JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Analysis of O,S-Dimethyl Hydrogen Phosphorothioate in Urine, a Specific Biomarker for Methamidophos

ELIZABETH TOMASZEWSKA AND VINCENT R. HEBERT*

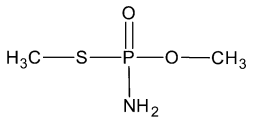
Food and Environmental Quality Laboratory, Washington State University, 2710 University Drive, Richland, Washington 99352

A rugged and sensitive method was developed to monitor urinary concentrations of O,S-dimethyl hydrogen phosphorothioate (O,S-DMPT), a specific biomarker of exposure to the organophosphate insecticide methamidophos. After pH adjustment and C18 solid phase extraction column cleanup, the urine was lyophilized at a low temperature to prevent loss of possibly highly volatile and unstable O,S-DMPT metabolite. The dried residue was derivatized using N-methyl-N-(*tert*-butyldimethylsilyl)-trifluoroacetamide and 1% *tert*-butyldimethylchlorosilane (MTBSTFA + 1% TBDMCS) in acetonitrile. After it was filtered, the derivatized product was analyzed and quantified by gas chromatography using a pulse flame photometric detector specific for phosphorus compounds. The limit of detection for this method was 0.004 ppm with a limit of quantitation of 0.02 ppm of urine. The mean recovery value for O,S-DMPT from 17 urine samples fortified at varying concentrations was 108% with a standard deviation of 12%.

KEYWORDS: O,S-Dimethyl hydrogen phosphorothioate; methamidophos; acephate; biomonitoring; pesticides; chromatography

INTRODUCTION

Organophosphate (OP) insecticides have a common mechanism of toxicity in vertebrates and invertebrates; thus, they often lack selectivity between nontarget organisms and insect pests. Their continued widespread use in agriculture and urban areas requires sensitive and reliable analytical methods to establish human exposure, especially to children. Exposure can be estimated by measuring food and environmental residues, but measuring parent pesticides and/or their metabolites in urine provides information about direct body doses (*1*). Numerous studies have measured the concentration of alkyl phosphate hydrolysis products of the most commonly used OP insecticides, including diazinon, chlorpyrifos, methyl parathion, and azinphos-methyl (*2*−*4*). The parent OPs methamidophos and acephate (**Figure 1**) have been monitored in worker's urine (*5*), but research so far has not characterized human excretion of phosphorothioate metabolites of these two pesticides. A major detoxication route for methamidophos in mammals is by mixed function oxidation deamination resulting in formation of the dimethyl phosphorothioate metabolite (O,S-dimethyl hydrogen phosphorothioate, O,S-DMPT) through cleavage of the P−N bond and replacement of amine by the hydroxyl group (*6*, *7*). Because transformation of acephate in the environment may yield methamidophos, O,S-DMPT can also be considered a specific biomarker for measurement or monitoring of human exposure to acephate.



Acephate

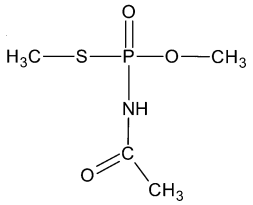


Figure 1. Structures of methamidophos and acephate.

Methamidophos is highly toxic by oral (female rodent LD_{50} = 13 mg/kg) and dermal routes (rabbit LD_{50} = 118 mg/kg) (*8*). Acephate has moderate acute oral toxicity (female rodent LD_{50} = 1000 mg/kg) and low acute dermal toxicity (rabbit LD_{50} > 10 000 mg/kg) (*9*). Although both of these OP insecticides are rapidly degraded in the environment, both workers and bystanders near cropland may be exposed during application.

O,S-DMPT has been detected in rodent urine following methamidophos exposure and also in small amounts in rodent urine exposed to very high doses of acephate (400 mg/kg) (*9*, *10*). Parent methamidophos has not been found in rodent urine (*8*, *10*) or in the urine of workers highly exposed to acephate (*5*). Thus, unlike acephate, methamidophos itself is not a good biomarker for estimating whole body exposure.

* To whom correspondence should be addressed. E-mail: vhebert@tricity.wsu.edu.

10.1021/jf030405d CCC: $25.00
Published on Web 09/06/2003

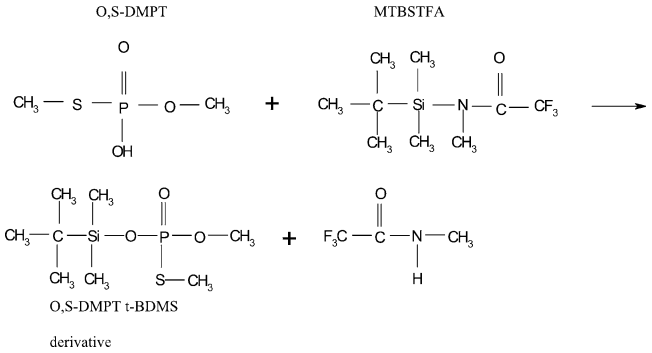


Figure 2. Silylation of O,S-dimethyl hydrogen phosphorothioate with MTBSTFA in acetonitrile.

