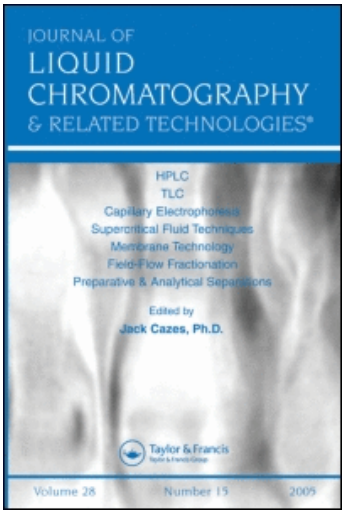

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DETERMINATION OF SULFADIAZINE AND TRIMETHOPRIM IN MARINE SEDIMENT BY LC-APCI-MS

L. K. Sørensen[a]; H. Hansen[a]
[a] Steins Laboratorium, Brørup, Denmark

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DETERMINATION OF SULFADIAZINE AND TRIMETHOPRIM IN MARINE SEDIMENT BY LC-APCI-MS

L. K. Sørensen* and H. Hansen

Steins Laboratorium, Ladelundvej 85, DK-6650 Brørup, Denmark

ABSTRACT

A liquid chromatographic method based on mass spectrometric detection using atmospheric pressure chemical ionisation (LC-APCI-MS) was developed for simultaneous determination of sulfadiazine and trimethoprim in marine sediment. Sediment was extracted with acetonitrile and cleaned up by solid phase extraction (SPE) on cationic and polymeric sorbents. Ion monitoring was performed using the protonated molecular ions for sulfadiazine (m/z 251) and trimethoprim (m/z 291). The limit of detection was 4 μg/kg for sulfadiazine and 0.9 μg/kg for trimethoprim. The recoveries were $72 \pm 3\%$ (mean $\pm$ SD) for sulfadiazine at a level of 100 μg/kg and $83 \pm 4\%$ for trimethoprim at a level of 25 μg/kg. The relative repeatability standard deviation was less than 6% at levels of 50 μg/kg for sulfadiazine and 20 μg/kg for trimethoprim.

*Corresponding author. E-mail: lks@steins.dk

INTRODUCTION

Sulfadiazine and trimethoprim are active against a broad range of Gram-positive and Gram-negative bacteria and are widely used in human and veterinary medicine. They are also commonly used for the treatment of infectious diseases in fish farming. They are normally applied in combination because of their synergistic inhibition of the folic acid biosynthetic pathway. The typical administered ratio of sulfadiazine to trimethoprim is 5 : 1. For application in aquaculture the drugs are administered as ingredients in feed pellets, which may result in a significant loss to the environment. Generally, contamination of the environment with drugs is of increasing concern because the drugs may enter the food chain and introduce a health risk to consumers through allergic reactions or through induction of antibiotic resistance in pathogenic organisms.

Some liquid chromatographic (LC) methods have been reported for determination of sulfadiazine (1–7) and trimethoprim (2–4,6,8) in matrices related to the aqueous environment. These include methods for fish tissues (1,4,6,7), fish feed (2,3), water (8), and waste waters (5) based on UV detection (1–4,6), postcolumn derivatization, and fluorescence detection (7) or tandem mass spectrometry (MS-MS) (5,8). In some cases, chromatography was performed directly on the sample extract (2,3,6) or after pre-concentration of the sample (5,8). In other cases, the sample extract was cleaned up by solid phase extraction (SPE) (1,4) or liquid–liquid partitioning (7) prior to LC analysis.

This paper describes a sensitive LC method for simultaneous determination of sulfadiazine and trimethoprim in marine sediment using SPE for sample clean-up and atmospheric pressure chemical ionisation (APCI) MS for detection.

EXPERIMENTAL

Chemicals and Reagents

Sulfadiazine and trimethoprim were purchased from Sigma (St. Louis, MO, USA). Acetonitrile of chromatography grade, methanol, phosphoric acid, oxalic acid dihydrate, sodium acetate anhydrous, sodium hydroxide, acetic acid, ammonium acetate, and calcium chloride dihydrate were obtained from Merck (Darmstadt, Germany). Water was purified through a Millipore Milli-Q Plus system (Bedford, MA, USA).

The mobile phase for LC was prepared by diluting 100 mL acetonitrile to 1000 mL with acetate buffer. The acetate buffer was prepared from 15 mM ammonium acetate solution adjusted to pH 4.2 with acetic acid.

