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A Sensitive Method for Detection of Sulfamethazine and N^4-Acetylsulfamethazine Residues in Environmental Samples Using Solid Phase Immunoextraction Coupled with MALDI-TOF MS

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Sulfamethazine (SMT) and its major metabolite, N^4-acetylsulfamethazine (NA-SMT), were each recovered from spiked water (0.1 ppb) and 10% (w/v) aqueous suspensions of soil (1 ppb) or composted manure (1 ppb), by using a three-stage solid phase immunoextraction (SPIE) system, followed by detection with matrix-assisted laser/desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Sulfonamide recovery rates are reported for separate stages of the SPIE system and for trace-level sulfonamide SPIE extraction from the environmental samples. SPIE MALDI-TOF MS is a rapid and definitive technique with potentially better efficiency relative to other established trace-level sulfonamide analytical methods. SPIE MALDI-TOF MS required 1.5 h per batch (8−24 samples/batch) for sample enrichment, 5 min per batch for probe preparation, and 5 min per sample to acquire and process the spectrum. This is the first time MALDI-TOF MS has been reported as a potential means of detecting trace-level drug residues in complex environmental samples.

KEYWORDS: MALDI-TOF MS; immunoaffinity; solid phase extraction; polyclonal antibody; sulfonamides; drug metabolites; water; soil; manure; residue analysis; antibiotic resistance

INTRODUCTION

Human or veterinary pharmaceutical residues can reach the water and soil in the environment as a constituent of urine, feces, or manure that enter the sewage system or are spread directly onto agricultural fields as fertilizer. Sulfamethazine (SMT) is a commonly used broad spectrum antibacterial compound which plays an important role in agriculture, especially for its routine addition to swine and poultry feeds to improve overall feed conversion efficiency (*1*). There is growing concern, backed by accumulating evidence, that agricultural use of antibacterial drugs, such as sulfonamides, may be contributing to the increasing problem of drug resistance in human and veterinary medicine (*2*, *3*). Other concerns surrounding SMT include both its allergenicity and potential toxicity (*4*, *5*). Many agricultural and pharmaceutical drugs, including SMT, have already been detected in some fields and waters suspected to be contaminated by agricultural practices (*6*, *7*). These compounds have now become part of the much wider debate of water purity, and governments are challenged to establish reasonable policies for their safe or permissible limits in water (*8*).

Sulfonamides are synthetic compounds that are competitive antagonists of prokaryotic folic acid synthesis and require a free primary aromatic amino group for their bacteriostatic activity (*9*). Specific animal liver enzymes convert SMT to various metabolites, such as the microbially inactive compound N^4-acetylsulfamethazine (NA-SMT), which is the main form of the drug excreted via the urine (*10*) (**Figure 1**). Hydrolysis of the acetyl group is readily catalyzed in vitro via acid or base mechanisms and is an important reaction for freeing the parent compound prior to its diazotization in the Bratton−Marshall colorimetric analysis (*11*). These nonacetylated, biologically active sulfonamides are chemically very stable in vitro (*12*) because of the stable sulfonamide bond, and since they are

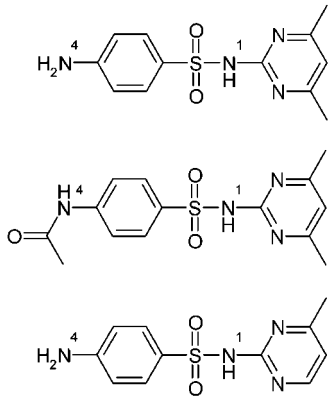

Figure 1. Chemical structures of sulfamethazine, N^4-acetylsulfamethazine, and sulfamerazine, from top to bottom.

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unlikely to be biodegraded quickly in the environment, they may have the potential to be persistent environmental pollutants. It is important that analytical methods be able to monitor both the parent sulfonamide compounds and their main metabolites, because very little is known about the stability of either acetylated or parent compounds in the environment.

The motivation of this research is to address a technological gap in environmental residue analysis, namely, the need for increased cost and time efficiency, sacrificing neither sensitivity nor accuracy. Several common methods of sulfonamide analysis have been established, but are subject to certain limitations. Colorimetric assays such as the Bratton−Marshall reaction are quick and simple, but lack specificity and can be subject to sample color interferences (*13*). Enzyme-linked immunosorbent assay (ELISA) is simple, fast, very cost efficient, and capable of a high degree of sensitivity in detecting sulfonamides (*14*). However, false positives are often associated with this technique due to sample matrix effects in biological samples (*15*), and an initial cleanup step is often required to gain more reliability. Significant cross-reactivity can occur in ELISA between sulfonamides of similar structure, even when monoclonal antibodies are used (*16*). For these reasons, although antibodies can be selected for a certain degree of specificity, the ELISA technique lacks definitiveness and is usually used as an initial screening tool prior to more confirmatory methods. Microbiological methods for sulfonamide detection are based on either microbial growth inhibition (*17*) or sulfonamide-binding microbial receptors such as the commercial CHARM II test (Charm Sciences, Inc., Malden, MA). These tests are sensitive, but have no ability to differentiate between sulfonamides, and growth inhibition assays cannot detect the microbially inactive N^4-acetyl form. Various mass spectrophotometric methods, coupled with solid phase extraction and liquid or gas chromatography (*18*−*20*) have demonstrated the best performance to date in both accuracy and sensitivity for sulfonamide analysis, yet have significant disadvantages of cost and laborious sample extraction and cleanup steps in addition to the already time-consuming chromatographic separation itself.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is capable of detecting many antibacterial compounds simultaneously and with high sensitivity. Ling et al. report detecting SMT to a limit of 200 ppb in methanol without concentration (*21*). Unlike other types of mass spectrometry that require extensive purification by methods such as high performance liquid chromatography (HPLC) prior to analysis, MALDI-TOF MS is relatively tolerant of sample matrix contaminants (*22*, *23*). Solid phase extraction is often employed before MALDI-TOF MS as a quick and nonspecific cleanup step to enrich and purify drugs from water (*24*); however, trace drug residue analysis in complex biological samples requires more selective purification.