The main reason for the large difference in toxicity between acephate and methamidophos lies in the carboxyamidase enzyme inhibiting properties of methamidophos, which in consequence limits the metabolic transformation of acephate into methamidophos (*10*). Therefore, much greater amounts of O,S-DMPT are expected to be excreted following exposure to methamidophos than acephate (*8*, *10*). Nevertheless, methamidophos residues are found on crops when acephate is used (*11*−*13*), and they have been detected in environmental samples following acephate use (*14*−*16*). Humans may be inadvertently exposed to methamidophos following acephate use, suggesting that biomonitoring of O,S-DMPT may also be worthwhile following the use of acephate.

This study was designed to specifically evaluate human exposure to methamidophos during and after application of the formulation monitor 4E to potato fields that were adjacent to a small residential community. Various methodologies currently exist for isolating many OP alkyl phosphate metabolites from urine. These include liquid/liquid solvent extraction (*17*−*19*), the use of anion exchange resins (*20*−*22*), azeotropic distillation (*23*−*25*), solid phase extraction (*26*), and freeze drying (*27*). The extracted alkyl phosphate moieties may be derivatized with pentafluorobenzyl bromide (*19*, *21*, *23*−*25*), diazomethane (*17*, *26*), diazoethane (*22*), diazopentane (*18*, *20*), or 1-(4-nitrobenzyl-3-(4-tolyl)triazene (*27*). The derivatized alkyl phosphate is quantified by gas chromatography (GC) with either flame photometric or nitrogen−phosphorus detection. Although the above methods are satisfactory for extracting and quantifying the majority of OP alkyl phosphate metabolites, we observed poor recovery efficiencies from fortified control urine during our investigations of O,S-DMPT. The chemical structure and polarity of O,S-DMPT differ significantly from the other broadly investigated alkyl phosphates (**Figure 2**). We hypothesized that the high water solubility, chemical and/or thermal instability, and possibly high vapor pressure adversely affected the isolation of O,S-DMPT by the traditional wet and solid phase extraction methods that had been applied to other alkyl phosphates including O,O-dimethyl phosphorothioate (referred to in the literature as DMPT (*23*, *27*) or DMTP (*18*−*21*, *24*, *25*)). However, by subjecting urine to solid phase extraction cleanup