Wash solution for cationic exchange SPE was prepared by mixing acetonitrile and 10 mM phosphoric acid solution (1 : 1). Elution solvent for

cationic exchange SPE was prepared by mixing 30 mL 1 M sodium acetate, 10 mL 1 M calcium chloride, and 120 mL methanol. This solution was prepared just before use.

Standard Solutions

Stock solution of sulfadiazine was prepared at a concentration of 1000 μg/mL by dissolving the pure substance in 5 mL 1 M NaOH solution, followed by dilution to 50 mL with water. A 1000 μg/mL stock solution of trimethoprim was prepared in methanol. The stock solutions were stable for at least one month when stored at $5 \pm 2^{\circ}$C. A combined standard solution containing 10 μg/mL of sulfadiazine and 2.5 μg/mL of trimethoprim was prepared by diluting the stock solutions with water. Calibration standards containing 20, 100, 200, 500, and 1000 ng/mL of sulfadiazine and four times less of trimethoprim were prepared by diluting aliquots of the combined standard solution with mobile phase. The calibration standards were stable for at least two weeks when stored at $5 \pm 2^{\circ}$C.

Materials

Strong cationic exchange (SCX) cartridges, Bakerbond aromatic sulphonic acid 500 mg, were obtained from Baker (Phillipsburg, NJ, USA). Polymeric sorbent cartridges, Oasis HLB (divinylbenzene-co-N-vinylpyrrolidone polymeric sorbent) 60 mg, were obtained from Waters (Milford, MA, USA). Top-capped centrifugal-driven ultrafiltration units of 2 mL capacity with 30,000 nominal molecular weight regenerated cellulose membranes were purchased from Millipore (Bedford, MA, USA). Centrifuge tubes of 15 mL and 50 mL capacity were made of polypropylene (Sarstedt, Nümbrecht, Germany). Marine sediments covering a range from sandy to muddy texture were used for method validation.

Instrumentation and Chromatographic Conditions

The instruments used were a Sigma centrifuge model 4K15 (Osterode, Germany), a VF2 test tube shaker (IKA, Staufen, Germany), a HS500 horizontal shaker (IKA), a vacuum manifold for SPE cartridges (Waters), a pH meter PHM 93 (Radiometer, Copenhagen, Denmark), and a temperature-controlled heating block with a manifold for nitrogen flow (Mikrolab Aarhus, Aarhus, Denmark).

The liquid chromatography system consisted of LC200 micro pumps (Perkin Elmer, Shelton, CT, USA), a series 200 autosampler (Perkin Elmer), a Sciex API 150 MCA mass spectrometer (Applied Biosystems, Foster City, CA,

USA), and a Heated Nebulizer for APCI (Applied Biosystems). Reverse-phase LC was carried out on an XTerra C18 column (3.5 µm, 150 mm × 4.6 mm I.D.) (Waters). The acquired data were processed with either MultiView 1.4 or MacQuan 1.6 software (Applied Biosystems).

The injection volume was 100 µL and the mobile phase flow rate was set at 0.8 mL/min. The column temperature was kept at 25 ± 1°C.

The chromatographic system was run isocratically with 25 min between injections. The mobile phase was allowed to drain for the first 6 min after injection, using a post-column switch.

Monitoring was performed in selected positive ion mode based on the protonated molecular ions m/z 251 for sulfadiazine and m/z 291 for trimethoprim. The nebulizer was heated to 510°C and the Corona pin voltage was set at 2.0 kV. The orifice and focusing ring voltages were set at 18 V and 120 V respectively. The high pressure entrance quadrupole (Q0) was set at −4 V.

Sample Preparation

A mass of 5.0 g sample was weighed into a 50 mL centrifuge tube. Volumes of 20 mL acetonitrile and 1.00 mL 10 mM phosphoric acid were added, and the tube was shaken horizontally (250 strokes/min) for 20 min. The mixture was centrifuged at 3000 g for 5 min and the supernatant was collected. The extraction was repeated with 20 mL acetonitrile and the supernatants were combined and diluted to 50 mL with water.

A volume of 20 mL extract was diluted to 100 mL with 50 mM oxalic acid solution. An SCX cartridge was conditioned with 3 mL methanol followed by 3 mL water. The sample solution was pulled through the column at a flow rate of max. 5 mL/min. The cartridge was washed with 2 mL wash solution followed by 1.5 mL water. The cartridge was then eluted with 4.0 mL elution solution.