The principle of immunoaffinity chromatography (IAC) is commonly used for purifying and concentrating nonimmunogenic small molecules (haptens) from complex samples (*25*−*27*). IAC has been used for sulfonamides (*28*, *29*), although these particular attempts have resulted in low column capacities of 2 μg sulfonamide/mL gel due to the use of nonpurified polyclonal IgG (specific and nonspecific IgG populations). These low column capacities can be critically limiting or impractical in residue analysis when attempting to extract enough hapten and to elute it with a small volume of solvent. The potential of IAC for enrichment prior to MALDI-TOF MS analysis has been explored (*30*−*32*), yet aside from the work reported from this laboratory on the detection of glycoalkaloids in serum (*33*), none have used immunopurification of small haptens such as sulfamethazine in conjunction with MALDI-TOF MS.

By making a high-capacity, hapten-specific IAC column, and combining this with the fast, automatable, and simple analytical features of MALDI-TOF MS, it was our goal to develop an efficient method for the definitive detection of trace levels of drugs and their metabolites in complex environmental samples. As an adaptable model for other drugs and haptens, we describe the recovery and detection of trace levels of sulfamethazine and its N^4-acetyl metabolite from water, and from soil or manure suspensions, using solid phase immunoextraction (SPIE) coupled with MALDI-TOF MS.

MATERIALS AND METHODS

Materials and Reagents. All water used in this study was deionized by a Milli-Q water purification system (Millipore Corp., Bedford, MA). Acetic anhydride was obtained from Caledon Laboratories (Georgetown, ON). Free acids of sulfamethazine (SMT), sulfamerazine (SMR), and tris(hydroxymethyl)aminomethane (TRIS) were obtained from Sigma Chemical Co. (St. Louis, MO). Hyflo Super Cel Celite, washed sea sand, 2,5-dihydroxybenenzoic acid (DHB), glacial acetic acid, and dimethyl sulfoxide (DMSO) were obtained from Fisher Scientific (Ottawa, ON). Anhydrous ethanol was obtained from Commercial Alcohols (Winnipeg, MB). All other chemicals and their sources for associated protocols are described previously by research from this laboratory (*33*−*37*).

Synthesis and Characterization of NA-SMT. NA-SMT was synthesized according to a method described by Whelpton et al. (*38*). NA-SMT was recrystallized from a 10% (w/v) solution in a solvent made of 1:1 DMSO/water (v/v), and the resultant crystals were washed extensively with water in a sintered glass funnel, dried at 100 °C in a forced air oven, and stored in a desiccator. The recrystallized NA-SMT was a fine, white powder and had a melting point between 247.5 and 249.5 °C. An Inova 600 spectrometer (Varian, Palo Alto, CA) was used to perform 1D proton and HMQC proton-carbon correlation and APT (attached proton test) experiments on NA-SMT dissolved in DMSO, which revealed spectra consistent for its structure (*39*). The carbon, hydrogen, nitrogen, and sulfur content of recrystallized NA-SMT was determined by the University of Alberta's Chemistry department service and were found to be 52.71%, 4.82%, 17.21%, and 10.34%, respectively (expected theoretical values are 52.49%, 5.07%, 17.50%, and 10.01%).

Preparation of Anti-SMT Antibodies. *Immunization and Conjugation.* Antibodies were produced by immunizing rabbits with SMT-*Limulus polyphemus* hemolymph conjugate. Protocols for rabbit immunizations and diazo conjugate production are described by Sheth and Sporns (*37*).

Preparation of SMT-BSA Immunoaffinity Chromatography (IAC) Column. A 15 mg sample of lyophilized SMT conjugated to bovine serum albumin (BSA) was reacted with 5.0 mL of swollen CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) in 0.1 M $NaCO_3$ pH 8.3 + 0.5 M NaCl (coupling buffer), as recommended by the manufacturer. The SMT-BSA affinity gel was equilibrated to phosphate-buffered saline (PBS = 0.05 M sodium phosphate + 0.9% NaCl, adjusted to pH 7.2) + 0.05% (w/v) sodium azide and stored at 4 °C in a glass vial. When required for immunoextraction of antibodies, the SMT-BSA affinity gel was transferred to a 5 mL pipet tip fitted with a 2 mm × 1/8 in. o.d. frit (Supelco, Bellefonte, PA) wedged in the tip (**Figure 2**).

Protein G and SMT-BSA Immunoaffinity Purification of Anti-SMT Antibodies. The IgG fractions of immunoglobulins from immunized rabbit sera were purified by HiTrap Protein G sepharose HP (Amersham Pharmacia Biotech, Uppsala, Sweden), followed by SMT-BSA IAC. The protocol recommended by the manufacturer was used for the initial Protein G purification step, and elution of the antibodies was monitored by absorbance at 280 nm. To enrich the fraction of IgG specific for SMT, 20−25 mg batches of Protein G-purified antibodies were dialyzed to PBS and centrifuged, and then the collected supernatant was applied

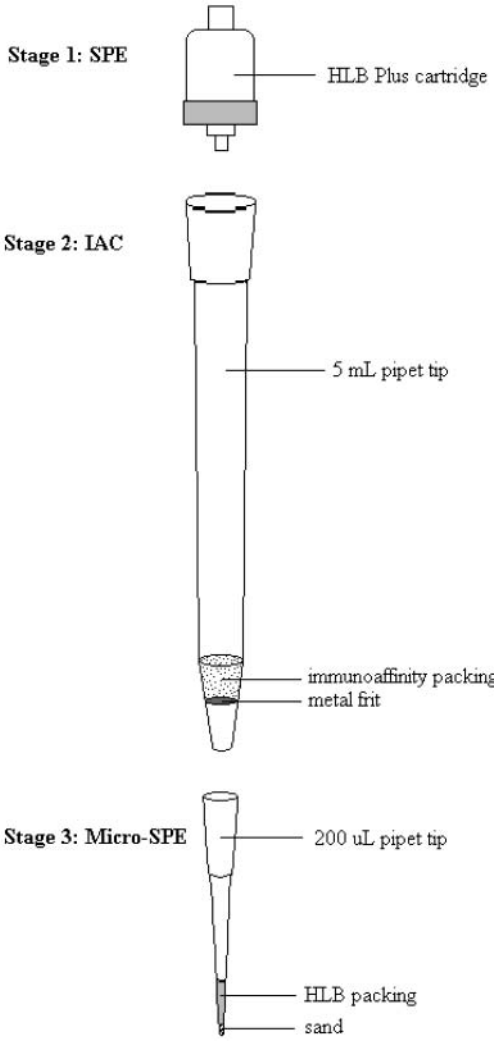

Figure 2. Schematic diagram of the SPIE system showing the three stages of sulfonamide enrichment achieved by solid phase extraction (SPE), anti-sulfamethazine specific immunoaffinity chromatography (IAC), and the micro-SPE column.