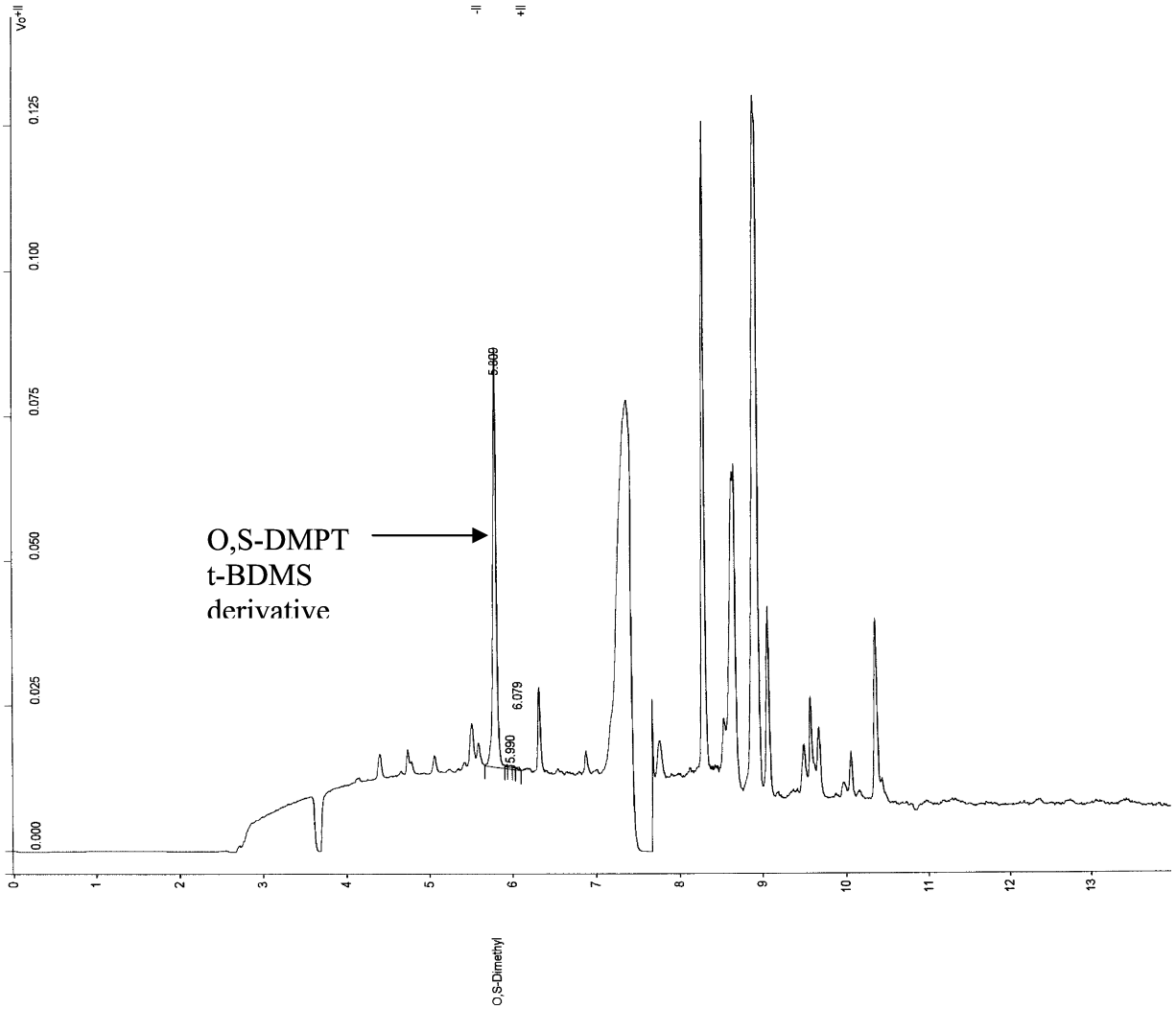


Figure 3. Fortified urine sample (0.2 ppm).

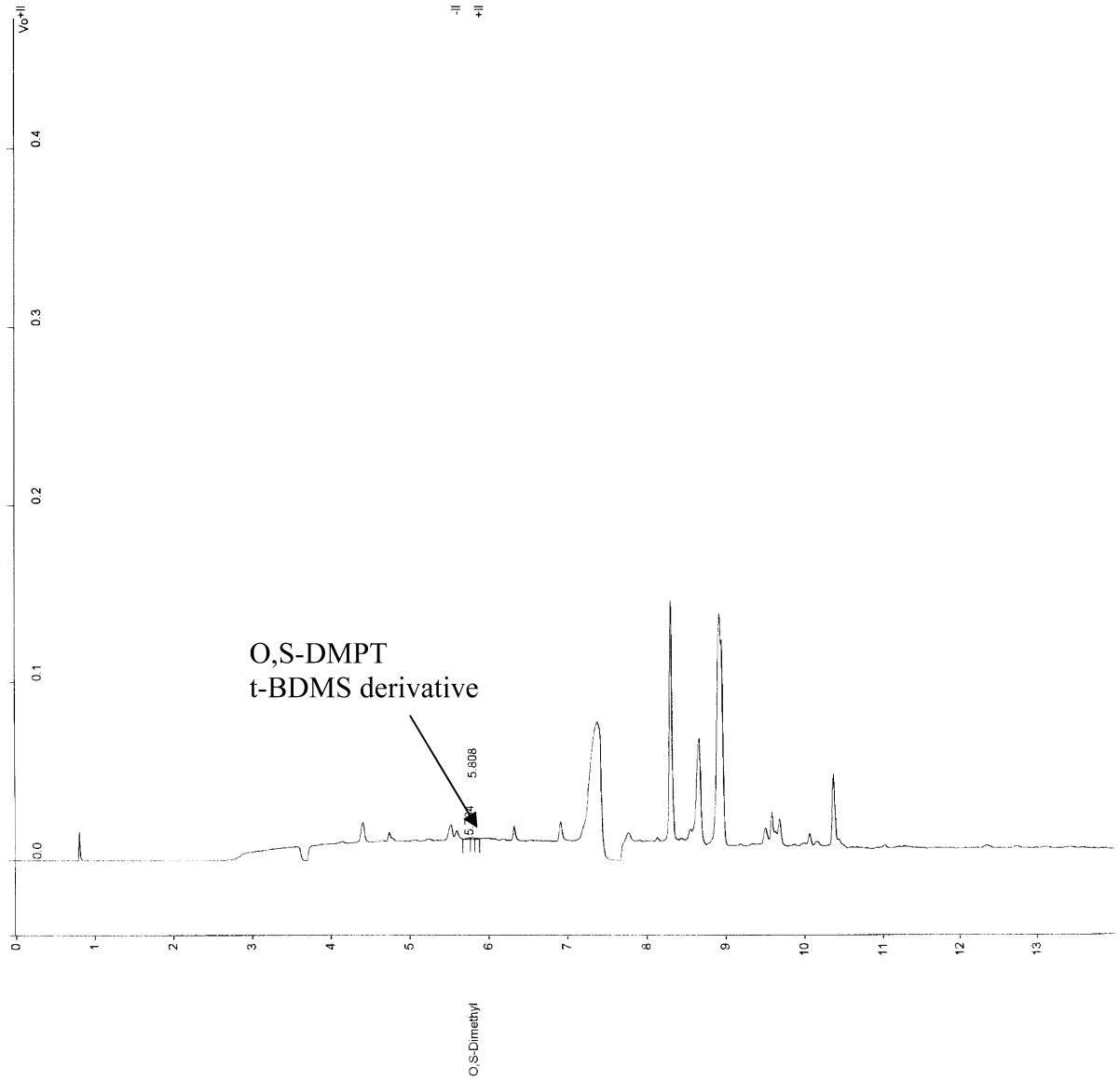


Figure 4. Control urine sample.

followed by lyophilization and then *tert*-butylsilyl derivatization, we were able to develop a rugged, reproducible, and sensitive residue method for the determination of O,S-DMPT using GC with pulsed flame photometric detection (PFPD).