The eluate was diluted with 60 mL water and mixed with 100 µL acetic acid. A polymeric sorbent cartridge was washed with 5 mL methanol followed by 2 mL water. The solution was pulled through the SPE cartridge at a flow rate of max 10 mL/min. The cartridge was dried by suction for 1 min and eluted with 4.0 mL acetonitrile. The eluate was evaporated to bare dryness at 50–55°C under a stream of nitrogen. The residue was redissolved in 100 µL acetonitrile, diluted with 900 µL acetate buffer for mobile phase and ultrafiltered at 4000 g for 10 min.

Ruggedness

The extraction yield obtained from each of three successive extractions with 20 mL acetonitrile was determined on samples spiked with sulfadiazine and trimethoprim to a level of 500 µg/kg.

The effect of acetonitrile concentration and flow rate on adsorption of sulfadiazine and trimethoprim to SCX cartridges was investigated by connecting two equal cartridges in series and applying extract from sediment spiked with sulfadiazine and trimethoprim to levels of 2000 μg/kg. The amounts of analyte adsorbed to each cartridge were determined according to the procedure.

The necessary dilution of the eluate from the SCX cartridge for complete retention of sulfadiazine and trimethoprim on the polymeric sorbent and the maximum flow rate, which could be applied, were investigated in a similar way.

The elution profile from SPE cartridges was determined on sediments spiked with sulfadiazine and trimethoprim to levels of 500 μg/kg. Aliquots of 1.0 mL eluent were used for elution.

The method was tested for matrix-induced effects on signal intensity. Mixed standards and final sample extracts spiked with 100 ng of sulfadiazine and 25 ng of trimethoprim were analysed in attenuated order.

The effect of mobile phase composition was investigated by response surface modelling (RSM) using standards containing 100 μg/L of sulfadiazine and 25 μg/L trimethoprim. The ammonium concentration was varied from 2 to 20 mM and the pH was varied from 4.0 to 6.3. Peak height and retention time were used as responses. A central composite face-centred (CCF) design with quadratic modelling was used for the experiment.

The stability at 5–7°C of calibration standard solutions and final extracts of sediments containing sulfadiazine at levels of 10 and 100 μg/kg and trimethoprim at levels of 2.5 and 25 μg/kg, was tested over a period of two weeks.

Limits of Detection

The limits of detection (LODs) were determined on 20 different blank control sediments. To obtain realistic LODs, the samples were spiked prior to extraction with sulfadiazine and trimethoprim to a peak height on chromatograms corresponding to ca. three times the short term baseline variation. The samples were, thus, spiked with sulfadiazine to a level of 2.5 μg/kg and trimethoprim to a level of 0.63 μg/kg. The spiked samples were mixed and stored 16–20 h at 5–7°C before extraction. The detection limits were determined as the mean results plus three times the standard deviation (SD) of the 20 measurements.

Precision and Recovery

The repeatability standard deviation (i.e. the variability of independent analytical results obtained by the same operator, using the same apparatus under the same conditions on the same test sample, and in a short interval of time) and

the intra-laboratory reproducibility standard deviation (i.e., the variability of independent analytical results obtained on the same test sample in the same laboratory by different operators under different experimental conditions) were determined on contaminated sediments containing sulfadiazine at levels of 20 and 70 μg/kg and trimethoprim at a level of 4 μg/kg. Precision was also determined on sediments spiked to levels of 10, 100, and 500 μg/kg with sulfadiazine and levels of 2.5, 25, and 125 μg/kg with trimethoprim. The samples were analysed in duplicate on each of eight days. Calculation of repeatability was done in accordance with ISO standard 5725-2, 1994 (9). The intra-laboratory reproducibility was calculated by the same principle used for determination of reproducibility (9). The recovery was determined on 20 different blank control sediments spiked to levels of 10 μg/kg and 100 μg/kg with sulfadiazine and levels of 2.5 μg/kg and 25 μg/kg with trimethoprim. The spiked samples were mixed and stored 16–20 h at 5–7°C before extraction.

Determination of Total Solids and Organic Matter

A mass of 5 g sample was transferred to a porcelain crucible and dried at 105 ± 3°C for 20 h to determine the content of total solids (TS). The residue was then ignited at 550 ± 25°C for 2 h to give the content of fixed residue. The difference was defined as the content of organic matter in total solids (OMTS).