by gravity to a 1 mL SMT-BSA column equilibrated to PBS. The column was washed with 10 column volumes of PBS, and then immunoglobulins were eluted using 0.1 M glycine-HCl buffer (pH 2.7) and 1.0 mL fractions were collected into test tubes containing 25 μL of 1.0 M TRIS buffer at pH 9.0. Elution fractions having an A_{280} > 0.3 were pooled, and then IgG was precipitated with ammonium sulfate (50% saturated solution at 4 °C) and stirred overnight at 4 °C. The resulting suspension was centrifuged at 5000*g* for 30 min, the supernatant discarded, and the pellet resuspended in PBS for storage at −75 °C, or resuspended in coupling buffer to a concentration of 5−10 mg/mL.

Preparation of Solid Phase Immunoextraction (SPIE) Column System. Oasis HLB Plus cartridges obtained from Waters Corp (Milford, MA) contained 225 mg of solid phase material and were used as is.

Production of Anti-SMT Immunoaffinity Chromatography (IAC) Column. The binding of purified anti-SMT antibodies to CNBr-activated Sepharose was carried out as recommended by the manufacturer. Frozen anti-SMT antibodies were quickly thawed in warm water and then dialyzed at 4 °C to coupling buffer prior to reacting with CNBr-activated Sepharose. Ten milligrams of antibody was reacted per milliliter of the swollen CNBr-activated gels. Incorporation of the antibody into the gel was monitored by measuring the A_{280} of the supernatant over the gel suspension. The anti-SMT affinity gel was equilibrated to PBS + 0.05% (w/v) sodium azide and stored at 4 °C in a glass vial. When required for immunoextraction of SMT, 0.4 mL of the anti-SMT immunoaffinity gel was applied to a 5 mL pipet tip fitted with a frit (**Figure 2**) and equilibrated to PBS. Buffer and samples were applied to the columns at a rate of approximately 1 mL/min using a manifold system capable of handling several columns, each adapted with rubber stopper attachments and hooked to a peristaltic pump.

Production of Micro-SPE Columns. Micro-solid-phase extraction (micro-SPE) columns were fashioned out of 200 μL beveled pipet tips (**Figure 2**). The tips were plugged with a small quantity of sand, and then approximately 5 mg of Oasis HLB Plus packing material was added.

Preparation of Water, Soil, and Composted Manure Samples. *Collection and Storage.* Two soil samples (3 kg) were collected from the top 20 cm of earth at two sites, one from a deciduous forest on the south bank of the North Saskatchewan River valley adjacent to the University of Alberta, Edmonton, Canada, approximately 15 m higher than the river (soil 1), and the other at a cultivated agricultural field approximately 15 km south of Edmonton (soil 2). Samples were mixed well, sealed in plastic bags, and stored at −20 °C until thawed for analysis. Composted manure was obtained from the Bioresource Engineering Group of the Department of Agricultural, Food and Nutritional Science at the University of Alberta and was a mixture of dairy cow, poultry, and swine manures and beddings, containing approximately 40% carbon and 2.5% nitrogen by dry weight. There was no history of sulfonamides used in the feed rations of the animals that produced manure resources in this study (personal communications). Moisture contents of the soil and manure samples were determined by the average of differential weighing of duplicate 10 g samples before and after heating for 20 h at 80 °C in a forced air oven.

Pre-SPIE Sample Processing. Water (1000 mL), acidified with 5.0 mL of 1.0 M acetic acid adjusted to pH 5.0, was added to a glass 1.2 L narrow mouth reagent bottle. Zero or 100 g of soil or composted manure sample was added by funnel to each glass container. SMT and NA-SMT (3.60 nmol each) were added to each sample by adding 50.0 μL of a 72.0 μM SMT stock solution in DMSO and 53.7 μL of a 67.0 μM stock solution of NA-SMT in DMSO. These spiked samples were equivalent to 1.00 ppb SMT and 1.15 ppb NA-SMT based on 1 L of water added. SMT and NA-SMT (0.36 nmol each) were added to 1 L water samples (equivalent to 0.100 ppb SMT and 0.115 ppb, respectively) by adding 5.0 μL of a 72.0 μM SMT stock solution in DMSO and 5.4 μL of a 67.0 μM stock solution of NA-SMT in DMSO. DMSO (100.0 μL) was added to all unspiked controls. Large magnetic stirbars (7.5 cm long, Teflon-coated) were added to each sample, and samples were stirred on magnetic stirring plates for at least 1 h at room temperature before being allowed to settle for 5 min. The liquid suspension from each sample was then decanted into centrifuge bottles and centrifuged at 5000*g* for 20 min. Supernatants were dispensed into 1.2 L clean glass narrow mouth reagent bottles and applied immediately to SPIE for concentration and purification of sulfonamides. Included in this report are the results from three separate experiments on different days investigating the SPIE-MALDI TOF detection of spiked SMT and NA-SMT in water, soils, and composted manure. Each spiked or unspiked sample was processed by SPIE in duplicate or triplicate and analyzed by MALDI-TOF MS in duplicate.

Solid Phase Immunoextraction (SPIE) of Samples. The SPIE system used to purify and concentrate the sulfonamides was comprised of three solid phase extraction steps used in tandem as follows: a primary concentration and crude purification step using HLB Plus cartridges, a secondary anti-SMT IAC chromatography step for further purification, and a final micro-SPE column concentration step (**Figure 2**).

