98.9 ± 34.4	41	89.7 ± 130	81
Anhydro-tetracycline	98.2 ± 19.3	40	71.3 ± 29.9	81
Meclocycline (SS)	101 ± 18.8	40	94.2 ± 34.5	78
Simetone (IS)	--	--	--	--

[1] Average percentage variation of spiked sample concentrations normalized to theoretical spiked concentration ± relative percentage standard deviation analyzed between 2000–2003.

Table 10. Comparison of concentrations in 44 surface-water samples analyzed using three analytical methods.

[SPE-LC/MS, solid-phase extraction liquid chromatography/mass spectrometry; <, less than indicated method reporting limits]

Sample number	Concentration, in micrograms per liter					
	Sulfamethoxazole			Trimethoprim		
	Offline tandem solid-phase extraction	Online solid-phase extraction	Pharmaceutical SPE-LC/MS (M3 method)	Offline tandem solid-phase extraction	Online solid-phase extraction	Pharmaceutical SPE-LC/MS (M3 method)
1	<0.05	<0.05	<0.023	<0.01	<0.05	<0.014
2	.08	<.05	.07	<.01	<.05	<.014
3	.04	<.05	.03	<.01	<.05	<.014
4	<.05	<.05	.01	<.01	<.05	<.014
5	<.05	<.05	<.023	<.01	<.05	<.014
6	.25	.29	.11	.05	<.05	.04
7	.02	<.05	.07	.01	<.05	.01
8	.02	<.05	.03	.01	<.05	.01
9	<.05	<.05	<.023	<.01	<.05	<.014
10	<.05	<.05	<.023	<.01	<.05	<.014
11	.18	.51	.20	.04	<.05	.04
12	.08	<.05	.10	.02	<.05	.02
13	.13	<.05	.16	.02	<.05	.03
14	<.05	<.05	<.023	<.01	<.05	<.014
15	<.05	<.05	<.023	<.01	<.05	<.014
16	<.05	<.05	<.023	<.01	<.05	<.014
17	<.05	<.05	<.023	<.01	<.05	<.014
18	<.05	<.05	.16	<.01	<.05	.10
19	<.05	<.05	<.023	.01	<.05	.02
20	.02	<.05	.02	.01	<.05	.01
21	<.05	<.05	<.023	<.01	<.05	<.014
22	.11	<.05	.07	.04	<.05	.03
23	<.05	<.05	<.023	<.01	<.05	<.014
24	<.05	<.05	<.023	<.01	<.05	<.014
25	<.05	<.05	<.023	<.01	<.05	<.014
26	.07	<.05	.18	<.01	<.05	<.014
27	.05	<.05	.04	<.01	<.05	<.014
28	.02	<.05	.02	<.01	<.05	<.014
29	<.05	<.05	<.023	<.01	<.05	<.014
30	.53	1.39	.62	.40	.57	.40

Table 10. Comparison of concentrations in 44 surface-water samples analyzed using three analytical methods.—Continued

[SPE-LC/MS, solid-phase extraction liquid chromatography/mass spectrometry; <, less than indicated method reporting limits]

| Sample number | Concentration, in micrograms per liter | | | | | |
| | Sulfamethoxazole | | | Trimethoprim | | |
	Offline tandem solid-phase extraction	Online solid-phase extraction	Pharmaceutical SPE-LC/MS (M3 method)	Offline tandem solid-phase extraction	Online solid-phase extraction	Pharmaceutical SPE-LC/MS (M3 method)
31	0.79	0.72	0.84	0.55	0.55	0.45
32	.39	<.05	.31	.08	.13	.09
33	<.05	<.05	<.023	.01	<.05	.01
34	.29	.36	.32	.01	<.05	.03
35	.08	<.05	.10	.01	<.05	.01
36	.09	.15	.09	.01	<.05	.02
37	<.05	<.05	.10	<.01	<.05	.02
38	.23	.58	.10	.03	.18	.02
39	.21	.49	.14	.02	.19	.05
40	.20	.48	.19	.02	.14	.04
41	.45	.93	.58	.21	<.05	.14
42	.15	.76	.36	.05	<.05	.14
43	<.05	1.14	.23	.08	<.05	.08
44	<.05	.92	.21	.05	<.05	.06