RESULTS AND DISCUSSION

The instrumental parameters were optimised by injection of 20 μL volumes of 1 μg/mL standards into the flow system bypassing the analytical column. The optimal MS conditions for sulfadiazine and trimethoprim appeared to be rather equal. Common conditions were, therefore, selected for both compounds. The mass spectra contained only the protonated molecules $[M + H]^+$ and no fragment ions of significant intensity.

The effect of mobile phase composition on signal intensity and retention time was investigated by RSM with ammonium concentration and pH as factors. Figure 1 shows the contour plots for sulfadiazine and trimethoprim obtained by injection of standards on the analytical column. The retention time of sulfadiazine increased with decreasing pH, whereas the retention time of trimethoprim decreased. The optimal isocratic conditions resulting in sufficiently long retention time for sulfadiazine and a relatively short retention time for trimethoprim were then obtained at low pH. The retention time was not significantly influenced by the ammonium acetate concentration. The signal intensity measured from peak height was not only affected by the pH (retention time) but also by the molar

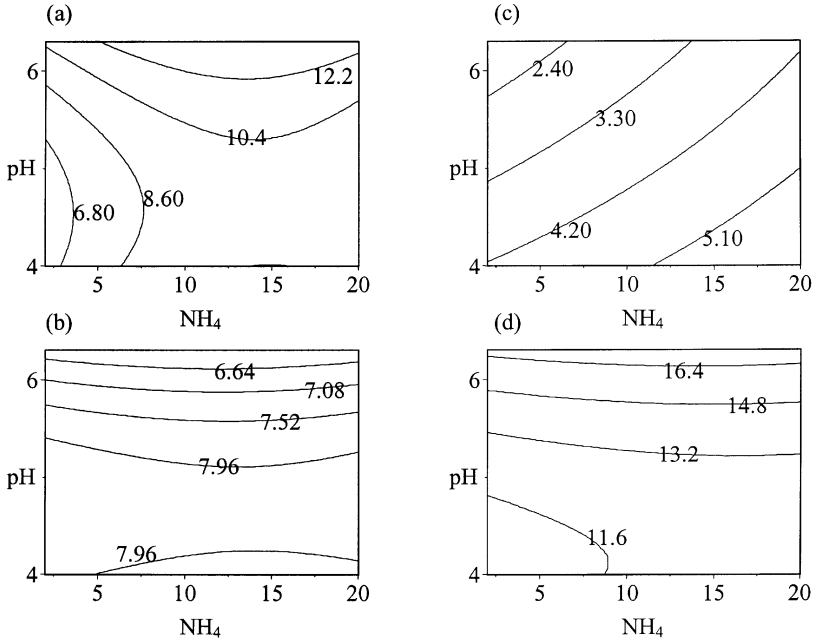

Figure 1. Contour plots showing the effect of mobile phase composition (pH and ammonium acetate concentration) on peak height (a) and retention time (b) of sulfadiazine and peak height (c) and retention time (d) of trimethoprim.

concentration of ammonium acetate. The dependence was easiest to model in the case of trimethoprim. At low pH, a relatively high molar concentration of ammonium acetate was optimal for the signal responses of both compounds.

The signal intensity obtained when standard solutions were injected by-passing the analytical column, did not change much when acetonitrile was replaced by methanol. However, the retention of trimethoprim, but not sulfadiazine, was prolonged dramatically on the analytical column, making it difficult to analyse both compounds under isocratic conditions.

Chromatograms of a typical blank control sediment spiked with sulfadiazine and trimethoprim to levels of 2.5 μg/kg and 0.6 μg/kg, respectively are shown in Figure 2. Chromatograms of a contaminated sediment are shown in Figure 3. Generally, no interfering peaks were observed on chromatograms.