HLB Plus Cartridges. Cartridges were preconditioned with 5 mL of ethanol followed by 10 mL of water. The cartridges were then fitted into centrally bored holes in #7 rubber stoppers, two-way stopcocks were fitted to the other cartridge end, and then these assemblies were mounted onto 1 L vacuum flasks. The filtrate or supernatant samples in 1.2 L glass containers were connected by flexible tubing (Tygon, Akron, OH) to the HLB Plus cartridges via the stopcocks. Samples were drawn through cartridges by manifold vacuum filtration in 30−45 min, and the stopcocks were adjusted to ensure approximate flow

rate equivalency among samples. The cartridges were removed and washed with 10 mL water, and then ethanol was applied through the cartridges, and the first 3 mL was collected for further purification.

Anti-SMT Immunoaffinity Chromatography (IAC) Columns. Each 3 mL of ethanol eluant from an HLB Plus cartridge was diluted by adding it to 12 mL of PBS, and then this diluted sample was applied to an anti-SMT IAC column. IAC columns were washed with 5 mL of PBS, sulfonamides were eluted with 4 mL of 10 mM HCl applied to the IAC columns, and the acidic eluants were collected into test tubes containing 3.0 μL of 0.379 mM SMR in DMSO. IAC columns were regenerated with 10 mL of 10 mM HCl followed by 10 mL of PBS. An aliquot comprised of the final 4 mL of acidic eluant used for column regeneration was collected and analyzed in the same manner as the samples to verify the removal of all sulfonamides from the column.

Micro-SPE Columns. Micro-SPE columns were inserted into a filtration manifold made from a #14 rubber stopper bored with twelve 4 mm holes inserted into a 400 mL beaker and connected to a vacuum. One milliliter pipet tips were inserted into the top of the micro-SPE columns to provide a holding reservoir for solvent and sample addition, and then the columns were conditioned with 1 mL of ethanol followed by 1 mL of water. The acidic eluants, containing SMR internal standards, were applied to the preconditioned micro-SPE columns and rapidly drawn through by vacuum. The micro-HLB columns were washed with 1 mL of water and detached from the rubber vacuum manifold after they were dry. Ethanol (70 μL) was added to each column and forced through with a positive displacement pipetor, and the eluant was collected in a glass vial. Ethanol eluants were evaporated to dryness with a stream of nitrogen gas.

Sample Analysis. *Anti-SMT ELISA.* An indirect, competitive ELISA was used to monitor the progression of the immunizations and was a sensitive way to monitor the performance of the SPIE extraction columns when the sensitivity of the Bratton−Marshall assay was limiting. The same chemicals, characterization, and format for the anti-SMT ELISA were used as previously described by Thomson and Sporns for the detection of sulfathiazole (*35*).

Bratton−Marshall Assay. The Bratton−Marshall assay is based on reaction of *N*-1[naphthyl]ethylenediamine dihydrochloride (NED) with the aromatic primary amine of SMT, originally described by Marshall (*11*), and was used to estimate sulfonamide recoveries from solid phase extraction columns relative to sulfonamide standards during SPIE system development. This method was miniaturized from the procedure as described by Low et al. (*36*) by using 96-well, flat-bottomed microtiter plates (Costar, Corning Inc., Corning, NY) instead of cuvettes. Sample-to-reagent volume ratios were unchanged, whereas the total reaction volume was reduced to 250 μL. The acetyl group was hydrolyzed from the NA-SMT by placing the sample in a sealed glass vial with 2 M NaOH in a 5:2 ratio and then placing it in a boiling water bath for 30 min. Development of color was measured by a SpectraMax 190 microtiter plate reader (Molecular Devices Corp., Sunnyvale, CA) at an absorbance of 545 nm.

MALDI-TOF MS. Ten-position target probes were made of highly polished stainless steel (Bruker Analytical Systems Inc., Billerica, MA). A matrix solution of 2,5-dihydroxybenzoic acid (DHB) was prepared (approximately 10 mg/mL) in 50% aqueous ethanol. Dried micro-HLB column eluants were resuspended with 10 μL of DHB solution, and approximately 1 μL was applied to each of three spots on the probe. The matrix and sample were cocrystallized on the probe by allowing the solvent to evaporate under a fan in ambient conditions. Spectra were acquired on a linear Bruker Proflex III instrument (Bruker Analytical Systems, Billerica, MA) equipped with a nitrogen laser (337 nm). Mass spectra were obtained in positive ion mode with an extraction potential of 20 kV and a delay of 6000 ns. All mass spectra were generated by collecting 100 laser shots. Laser strength and detector voltage were adjusted to obtain optimal signal-to-noise ratios and high resolution. Three spectra were generated for each sample, and the analyte responses were averaged. The MALDI-TOF MS response for an analyte was determined as the sum of peak heights from proton, sodium, and potassium adducts together with any fragments. MALDI-TOF MS peaks associated with an analyte were used for the response calculation only if they were 3 times greater than the general background noise in the spectral region of interest.

The MALDI-TOF MS response factors of SMT and NA-SMT relative to the internal standard SMR were determined on the basis of the quotient of the MALDI-TOF MS response of SMT or NA-SMT to that of SMR. SMT, NA-SMT, and SMR stock solutions (1.00 ± 0.01 μL each) in DMSO (3.60, 3.35, and 3.79 mM, respectively) were mixed with PBS to a volume of 3.00 mL, and then this was processed normally through a micro-SPE column and analyzed by MALDI-TOF MS three times to generate triplicate spectra. The molar response ratio for SMT or NA-SMT was determined on the basis of an average of these experiments according to eq 1.

$$\text{molar response ratio} = AD/BC \quad (1)$$

where A = sum of peak heights of SMT or NA-SMT analyte adducts/fragment; B = sum of peak heights of SMR adducts/fragment; C = moles of SMT or NA-SMT analyte processed through micro-HLB column; and D = moles of SMR internal standard processed through micro-SPE column. Response ratios are 0.99 ± 0.12 and 0.42 ± 0.06 for SMT/SMR and NA-SMT/SMR, respectively (n = 3).