MATERIALS AND METHODS

Chemicals. *Standard.* An O,S-DMPT standard of 92.9% purity was supplied and chemically characterized by Bayer Crop Science (Kansas City, MO). Working standard solutions used for urine fortifications and linearity standards were prepared by diluting 1 mg/mL of stock solution in methanol to 100 and 10 μg/mL in methanol. Standard solutions and their dilutions were stored frozen and equilibrated at room temperature prior to use.

Reagents. The derivatization reagent N-methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide and 1% *tert*-butyldimethylchlorosilane (MTBSTFA + 1% TBDMCS) was acquired from Pierce. High-performance liquid chromatography (HPLC) grade acetonitrile (Fisher Scientific) and octadecyl C18 disposable extraction cartridges (500 mg, Baker Bond SPE) were used throughout the study.

Apparatus. *GC.* A Varian Star 3400CX gas chromatograph with PFPD and 8200CX autosampler controlled by Varian Star Chromatography Workstation software was used for quantification of the derivatized metabolite.

Freeze Drier. A Virtis Unitop 600SL shelf unit lyophilizer (New York) was used to freeze dry the urine samples.

Shaker. An Eberbach (Michigan) shaker was used to agitate samples during derivatization.

Centrifuge. A Fisher Scientific Marathon 13K/M centrifuge was used to filter derivatized samples.

Sample Preparation. *Sample Preparation without a Cleanup Step.* An aliquot of the O,S-DMPT working standard was added to a clean 10 mL glass vial. A 5 mL aliquot of control urine was added to the vial, and the content was vortexed. The urine sample was adjusted to pH 7−8 with a 0.4 N solution of sodium hydroxide and then capped tightly and frozen at −80 °C for storage prior to further handling.

Sample Preparation with a Cleanup Step. An aliquot of O,S-DMPT working standard solution was added to a clean 10 mL vial followed by addition of a 5 mL aliquot of control urine. The content was vortexed and poured through a C18 SPE column (500 mg of sorbent, 6 mL volume) previously conditioned with one column volume of methanol and one column volume of deionized water. The column was washed with 2−3 mL of deionized water. The eluted sample and the column wash were combined in a glass vial. The urine samples were then adjusted to pH 7−8 with 0.4 N sodium hydroxide solution, capped tightly, and then frozen at −80 °C.

Sample Preparation for Precleaned and Unclean Samples. The frozen sample was placed into a −40 °C, airtight freeze drier chamber.