Residual polymeric compounds retained in the final extract were removed by ultrafiltration. If ultrafiltration was omitted, the initial switch of column effluent to drain was essential in order to avoid contamination of the MS orifice. When the complete clean-up procedure was followed, the matrix effect on signal

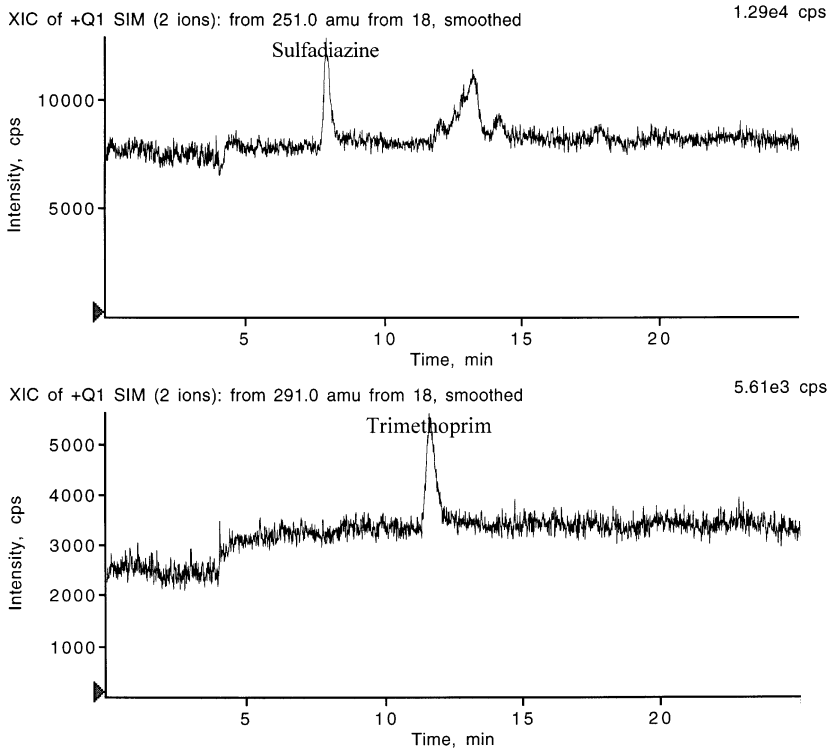

Figure 2. Chromatograms of blank control muddy sediment (3% OMTS and 32% TS) spiked with sulfadiazine and trimethoprim to levels of 2.5 μg/kg and 0.6 μg/kg, respectively.

response was negligible. The recoveries (mean ± SD) obtained from sample extracts spiked just before ultra-filtration were 101 ± 2% for sulfadiazine and 104 ± 2% for trimethoprim (n = 20).

The sample was extracted twice. The relative extraction yield obtained from the second extraction was 8% for sulfadiazine and 3% for trimethoprim (n = 4). Less than 1% was recovered by a third extraction.

Dilution of 20 mL sample extract to 100 mL with oxalic acid was sufficient to retain sulfadiazine and trimethoprim on the SCX cartridge when the flow rate did not exceed 5 mL/min (n = 6). The capacity of the cartridge was sufficient for at least 120 mL diluted sample extract (n = 6). If the dilution was performed with 60 mL oxalic acid or the flow rate was increased to 7 mL/min, then 3% of the sulfadiazine, but no trimethoprim, was lost.