SPIE Recovery Estimation. MALDI-TOF MS response ratios of SMT and NA-SMT relative to SMR were used to estimate their recoveries from the first two steps of SPIE in spiked samples relative to the SMR internal standard, and the Bratton−Marshall (BM) assay was used to estimate micro-SPE column efficiency. Percent recoveries of sulfonamide from SPIE were estimated by MALDI-TOF MS using eq 2.

$$\%\ \text{recovery of analyte from SPIE} = 100 \times ADF/BCE \quad (2)$$

where A = sum of MALDI-TOF MS spectral peak heights associated with $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$, and $[M - SO_2 + H]^+$ ions (M = parent compound of the analyte SMT or NA-SMT); B = sum of MALDI-TOF MS spectral peak heights associated with $[SMR + H]^+$, $[SMR + Na]^+$, $[SMR + K]^+$, and $[SMR - SO_2 + H]^+$ ions where SMR is the internal standard; C = response ratio determined by eq 1 for the analyte SMT or NA-SMT; D = moles of internal standard SMR added to system (1.14 nmol); E = moles of analyte, SMT or NA-SMT, added originally to sample (3.60 nmol); and F = average colorimetric determination of sulfonamide (SMT, NA-SMT, and SMR) fractional recovery for micro-SPE column (F = 0.81 ± 0.01; n = 3). Factor F is necessary to compensate for loss at the micro-SPE column step, because the MALDI-TOF MS internal standard was added just before this last step and so only estimates the recovery of the first two stages of the SPIE system. All errors reported in this paper are the standard error of the mean.

RESULTS AND DISCUSSION

The goal of this research was to use solid phase immunoextraction (SPIE) coupled with MALDI-TOF MS to rapidly recover and detect trace levels of sulfamethazine and its N^4-acetyl metabolite from water and from soil or manure suspensions. We chose SMT as a drug model to represent the potential of SPIE-MALDI TOF because SMT has been used extensively in animal agriculture for decades, and it is possible that this compound may be detected in ecosystems associated with intensive agriculture. We developed a protocol that could be used for a variety of agriculture environmental samples, such as water samples with unknown amounts of dissolved material, manure wastes from animals, or soil samples.

Although our methods development showed that HLB Plus solid phase extraction (SPE) cartridges were adequate by themselves for concentration and MALDI-TOF MS detection of SMT in water (data not reported), the cartridges alone were unable to provide enough purification for SMT detection in complex biological suspensions such as manure or soil. When we used immunoaffinity chromatography (IAC) in conjunction with HLB Plus cartridges, we were able to rapidly purify ppb levels of sulfonamides from both water and complex biological suspensions using a batch process and thus fulfill our objectives. MALDI-TOF MS analysis required on-probe sulfonamide

Table 1. Percent Recovery of Sulfonamides from Each Stage of the SPIE System[a]

stage	description	SMT	NA-SMT
1	solid phase extraction (SPE)	57.3 ± 9.1	42.7 ± 4.3
2	immunoaffinity chromatography (IAC)	95.3 ± 4.7	101.6 ± 1.9
3	micro-SPE	80.2 ± 2.6	78.9 ± 3.5
	expected total SPIE recovery[b]	44	34

[a] 10 nmol of each sulfonamide was spiked into 1 L of water, 15 mL of PBS with 20% v/v ethanol, or 4 mL of 10 mM HCl for the SPE, IAC, or micro-SPE stages, respectively. Recoveries were determined colorimetrically using the Bratton–Marshall assay. Values represent the average of 2 trials. [b] Theoretical values were determined by multiplying recovery rates from stages 1–3.

concentrations of pure sulfonamide in the ppm range to generate substantial and unequivocal responses. Despite the excellent binding capacity and recoveries for our IAC column under low sample volume conditions (2−15 mL), our initial attempts to use IAC alone to concentrate ppb levels of sulfonamides from water were not successful. The large sample volumes required to collect enough analyte for MALDI-TOF MS analysis (200−1000 mL) exceeded the breakout volume of the IAC column. This breakout phenomenon is one limitation in determining the practical limits of detection in a residue analysis. Although low concentrations of herbicides in 1 L water have been immunoaffinity extracted without significant breakout (*40*), this is a function of the particular antiserum and its binding constant with the hapten. A preliminary concentrating step was necessary before immunoaffinity purification of SMT; however, commercial Sep Pak C_{18} cartridges (Waters Corp., Milford, MA), most commonly used for this purpose in residue analysis, exhibited breakout phenomenon at about 200 mL of water, similar to the observations with our IAC column. Instead we used a solid phase support of HLB Plus cartridges, which were superior in total capacity, were resistant to breakout, and maintained performance even if they ran dry during use.

In developing the IAC column, HiTrap Protein G-purified antibodies were added to the SMT-BSA IAC column to purify the SMT-specific population of IgG. As determined by UV spectrophotometry, 94% of the IgG applied to the SMT-BSA column was accounted for in the flow-through and eluted fractions. Seventeen percent of IgG was recovered in the acidic eluant, inferring that at least 17% of the original rabbit IgG could bind SMT. ELISA analysis of SMT-BSA column fractions determined that the SMT binding activity had been conserved in the SMT-BSA acid eluant with negligible activity remaining in the flow-through fractions. Anti-SMT antibodies (9.3 mg) were then incorporated per milliliter of CNBr-activated Sepharose gel, which had a binding capacity of 71% (25 μg/mL gel) and 57% (23 μg/mL gel) of the maximum theoretical values for SMT and NA-SMT, respectively, as determined by the Bratton−Marshall assay relative to sulfonamide standards. The immunoaffinity binding capacity is exceptional for a specific-hapten IgG IAC column, especially when one considers there is no control over orientation of IgG attachment to the solid phase with this attachment method, and also because one expects some denaturation and breakage of IgG during handling. IAC recoveries of nonsaturating concentrations of SMT and NA-SMT from 15 mL of PBS with 20% w/w ethanol were 95.3 ± 4.7% and 101.6 ± 1.9% respectively (**Table 1**). No loss of hapten binding capacity was observed for the IAC columns despite reusing them more than 20 times. Assil et al. (*41*) reported the ELISA detection of sulfonamides using affinity-purified polyclonal antibodies, yet their percentage yield estimate for the specific IgG fraction was strikingly low at 0.2%, even though the proportion of specific antibodies in the antiserum was likely much higher. We are continuing to investigate factors that affect the performance of the sulfonamide-IAC column, such as the selection of a linker arm for the hapten−protein conjugate immunogen used for making the polyclonal antibodies, the subsequent antibody−hapten binding constants and sulfonamide cross-reactivities, and the method used to purify the antibodies.