Sulfamethoxazole was detected in 24, 13, and 30 of the samples, and trimethoprim in 23, 6, and 25 of the samples for the offline tandem SPE, online SPE, and M3 methods, respectively, in the USEPA-USGS study (table 10). The reporting levels for the offline tandem SPE method were 0.05 and 0.01 µg/L; online SPE method, 0.05 and 0.05 µg/L; and M3 method, 0.023 and 0.014 µg/L, for sulfamethoxazole and trimethoprim, respectively. Two of the 30 sulfamethoxazole detections reported by the M3 method were five or more times less than the reporting level and were not detected by the offline tandem SPE method. The offline tandem SPE method agreed with 24 of 28 (86 percent) of the sulfamethoxazole detections using the M3 method and 23 of 25 (92 percent) of the trimethoprim detections. Seven of the 30 sulfamethoxazole and 16 of the 25 trimethoprim detections using the M3 method were less than the reporting level of the online SPE method. The online SPE method agreed with 13 of 23 (57 percent) of the sulfamethoxazole detections using the M3 method and 5 of 9 (56 percent) of the trimethoprim detections. Also, two of the trimethoprim detections using the M3 method that were less than the reporting level of the online SPE method were detected at concentrations greater than the reporting level using the online SPE method. Five of the 24 sulfamethoxazole and 15 of the 23 trimethoprim detections using the offline tandem SPE method were less than the reporting level of the online SPE method. The online SPE method agreed with 11 of 19 (58 percent) of the sulfamethoxazole detections using the offline tandem SPE method and 4 of 8 (50 percent) of the trimethoprim detections. Also, there were four trimethoprim detections using the offline tandem SPE method that were less than the reporting level of the online SPE method but were detected at greater than the reporting level using the online SPE method, and there were two sulfamethoxazole detections using the online SPE method that were not detected using the offline tandem SPE method.
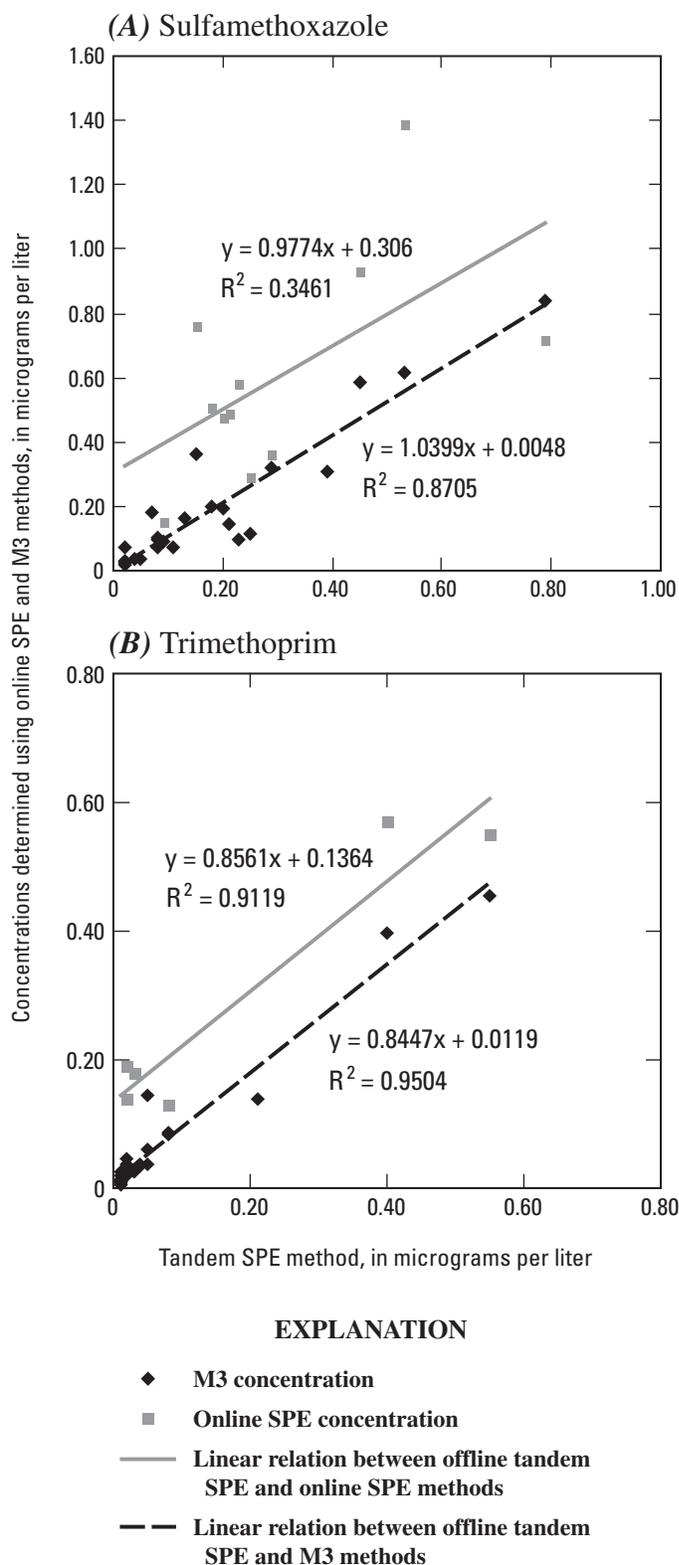
These data indicate that the detection frequencies of the offline tandem SPE and M3 methods for sulfamethoxazole and trimethoprim were similar and that the detection frequency of the online SPE method was 50 to 60 percent that of the offline tandem SPE and M3 methods when adjusted for differences in reporting levels. These data also show that many of the analyte detections using the offline tandem SPE and online SPE methods were near the reporting levels of the online SPE method (table 10). It may be that antibiotics analyzed using the online SPE method were detected in several of the samples, but they were detected at less than the method reporting level.