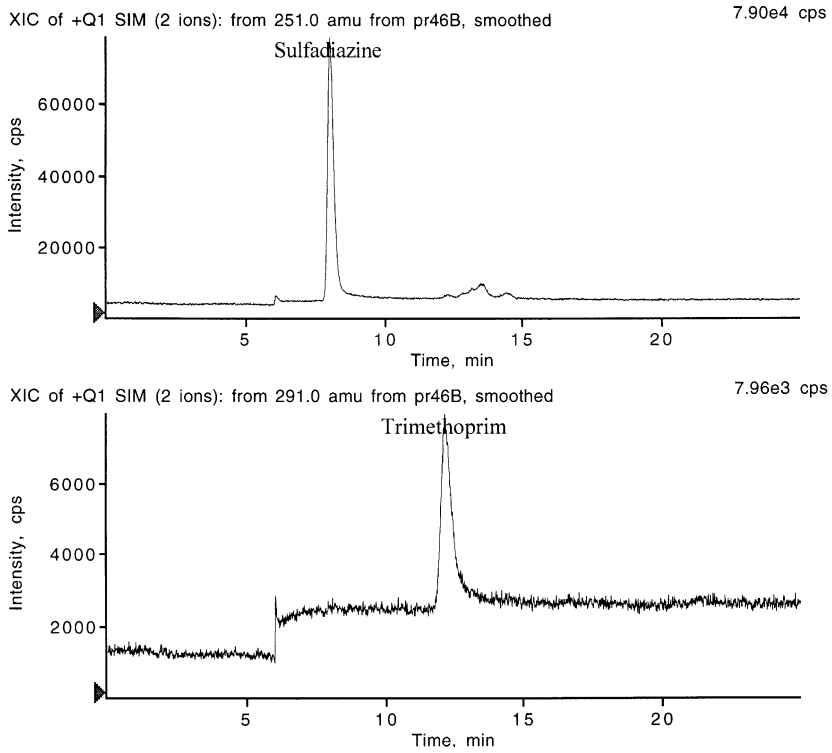

Figure 3. Chromatograms of a contaminated sediment (3% OMTS, 16% TS) from centre of fish farm containing 33 μg/kg sulfadiazine and 3 μg/kg trimethoprim as measured before recovery correction.

The SCX cartridge was washed with 50% acetonitrile in 10 mM phosphoric acid. If 20–30% acetonitrile was used, a more unstable detection was observed. A small loss of sulfadiazine was observed if the acetonitrile concentration was increased to 60%.

Elution of sulfadiazine and trimethoprim from the SCX cartridge required at least 3 mL elution solution ($n = 4$). Less than 1% of the total recovery was found in the 4th 1 mL volume of eluate.

Dilution of 4 mL eluate from the SCX cartridge with 60 mL water, followed by addition of 100 μL acetic acid to obtain a pH of ca. 4.5, was sufficient for complete adsorption of sulfadiazine and trimethoprim to the polymeric sorbent SPE cartridge ($n = 6$). The capacity of the cartridge was sufficient for at least 80 mL diluted eluate. If dilution was performed with 40 mL water, ca. 3% of

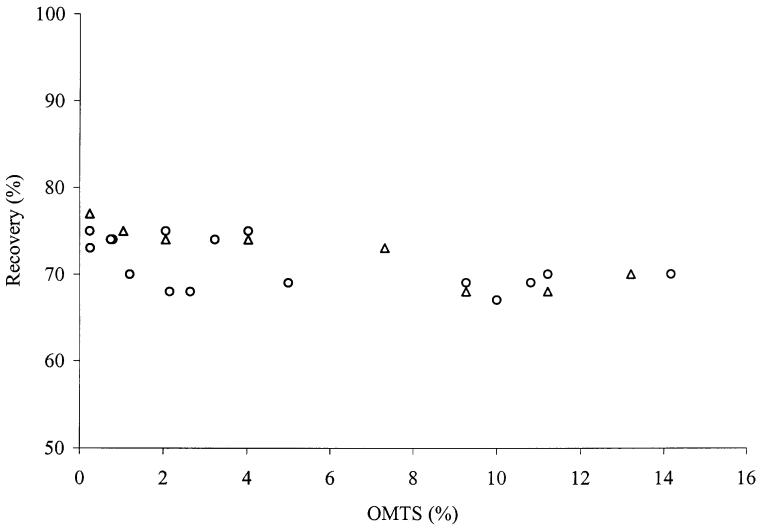

Figure 4. Correlation between OMTS and recovery from sediments spiked with sulfadiazine to levels of 10 µg/kg (Δ) and 100 µg/kg (○). A single determination was performed on each sample.

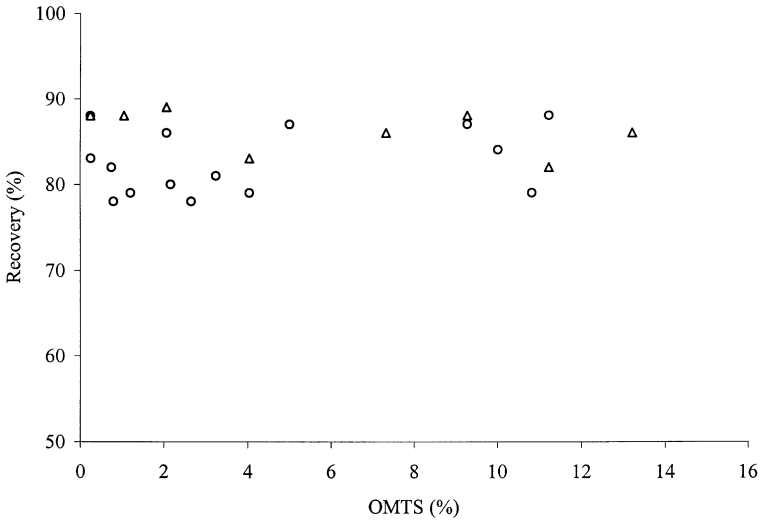

Figure 5. Correlation between OMTS and recovery from sediments spiked with trimethoprim to levels of 2.5 µg/kg (Δ) and 25 µg/kg (○). A single determination was performed on each sample.