Table 2. Percent Recovery of Sulfonamides from Spiked Environmental Samples Using the SPIE MALDI-TOF MS System

sample	spike level (nmol)[a]	number of trials	SMT[b]	NA-SMT[b]
water	3.6	3	44.2 ± 3.1	40.7 ± 2.9
water	0.90	2	39.0 ± 16.8	48.3 ± 9.2
water	0.36	2	91.3 ± 17.4	59.5 ± 4.7
soil 1	3.6	2	43.7 ± 4.4	37.9 ± 4.1
soil 2	3.6	3	24.3 ± 5.1	25.5 ± 4.8
manure	3.6	3	8.0 ± 6.3	20.0 ± 5.8

[a] Units refer to the quantity of each sulfonamide added to 1 L of water, aqueous soil suspension (10% w/v), or aqueous manure suspension (10% w/v). [b] Values were determined by MALDI-TOF MS based on eq 2.

We tried using commercial micro C_{18} solid-phase extraction columns (Zip Tip, Millipore, Bedford, MA) to concentrate the IAC column eluants, but like our investigations into the initial concentration of dilute solutions of SMT in large water samples, C_{18} had little binding capacity for the relatively large 4 mL volumes we put through the micro columns. Alternatively, we made micro-HLB columns that performed well, both in concentrating the IAC column eluant and in changing the buffer to ethanol, a solvent that could be easily concentrated further and supported the dissolution of the DHB matrix used for MALDI-TOF MS.

Sulfamerazine (SMR) was used as an internal standard for several reasons: it is structurally similar to SMT so is extracted similarly to other sulfonamides in HLB columns, it responds similarly to SMT in MALDI-TOF MS analysis, and it is not commonly used in animal husbandry so is unlikely to be found in environmental samples. SMR did not bind well to the anti-SMT IAC column and therefore could not be added at the beginning of SPIE. The antibody was made against the dimethylpyrimidine end of SMT, and SMR has one less methyl group compared to SMT or NA-SMT (**Figure 1**), resulting in poor antibody binding of SMR. SMR was added as an internal standard to the IAC column eluants to determine the recovery of spiked SMT and NA-SMT based on their relative MALDI-TOF MS responses to SMR (eq 2). Although the use of SMR as an internal standard allowed for sensitive estimation of sulfonamide SPIE recoveries from spiked samples, the large variations in recoveries among different samples would not allow for quantification of SMT and NA-SMT in environmental samples using SMR (**Table 2**). An internal standard capable of quantifying sulfonamides in this system must be added to the samples before the SPIE process, be recovered from the SPIE system similarly compared to the analytes, and have a different molecular weight than the analytes. Nonetheless, SMR served as a necessary positive control for each MALDI-TOF MS spectrum. One of the inherent difficulties with MALDI-TOF MS is that impurities in the sample can interfere with the crystallization on the probe or with the transfer of ionization energy to the analyte. A positive SMR response established that the MALDI-TOF MS process was working well for sulfonamides in each spectrum and that a negative response for the analyte of interest could be interpreted appropriately. Another important function of SMR was to establish the response ratio

Table 3. Isotopic Molecular Weights for Sulfonamide Parent Molecules (M), Cationic Adducts, and Fragment Ions

	M	$[M + H]^+$	$[M + Na]^+$	$[M + K]^+$	$[M - SO_2 + H]^+$
sulfamethazine ($C_{12}H_{14}O_2N_4S$)	278.1	279.1	301.1	317.1	215.1
N^4-acetylsulfamethazine ($C_{14}H_{16}O_3N_4S$)	320.1	321.1	343.1	359.1	257.1
sulfamerazine ($C_{11}H_{12}O_2N_4S$)	264.1	265.1	287.1	303.0	201.1

pattern for sulfonamide adducts in a given MALDI-TOF MS spectrum (**Table 3**). A consistent cation adduct response pattern among different sulfonamides was observed for standards, and this consistency gave a more definitive MALDI-TOF MS positive identification than would molecular weight information alone.

SMR and SMT had similar MALDI-TOF MS responses, yet the response of NA-SMT was less than half of SMT (0.99 ± 0.12 and 0.42 ± 0.06, response ratios for SMT/SMR and NA-SMT/SMR, respectively, $n = 3$). This finding, together with the variability associated with well-documented inconsistencies of analyte/matrix cocrystallization, emphasizes the need for accurate determination of response factors and the acquisition of replicate spectra during quantification using MALDI-TOF MS (*24*). Mass spectral peaks for DHB matrix alone did not conflict with theoretical isotopic molecular weights (*m*/*z*) for SMT, NA-SMT, or SMR, except for very minor peaks at *m*/*z* 215 and 317 (**Table 3**). Controls that omitted the addition of SMR internal standard showed no spectral peaks typical of SMR, whereas SMR peaks were easily seen in the spectra from samples including internal standard but not spiked with SMT or NA-SMT.