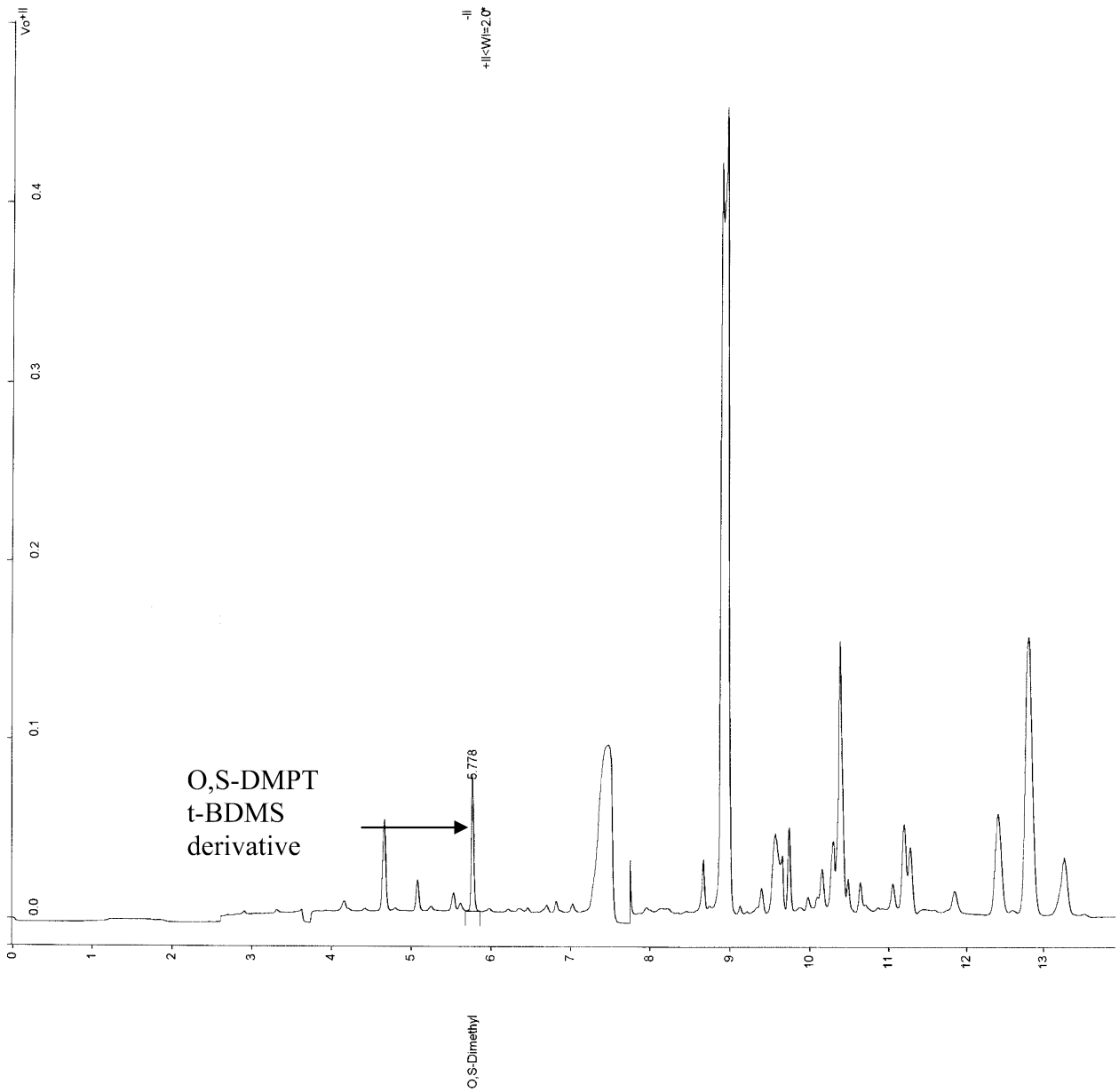


Figure 5. O,S-DMPT residue in a urine sample, 0.141 ppm.

Vacuum was applied, and the temperature was elevated to −7 °C. On average, 48 h was required to freeze dry a set of 50 5 mL urine samples. The freeze-dried samples were covered with 0.5 mL of acetonitrile followed by an addition of 0.5 mL of derivatization reagent. The samples were then vortexed and placed on a shaker for 2 h. After the derivatization was completed, samples were centrifuge-filtered at 12 000 rpm for 5 min in Costar spin-X centrifuge tubes with 0.2 μm nylon membranes (VWR). The supernatants were then transferred to GC autosampler vials for residue determination.

Standard Preparation. Derivatized standards were prepared by measuring the appropriate volumes of standard solutions into the vials and adding 0.5 mL of acetonitrile and 0.5 mL of derivatization reagent to each vial. The standard concentrations ranged from 0.1 to 5 μg/mL. For each analytical set, derivatized standards together with urine samples were placed into the GC vials and analyzed by GC.

GC Analysis. *Capillary Column.* An Alltech EC-1 megabore column of fused silica (100% methyl silicone, 0.53 mm i.d. × 30 m length, 1.20 μm film thickness) was used for quantifying O,S-DMPT.

GC Operating Conditions. Samples and standards were injected into the splitless programmable injector port. The initial temperature of the injector was programmed from 200 to 250 °C at a rate of 250 °C/min. The initial column temperature was held at 80 °C for 2 min and then programmed to 250 °C at a rate of 20 °C/min, and held for 3 min, programmed to 300 °C at a rate of 20 °C/min, and held at 300 °C for 3 min. The helium carrier gas flow rate was 15 mL/min. The injection volume for each sample and standard was 2 μL. The PFPD flame was supported by hydrogen at a flow rate of 20 mL/min and air at a rate of 44 mL/min. The detector temperature was 310 °C.

Quantification. The chromatographic data were integrated using Varian Star Chromatography Workstation version 5.3. O,S-DMPT residues were quantified on the basis of peak area using a multipoint external calibration with single concentration average during an analysis run. The calibration curve was constructed for every sample run using four concentrations of external standards that ranged from the method limit of quantitation (LOQ; 0.1 μg/mL, which is equivalent to 0.02 ppm in urine) to the anticipated highest residue concentration (5 μg/mL or 1 ppm in urine). Each set of derivatized standards was prepared on the day of GC analysis parallel to sample preparation.

Recovery Samples. Five milliliter aliquots of control urine were fortified with 0.1 (equivalent to an O,S-DMPT concentration in urine of 0.02 ppm w/v), 1 (0.2 ppm), and 5 μg (1 ppm) of O,S-DMPT in methanol. For each set of recovery samples, at least one control urine sample was prepared. A reagent blank sample was also analyzed routinely during the course of the study. The method was validated at