Table 1. Limits of Detection Determined on Blank Control Marine Sediments (n = 20) Spiked with Sulfadiazine and Trimethoprim to Levels of 2.5 μg/kg and 0.63 μg/kg, Respectively

	Measured Conc. (μg/kg) Mean ± SD	Mean recovery (%)	LOD (μg/kg)	LOD corrected for recovery (μg/kg)
Sulfadiazine	1.91 ± 0.27	75	2.7	4
Trimethoprim	0.51 ± 0.07	82	0.7	0.9

sulfadiazine and trimethoprim was lost. The highest application flow rate, which could be used without loss was 10–12 mL/min.

Elution of sulfadiazine and trimethoprim from the polymeric sorbent required at least 3 mL acetonitrile (n = 6). Less than 1% of the total recovery was found in the 4th 1 mL volume of eluate.

The mean recoveries of sulfadiazine obtained on spiked samples were 73 ± 6% (mean ± SD) and 72 ± 3% at levels of 10 μg/kg and 100 μg/kg, respectively. The corresponding recoveries of trimethoprim were 85 ± 4% (mean ± SD) and 83 ± 4% at levels of 2.5 μg/kg and 25 μg/kg, respectively. Because the matrix effect on signal response was negligible, these recoveries could be regarded as true extraction yield from spiked samples. The recovery of sulfadiazine, but not trimethoprim, tended to be slightly dependent on OMTS content (Figures 4 and 5).

Table 2. The Relative Repeatability Standard Deviation (RSD_r) and Intra-laboratory Reproducibility Standard Deviation ($RSD_{R,intra}$) Determined for Sulfadiazine on Contaminated and Spiked Samples

Measured conc. (μg/kg)[a]	TS (%)	OMTS (%)	RSD_r[d] (μg/kg)	$RSD_{R,intra}$[d] (μg/kg)
7.0[c]	69	1.6	10	17
16[b]	51	5.5	9.4	13
51[b]	15	19	5.6	8.7
75[c]	26	11	4.2	4.2
370[c]	20	13	6.1	6.3

[a]Not corrected for recovery.
[b]Naturally contaminated sample.
[c]Spiked sample.
[d]One duplicate analysis at each level was conducted on each of eight days.

Table 3. The Relative Repeatability Standard Deviation (RSD_r) and Intra-laboratory Reproducibility Standard Deviation ($RSD_{R,intra}$) Determined for Trimethoprim on Contaminated and Spiked Samples

Measured conc. (μg/kg)[a]	TS (%)	OMTS (%)	RSD_r[d] (μg/kg)	$RSD_{R,intra}$[d] (μg/kg)
2.3[c]	69	1.6	6.3	8.9
3.1[b]	15	19	8.5	9.4
21[c]	26	11	4.8	8.4
110[c]	20	13	3.0	4.2

[a]Not corrected for recovery.
[b]Naturally contaminated sample.
[c]Spiked sample.
[d]One duplicate analysis at each level was conducted on each of eight days.

The LODs estimated by a conservative model were 4 μg/kg for sulfadiazine and 0.9 μg/kg for trimethoprim (Table 1).

The relative repeatability standard deviation was less than 10% at a sulfadiazine level of 15 μg/kg and a trimethoprim level of 5 μg/kg. The imprecision was less than 6% at levels of 50 μg/kg for sulfadiazine and 20 μg/kg for trimethoprim (Tables 2 and 3).

The calibration curves were linear in the tested range up to 1000 ng/mL for sulfadiazine and 250 ng/mL for trimethoprim. The slope converting concentration (ng/mL) to peak area was $1.8 \times 10^4 \pm 0.1 \times 10^4$ (mean ± SD) for sulfadiazine and $3.2 \times 10^4 \pm 0.2 \times 10^4$ for trimethoprim in the precision study. The coefficients of determination (R^2) were 0.9993–1.0000.

Final sample extracts and calibration standards were stable for at least 14 days when stored at 5–7°C.

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