We did not expect to find sulfonamide residues naturally occurring in the soils or the composted manure because there were no records or awareness of manure having been spread in the area where soil was collected and no history of sulfonamides having been used in the feed of animals that provided manure in this study. No peaks associated with SMT and NA-SMT were identified for the unspiked water, soil 1, and manure samples, while small but significant peaks were consistently observed in the unspiked soil 2 sample, close to the limit of detection (**Figure 3**). These peaks at *m*/*z* 321.1 and 343.1, representing the $[M + H]^+$ and $[M + Na]^+$ adducts of NA-SMZ, respectively, corresponded to roughly 2 ppb on a dry weight basis, when calculated using the same rate of recovery as observed for NA-SMT in spiked soil 2 samples. It is unlikely that laboratory contamination can explain the presence of these peaks, because they appeared in no other unspiked samples analyzed. Also, SMT and NA-SMT were always added in equal mass ratios, and SMT inherently has more than a 2-fold better response relative to NA-SMT, so it seems unlikely that NA-SMT would appear as an isolated accidental contaminant. Repetition of sampling and analysis with other validated methods would be required to make any conclusions about the presence of NA-SMT residues in this soil. These anomalous signals exemplify the difficulty in trace residue analysis to establish adequate negative controls. When the limits of detection in residue analysis approaches sub-ppb levels (or lower), it is difficult to define a sample matrix that can be guaranteed to not contain measurable quantities of analyte. False positives become a real problem due to laboratory or sampling contamination, and confidence in the negative controls become all the more necessary.

Water samples and soil and manure suspensions were spiked with 3.60 nmol each of SMT and NA-SMT. In 1 L of water, this spike level corresponded to concentrations of 1.00 and 1.15 ppb, respectively. In soils 1 and 2 and composted manure

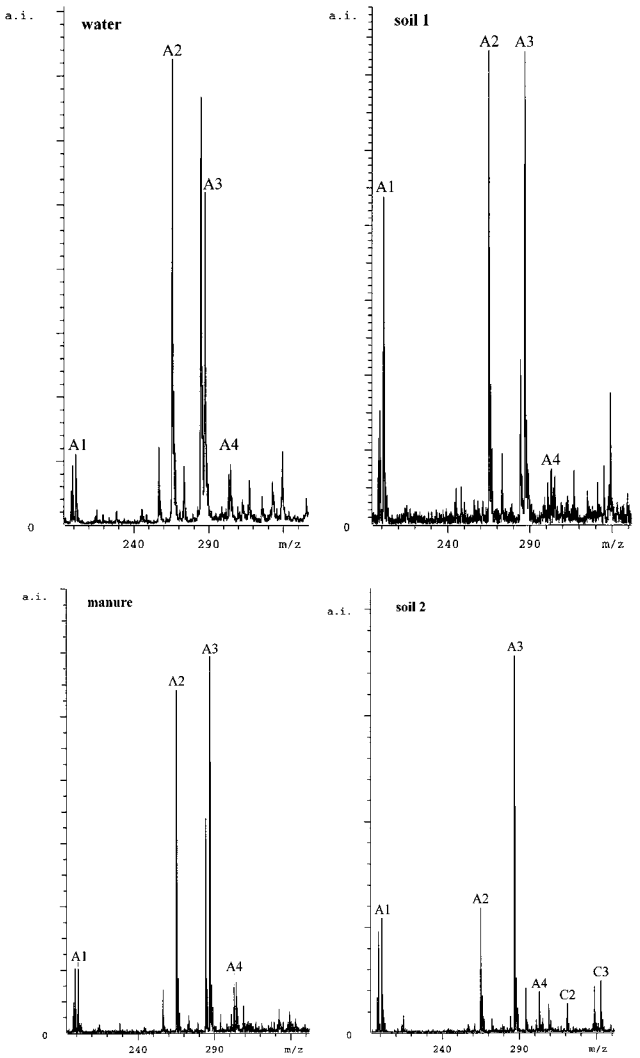

Figure 3. MALDI-TOF MS spectra of water and suspensions of soil 1, soil 2, and composted manure, processed through the SPIE system, with added SMR. Letters A and C represent peaks associated with SMR and NA-SMT, respectively, and numbers 1, 2, 3, and 4 represent sulfonamide fragment and proton, sodium, and potassium adducts, respectively. No peaks associated with sulfamethazine were observed.

samples, the sulfonamide concentrations on a dry weight basis (corresponding to 3.60 nmol sulfonamide per ~1 L suspension) were 12.2, 11.9, and 14.5 ppb, respectively, for SMT and 14.0, 13.7, and 16.6 ppb, respectively, for NA-SMT. These low initial concentrations, when concentrated and purified through the SPIE system, were able to produce strong signals in the mass spectra (**Figure 4**). On the basis of the high response and relatively low noise, detection of much lower initial sulfonamide concentrations is possible. Water samples spiked with 10 times less sulfonamide (0.360 nmol/L of both of SMT and NA-SMT, corresponding to 0.100 and 0.115 ppb, respectively) still showed significant SMT and NA-SMT signals more than 3 times above the average background noise (**Figure 5**), making the sensitivity