The overall agreement between the offline tandem SPE and M3 methods (96 samples) for sulfamethoxazole and trimethoprim was 96 and 98 percent, respectively, when considering the number of detections and nondetections

of the overlapping analytes from each method. The overall agreement between the online SPE and M3 methods for the 44 samples from Glassmeyer and others (2005) was 72 and 91 percent and between the online SPE and offline tandem SPE methods was 68 and 91 percent for sulfamethoxazole and trimethoprim, respectively, when considering the number of detections and nondetections of the overlapping analytes from each method. In addition, there was 100 percent agreement between the online SPE and offline tandem SPE methods for all of the other overlapping analytes, which were all nondetec-tions. The data from the surface-water reconnaissance study of Kolpin and others (2002) also show that the detection fre-quency between the offline tandem SPE, online SPE, and M3 methods for sulfamethoxazole and trimethoprim were similar when the differences in the reporting levels between the meth-ods were taken into account.

The relation of the analyte concentrations between meth-ods also was examined. Linear regression was used to assess whether the determined analyte concentrations were similar between the offline tandem SPE, online SPE, and M3 methods (fig. 6). Samples in which sulfamethoxazole and trimethoprim were detected by both the offline tandem SPE and M3 meth-ods and the online SPE and M3 methods were analyzed. The correlation coefficient (R^2) between the offline tandem SPE and M3 methods for sulfamethoxazole was 0.87 with a slope of 1.04. The R^2 between the offline tandem SPE and online SPE methods for sulfamethoxazole was 0.35 with a slope of 0.98, and the scatter clearly indicates that the concentrations of several samples were higher when the online SPE method of analysis was used than when the offline tandem SPE method was used. A similar result was obtained in a regression of data for the online SPE and M3 methods (not shown). These data also indicate that for several samples, the online SPE method provided concentrations two to four times higher than were obtained by the offline tandem SPE or M3 methods (fig. 6). For trimethoprim, the R^2 between the offline tandem SPE and M3 methods and the offline tandem SPE and online SPE methods were 0.95 and 0.91, respectively (fig. 6). The slopes were approximately 0.85 for both methods. The regressed data indicate that the slopes of the offline tandem SPE rela-tion between the online SPE and M3 methods are sufficiently parallel over the concentration range displayed for both sulfamethoxazole and trimethoprim to suggest that the online SPE method has a high bias relative to both the offline tandem SPE and M3 methods. Comparison of the distance between the slopes indicate that the online SPE method has positive regressed bias of approximately 0.3 and 0.13 μg/L for sulfa-methoxazole and trimethoprim, respectively, compared to the M3 and online SPE methods. The regressed data also indicate that the offline tandem SPE and M3 methods provided similar concentration data and had similar low y-intercepts.