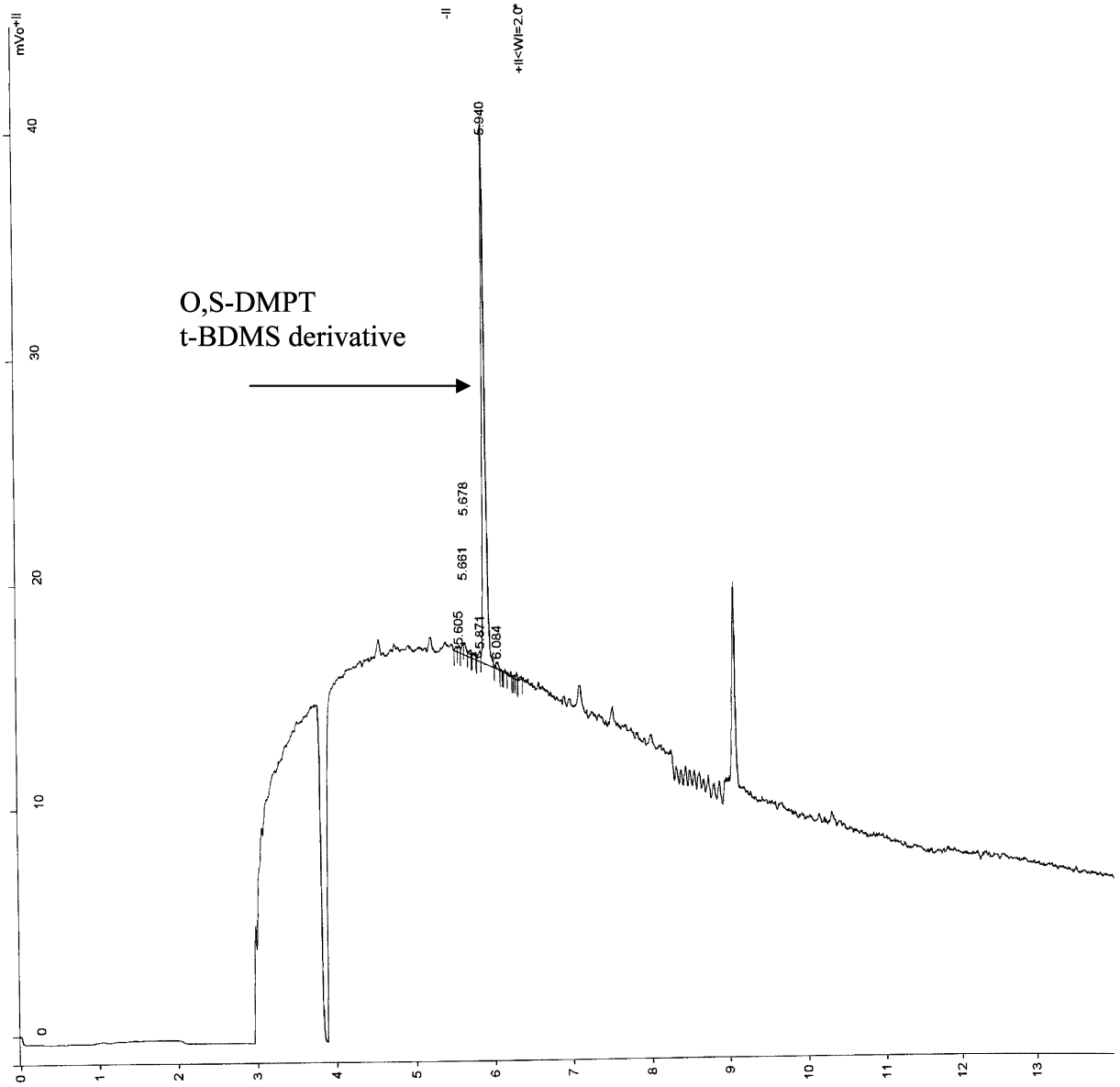


Figure 6. O,S-DMPT standard, concentration 0.1 μg/mL (equivalent to 0.02 ppm).

the above three residue concentrations in triplicate before proceeding with residue determination in actual urine samples.

RESULTS AND DISCUSSION

Several different approaches to extraction of O,S-DMPT from urine were investigated during this study. Liquid/liquid partitioning of acidified urine into a polar organic solvent (acetonitrile) with sodium chloride to force O,S-DMPT into the organic phase extracted only 10−20% from fortified samples. Several solid phase extraction mechanisms were also investigated that included reversed phase (C2, cyclohexyl, phenyl, and cyanopropyl), ion exchange, surface interaction (carbograph, XAD, styrene divinylbenzene copolymer: Bond Elut PPL and Oasis HLB), and mixed mode mechanisms (Nexus Abselut, Focus). None of the solid phase extraction methods isolated more than 20% of the O,S-DMPT metabolite. Many of the established methods worked well in recovering O,O-dialkyl phosphate metabolites but failed for O,S-DMPT. The relatively poor recovery of O,S-DMPT as compared with the other dialkyl phosphate metabolites may have been due to its higher polarity and its much higher affinity for the aqueous phase. Additionally, azeotropic vacuum distillation from acetonitrile, a highly successful method for recovery of O,O-dialkyl phosphate metabolites (*23*−*25*), recovered only 20% of fortified O,S-DMPT. Poor recovery of O,S-DMPT with vacuum distillation suggests that it is more prone to decomposition than the O,O-dialkyl phosphates. Preliminary attempts to freeze dry the fortified urine at room temperature markedly increased O,S-DMPT recovery to a range of 60−70% with high reproducibility. O,S-DMPT volatility losses were further reduced by maintaining a lower temperature (−7 °C) during lyophilization.