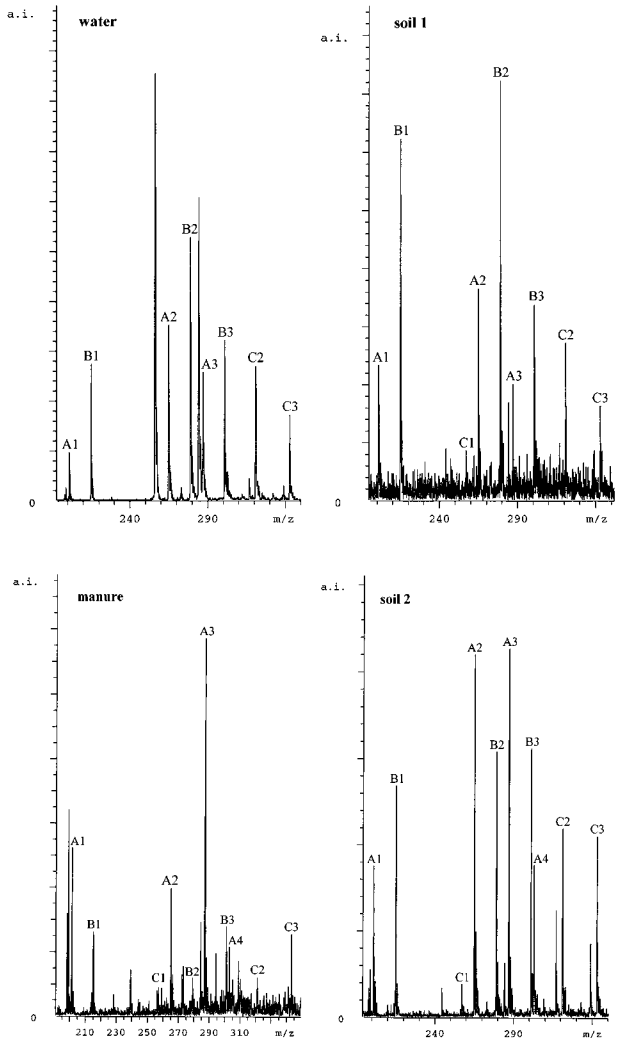

Figure 4. MALDI-TOF MS spectra of 1 L of water and suspensions of soil 1, soil 2, and composted manure (1 L of deionized water added to each), spiked each with 3.60 nmol of SMT and NA-SMT, processed through the SPIE system, with added SMR. Letters A, B, and C represent peaks associated with SMR, SMT, and NA-SMT, respectively, and numbers 1, 2, 3, and 4 represent sulfonamide fragment and proton, sodium, and potassium adducts, respectively.

of SPIE MALDI-TOF MS comparable to the performance reported for mass spectrometric methods in drug residue analysis (*15*, *17*).

Recoveries of SMT and NA-SMT for spiked samples in each stage of the SPIE system were determined colorimetrically using the Bratton−Marshall (BM) assay with buffers and volumes appropriate for each stage of SPIE (1 L of water for initial SPE, 15 mL of PBS with 20% ethanol for IAC, and 4 mL of 10 mM HCl for micro-SPE). As shown in **Table 1**, both sulfonamides exhibited similar recovery patterns through the system. Approximately half of the sulfonamide content was lost in the first solid phase extraction step. IAC was not selective for one sulfonamide over the other, and both were recovered from this stage at nearly 100%. There was a similar loss of approximately 20% of both sulfonamides in the final microextraction step. Overall, the colorimetric estimates of each stage of the SPIE system predict that SMT would be recovered at a rate of 44% through the whole system and NA-SMT would be recovered at a rate of 34%. However, despite losses, the SPIE system demonstrated enough concentration effect to allow confident

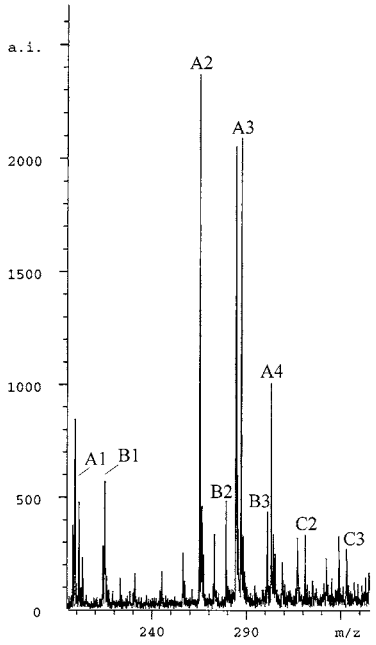

Figure 5. MALDI-TOF MS spectrum of 1 L of water spiked with 0.36 nmol each of SMT and NA-SMT, processed through the SPIE system, with added SMR. Letters A, B, and C represent peaks associated with SMR, SMT, and NA-SMT, respectively, and numbers 1, 2, 3, and 4 represent sulfonamide fragment and proton, sodium, and potassium adducts, respectively.

sulfonamide detection in samples with even sub-ppb initial sulfonamide concentrations.

Recoveries of SMT and NA-SMT from water, soil, and manure systems spiked at levels shown in **Table 2** were determined by MALDI-TOF MS with the addition of SMR as internal standard at the micro-SPE stage. An average recovery rate for SMT, NA-SMT, and SMR of 81 + 0.01% ($n = 3$) from the micro-SPE column (determined colorimetrically) was factored in (F in eq 2) to estimate recoveries using MALDI-TOF MS. The observed recovery rates for 3.6 nmol SMT and NA-SMT from 1 L of water (44.2 ± 3.1% and 40.7 ± 2.9%, respectively; $n = 3$) were close to the theoretical values reported in Table 2 (44% and 34%, respectively). As expected, in cases where sulfonamides were present in complex organic mixtures (i.e., soil or manure), the recoveries decreased. The lower recoveries for soil 2 and manure samples were likely due to greater sulfonamide losses at the initial SPE step, but we could not directly verify this colorimetrically due to large sample blank interferences at 545 nm absorbance. SPE cartridges are not selective like the IAC, and it is possible the HLB Plus cartridge became saturated with hydrophobic organic compounds such as lipids or proteins. We cannot explain the higher recoveries reported for the lowest concentration (0.36 nmol in 1 L) of SMT and NA-SMT in water (91.3 ± 17.4% and 59.5 ± 4.7%, respectively, $n = 2$) by column saturation dynamics because sample loading of 0.9, 3.6, and 10 nmol all exhibited similar recovery rates (**Tables 1** and **2**). The recovery of this lowest sulfonamide concentration represents data that were near the detection limits of the MALDI-TOF MS method, and large spectral variability associated with these low responses are likely responsible for an overestimation of the recovery rate.

We have demonstrated the capability of SPIE MALDI TOF to detect low ppb quantities of SMT and NA-SMT in environmental samples. Currently our SPIE process takes about 1.5 h per batch (8−24 samples/batch) for sample enrichment, 5 min per batch for probe preparation, and 5 min per sample to acquire

and process the spectrum. In comparison to other mass spectrometric techniques that require extensive purification and rely on a rate-limiting, serial process such as HPLC, SPIE MALDI-TOF MS is fundamentally better suited to high-throughput analysis. The "bottlenecks" inherent in solid phase extraction can be overcome by automated and large-scale parallel batch processing of samples.

With a future focus on developing an internal standard that can be recovered equivalently from SMT or NA-SMT (like N^4-propionyl SMT), it is our intention to develop and validate a SPIE MALDI-TOF MS system capable of rapidly quantifying sulfonamide residues from environmental samples. Coupled with rapidly evolving technologies such as automated sample addition and spectrum acquisition (*42*−*44*), SPIE MALDI-TOF MS could address future demands for rapid, sensitive, and definitive drug residue analysis in complex environmental samples.

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