These data show that the offline tandem SPE and M3 methods provided well-correlated regressed relations with slopes near unity and very low y-intercepts for sulfa-methoxaole and trimethoprim. These data also show that the online SPE method provided a good regressed relation for

(A) Sulfamethoxazole

$$y = 0.9774x + 0.306$$
$$R^2 = 0.3461$$

$$y = 1.0399x + 0.0048$$
$$R^2 = 0.8705$$

(B) Trimethoprim

$$y = 0.8561x + 0.1364$$
$$R^2 = 0.9119$$

$$y = 0.8447x + 0.0119$$
$$R^2 = 0.9504$$

Tandem SPE method, in micrograms per liter

EXPLANATION

◆ **M3 concentration**

■ **Online SPE concentration**

—— **Linear relation between offline tandem SPE and online SPE methods**

– – **Linear relation between offline tandem SPE and M3 methods**

Figure 6. Regression relations between sample concentrations deter-mined using offline tandem solid-phase extraction (SPE) method, online SPE method, and M3 pharmaceutical method for analysis of *(A)* sulfa-methoxazole and *(B)* trimethoprim.

trimethoprim and a poor relation for sulfamethoxazole with high y-intercepts and also displayed a high bias relative to the offline tandem SPE and M3 methods. The data also show that for sulfamethoxazole more scatter was observed between the offline tandem SPE and online SPE methods than was observed between the tandem SPE and M3 methods.

Conclusions

The offline tandem SPE method of analysis for antibiotics was used from 1999 to 2003 to analyze more than 1,000 surface- and ground-water samples from national stream-water reconnaissance studies, watershed and field-scale studies of antibiotic and antibiotic-resistant bacterial transport from urban and agricultural sources in multiple States, and wastewater outfall transport studies. The online SPE method of analysis for antibiotics was used from 2000 to 2003 to analyze more than 500 samples from similar studies.

The cumulative data from these studies show that the recoveries for most antibiotics in environment samples were within acceptable (greater than 60 percent) limits for quantitative analysis. The spiked reagent-water data showed that the offline tandem SPE and online SPE methods were stable during the course of their use. The application and variability of these methods for the analysis of multiple classes of antibiotics were demonstrated for a variety of surface- and ground-water samples. The data from the offline tandem SPE method indicate that the tetracycline antibiotics were subject to substantial matrix effects. A modified standard-addition technique allowed for the tetracyclines to be quantitated without extracting a spiked duplicate sample with every sample to be analyzed. The matrix-spiked data from the online SPE method also showed that some of the beta-lactam and tetracycline antibiotics could have high variability. None of the analytes were detected in any of the reagent-water blanks for the offline tandem SPE and online SPE methods during their use.

Comparison of the online SPE and offline tandem SPE for 44 to 96 samples, respectively, with an independently developed pharmaceutical method (M3) with overlapping compounds indicated agreements of 50 to 92 percent in sample detection and 72 to 98 percent in presence-absence comparison. Finally, regressed compound concentrations between methods indicated well-correlated relations between all three methods for trimethoprim and between the tandem SPE and M3 methods for sulfamethoxazole. The data also show that the online SPE method tended to have higher concentrations than the offline tandem SPE and M3 methods for both sulfamethoxazole and trimethoprim.

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