We found that the method of derivatization was also important in quantifying O,S-DMPT residues in urine. Pentafluorobenzyl bromide did not derivatize O,S-DMPT effectively, even at elevated temperatures and under prolonged derivatization times. Although O,S-DMPT was quantitatively derivatized with diazomethane in solution, this reagent did not react with O,S-DMPT in freeze-dried extracts. We found the derivatization of

Table 1. Recovery of O,S-Dimethyl Hydrogen Phosphorothioate from Fortified Urine Samples without C18 Cleanup Step

fortification level (ppm)	no. of replicates (*n*)	mean recovery (%)	recovery range (%)	SD (%)
0.02	8	109	91–122	10
0.2	6	107	100–112	5
1	7	123	117–128	4

Table 2. Recovery of O,S-Dimethyl Hydrogen Phosphorothioate from Fortified Urine Samples with C18 Cleanup Step

fortification level (ppm)	no. of replicates (*n*)	mean recovery (%)	recovery range (%)	SD (%)
0.02	4	111	93–134	17
0.2	6	110	103–127	11
1	7	104	95–109	6

O,S-DMPT using MTBSTFA + 1%TBDMCS to be reproducible and quantitative in both solution and in freeze-dried extracts.

We believe this is the first reported use of silylation as a method for derivatization of urinary alkyl phosphates. The derivatization process, which proceeds according to **Figure 2**, yields a strong and stable butyldimethylsilyl derivative product that is well-suited for quantitation by GC. Representative GC/PFPD chromatograms of urine extracts are shown in **Figures 3**−**5**. The O,S-DMPT butyldimethylsilyl derivative was found to be well-resolved with a retention time of 5.8 min (±0.1 min) when using the 100% methyl silicone capillary column. The control urine samples did not result in interferences within the chromatographic retention time window that would affect residue quantitation.

The linearity of the silylated standards was maintained over a range of five concentrations from 0.1 (**Figure 6**) to 5 μg/mL (R^2 ranging from 0.987 to 0.999). The limit of detection (LOD) for the method was established at 0.004 ppm with a reporting LOQ of 0.02 ppm. The sensitivity of the method for O,S-DMPT is comparable with LODs and LOQs achieved for other urinary dialkyl phosphates of OPs. Recoveries of fortified samples using control urine samples were satisfactory and consistent at three concentration levels from our reporting LOQ through the anticipated highest residues in urine (**Tables 1** and **2**).

Precleaning urine extracts by C18 SPE column chromatography was not originally employed in method development. Recoveries of the metabolite from fortified urine samples without this cleanup step varied from 91 to 128% with an average of 113% and a standard deviation (SD) of 10% (**Table 1**). Although the lack of preliminary cleanup step resulted in adequate quantitation, we found it more desirable to introduce the use of C18 SPE to provide more reliable chromatography. The recovery values for C18-cleaned samples ranged from 93 to 134% with an average of 108% and a SD of 12%. Use of C18 SPE retained nonpolar to moderately polar organic compounds allowing polar compounds such as O,S-DMPT to pass through unretained. The use of the C18 cleanup step improved chromatography of analyzed extracts particularly for the urine samples with high dry matter content. The samples were also easier to work with during derivatization. Furthermore, the presence of dry matter in urine requires additional volumes of derivatization reagent and acetonitrile, which lowers the sensitivity of the analysis. Using C18 SPE column cleanup prior to lyophilization remedies the need for additional reagents and solvents.

The assay described herein for determination of O,S-DMPT in urine using lyophilization as a method for removing water and MTBSTFA + TBDMCS as the derivatization reagent is sensitive, specific, and reproducible. Together with the determination of parent residues, this method of analysis of O,S-DMPT provides detection sensitivity appropriate for monitoring human exposure to the pesticides methamidophos and acephate. The method is also fast, which can be crucial to a routine monitoring program. One trained person can process 30−40 lyophilized urine samples in 1 day and complete the GC analysis in an automated overnight run.

ACKNOWLEDGMENT

We thank Kai Elgethun and Sarah Weppner, School of Public Health and Community Medicine, University of Washington, for collecting and delivering urine samples and Professor Allan Felsot, Washington State University, for useful information and valuable discussion throughout the study.

LITERATURE CITED

(1) Aprea, C.; Colosio, C.; Mammone, T.; Minoia, M.; Maroni, M. Biological monitoring of pesticide exposeure: a review of analytical methods. *J. Chromatogr. B* **2002**, *769*, 191−219.

(2) Loewenherz, C.; Fenske, R. A.; Simcox, N. J.; Bellamy, G.; Kalman, D. Biological monitoring of organophosphorus pesticide exposure among children of agricultural workers in Central Washington State. *Environ. Health Perspect.* **1997**, *105*, 1344−1353.

(3) Cocker, J.; Mason, H. J.; Garfitt, S. J.; Jones, K. Biological monitoring of exposure to organophosphate pesticides. *Toxicol. Lett.* **2002**, *134*, 97−103.

(4) Koch, D.; Lu, C.; Fisker-Anderson, J.; Joley, L.; Fenske, R. A. Temporal association of children's pesticide exposure and agricultural spraying: report of a longitudinal biological monitoring study. *Environ. Health Perspect.* **2002**, *110*, 829−833.

(5) Maroni, M.; Catenacci, G.; Galli, D.; Cavallo, D.; Ravazzani, G. Biological monitoring of human exposure to acephate. *Arch. Environ. Contam. Toxicol.* **1990**, *19*, 782−788.

(6) Eto, M. Organophosphorus pesticides. In *Encyclopedia of Agrochemicals*; Plimmer, J. R., Ed.; John Wiley & Sons: New York, 2003; pp 1150−1177.

(7) U.S. Environmental Protection Agency (EPA). Methamidophos. *Revised Product and Chemistry Chapters for the Registration Eligibility Decision*; Registration Case 0043; Environmental Protection Agency: Washington, DC, 2000; pp 16−17.

(8) Fort, F. Human health risks assessment. *Methamidophos*; U.S. Environmental Protection Agency, Office of Pesticide Programs: Washington, DC, 2000(a).

(9) Fort, F. Human health risk assessment. *Acephate*; U.S. Environmental Protection Agency, Office of Pesticide Programs: Washington, DC, 2000(b).

(10) Mahajna, M.; Quistad, G. B.; Casida, J. E. Acephate insecticide toxicity: safety conferred by inhibition of the bioactivating carboxyamidase by the metabolite methamidophos. *Chem. Res. Toxicol.* **1997**, *10*, 64−69.

(11) Nigg, H. N.; Reinert, J. A.; Fitzpatrick, G. E. Acephate and methamidophos residue behavior in Florida citrus. *Pestic. Monit. J.* **1979**, *12*, 167−171.

(12) Antonious, G. F. Analysis and fate of acephate and its metabolite, methamidophos, in pepper and cucumber. *J. Environ. Sci. Health B* **1995**, *30*, 377−399.

(13) USDA Pesticide Data Program. *Annual Summary Calendar, Year 2001*; USDA: Washington, DC, 2003.

(14) Szeto, S. Y.; et al. The fate of acephate and carbaryl in water. *J. Environ. Sci. Health B* **1979**, *14*, 635−654.

(15) Geen, G. H. Fate and toxicity of acephate (Orthene) added to coastal B. C. stream. *J. Environ. Sci. Health B* **1981**, *16*, 253−271.

(16) Sundaram, K. M. S. Partitioning and fate of acephate and its metabolite, methamidophos, from white spruce cones into soil and water. *J. Environ. Sci. Health B* **1993**, *28*, 29−66.
(17) Drevenkar, V.; Radić, Z.; Vasilić, Ž.; Reiner, E. Dialkylphosphorus metabolites in the urine and activities of esterases in the serum as biological indices for human absorption of organophosphorus pesticides. *Arch. Environ. Contam. Toxicol*. **1991**, *20*, 417−422.
(18) Shafik, T.; Bradway, D. E.; Enos, H. F.; Yobs, A. R. Human exposure to organophosphorus pesticides. A modified procedure for the gas−liquid chromatographic analysis of alkyl phosphate metabolites in urine. *J. Agric. Food Chem*. **1973**, *21*, 625−629.
(19) Hardt, J.; Angerer, J. Determination of dialkyl phosphates in human urine using gas chromatography−mass spectrometry. *J. Anal. Toxicol*. **2000**, *24*, 678−684.
(20) Lores, E. M.; Bradway, D. E. Extraction and recovery of organophosphorus metabolites from urine using an anion-exchange resin. *J. Agric. Food Chem*. **1977**, *25*, 75−79.
(21) Bradway, D. E.; Moseman, R.; May, R. Analysis of alkyl phosphates by extractive alkylation. *Bull. Environ. Contam. Toxicol*. **1981**, *26*, 520−523.
(22) Blair, D.; Roderick, H. R. An improved method for the determination of urinary dimethyl phosphate. *J. Agric. Food Chem*. **1976**, *24*, 1221−1223.
(23) Nutley, B. P.; Cocker, J. Biological monitoring of workers occupationally exposed to organophosphorus pesticides. *Pestic. Sci*. **1993**, *38*, 315−322.
(24) Moate, T. F.; Lu C.; Fenske, R. A.; Hahne, R. M. A.; Kalman, D. A. Improved cleanup and determination of dialkyl phosphates in the urine of children exposed to organophosphorus insecticides. *J. Anal. Toxicol*. **1999**, *23*, 230−236.
(25) Aprea, C.; Sciarra, G.; Lunghini, L. Analytical method for the determination of urinary alkyl phosphates in subjects occupationally exposed to organophosphorus pesticides and in general population. *J. Anal. Toxicol.* **1996**, *20*, 559−563.
(26) Muan, B.; Skare J. U. A method for the determination of the main metabolites of malathion in biological samples. *J. Agric. Food Chem*. **1989**, *37*, 1081−1085.
(27) Takada, D. Y.; Reynolds, J. M.; Nelson, J. H. 1-(4-Nitrobenzyl)-3-(4-tolyl)triazene as a derivatizing reagent for the analysis of urinary dialkyl phosphate metabolites of organophosphorus pesticides by gas chromatography. *J. Agric. Food Chem*. **1979**, *27*, 746−753.

Received for review May 30, 2003. Revised manuscript received July 15, 2003. Accepted July 17, 2003.

JF030405D