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Simultaneous analytical method for urinary metabolites of organophosphorus compounds and moth repellents in general population

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ABSTRACT

An analytical method was developed for simultaneous measurement of urinary metabolites in the general population exposed to organophosphorus compounds (insecticides, flame retardants and plasticizers) and moth repellents used in Japanese households. Fifteen metabolites, dimethylphosphate, dimethylthiophosphate, diethylphosphate, diethylthiophosphate, di-*n*-butylphosphate, diphenylphosphate, bis(2-ethylhexyl)phosphate, 2-isopropyl-6-methyl-4-pyrimidinol, 3,5,6-trichloro-2-pyridinol, 3-methyl-4-(methylthio)phenol, 3-methyl-4-nitrophenol, 2,4-dichlorophenol, 2,5-dichlorophenol, 1-naphthol and 2-naphthol, were extracted from hydrolyzed urine by using a sorbent (hydroxylated polystyrene-divinylbenzene copolymers), and then desorbed with methylacetate and acetonitrile, concentrated, and after transformation to their *tert*-butyldimethylsilyl derivatives, analyzed by gas chromatography/mass spectrometry in the electron impact ionization mode. They could be determined accurately and precisely (quantification limits: 0.8–4 μg/l). The collected urine samples could be stored for up to 1 month at −20 °C in a freezer.

1. Introduction

Many of us are exposed daily to a wide variety of environmental chemicals. Indoor air quality has a strong effect on our health because we spend much time indoors such as at home. Analysis of indoor air has revealed the presence of many organophosphorus compounds and chemicals diffusing from moth repellents, in addition to general indoor contaminants such as formaldehyde [1–11]. Organophosphorus insecticides are widely used indoors to control household pests. Many trialkylphosphates are used as flame retardants and plasticizers for various household products, and *p*-dichlorobenzene and naphthalene as moth repellents and toilet bowl deodorants [12,13]. Most organophosphorus compounds are known to cause cholinergic symptoms (headache, blurred vision, dizziness, fatigue, nausea, and vomiting, etc.) by inhibiting cholinesterase activities in nerve synaptic clefts of humans exposed to them [12]. In addition, the International Agency for Research on Cancer (IARC) has classified dichlorvos, one of the organophosphorus insecticides [14], *p*-dichlorobenzene [15] and naphthalene [16] as Group 2B (possibly carcinogenic to humans). Therefore, to evaluate any adverse effects on humans in indoor environments, it is important to know how much of these compounds are absorbed.

Generally, the amounts of chemicals absorbed are evaluated using their urinary metabolites. Most organophosphorus insecticides are mainly degraded to dialkylphosphates and phenolic metabolites in mammals [17]. To monitor human exposure to the insecticides, urinary dialkylphosphates have generally been measured in previous studies [18–28]. However, the parent compounds cannot be identified from urinary dialkylphosphates because they are common metabolites resulting from most organophosphorus insecticides. There are a few studies that have examined the urinary excretion of phenolic metabolites, which can be a help to identify the parent compounds, together with dialkylphosphates in the general population [26,29]. On the other hand, the main human urinary metabolites of many trialkylphosphates for flame retardants and plasticizers are presumed to be the corresponding dialkylphosphates based on previous reports concerning their metabolism in animals [30–32]. However, little work has been conducted on monitoring the general population for exposure to trialkylphosphates using their urinary metabolites, except for studies by Schindler et al. [32,33]. *p*-Dichlorobenzene [34] and naphthalene [35] are principally excreted in human urine as their phenolic metabolites.

The aim of this study was to develop an analytical method for measuring urinary metabolites to evaluate exposure to organophosphorus compounds (insecticides and trialkylphosphates) and moth repellents in the Japanese general population. The chemicals listed in Table 1 were expected to be absorbed through indoor air mainly by inhalation on consideration of the use of such chemicals [13,36,37] and our survey of marketed

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Table 1
Original chemicals and their urinary metabolites determined in the present study.

Original chemical	Objective urinary metabolite		
Organophosphorus insecticides			
Chlorpyrifos	Diethylphosphate (S, 99.5)	Diethylthiophosphate (H, 95.2)	3,5,6-Trichloro-2-pyridinol (S, 99.3)
Diazinon	Diethylphosphate	Diethylthiophosphate	2-Isopropyl-6-methyl-4-pyrimidinol (S, 99.0)
Dichlofenthion	Diethylphosphate	Diethylthiophosphate	2,4-Dichlorophenol (K, 99.9)
Dichlorvos	Dimethylphosphate (A, 98.8)		
Fenitrothion	Dimethylphosphate	Dimethylthiophosphate (H, 98.9)	3-Methyl-4-nitrophenol (T, 98.3)
Fenthion	Dimethylphosphate	Dimethylthiophosphate	3-Methyl-4-(methylthio)phenol (T, 98.8)
Isoxathion	Diethylphosphate	Diethylthiophosphate	
Malathion	Dimethylphosphate	Dimethylthiophosphate	
Pyridafenthion	Diethylphosphate	Diethylthiophosphate	
Tetrachlorvinphos	Dimethylphosphate		
Organophosphorus flame retardants and plasticizers			
Tributylphosphate	Di-*n*-butylphosphate (T, 98.7)		
Triethylphosphate	Diethylphosphate		
Trimethylphosphate	Dimethylphosphate		
Triphenylphosphate	Diphenylphosphate (T 99.8)		
Tris(2-ethylhexyl)phosphate	Bis(2-ethylhexyl)phosphate (W, 96.0)		
Moth repellents and toilet bowl deodorants			
p-Dichlorobenzene	2,5-Dichlorophenol (W, 99.2)		
Naphthalene	1-Naphthol (W, 100.0)	2-Naphthol (W, 99.8)	

Characters in parenthesis are purchase sources and purities (%) of the standard materials. Abbreviations of the manufacturers are as follows: A, Acros Organics (Geel, Belgium); H, Hayashi Pure Chemical (Osaka, Japan); K, Kanto Chemical (Tokyo, Japan); S, Sigma–Aldrich (MO, USA); T, Tokyo Kasei Kogyo (Tokyo, Japan); W, Wako Pure Chemicals (Osaka, Japan).

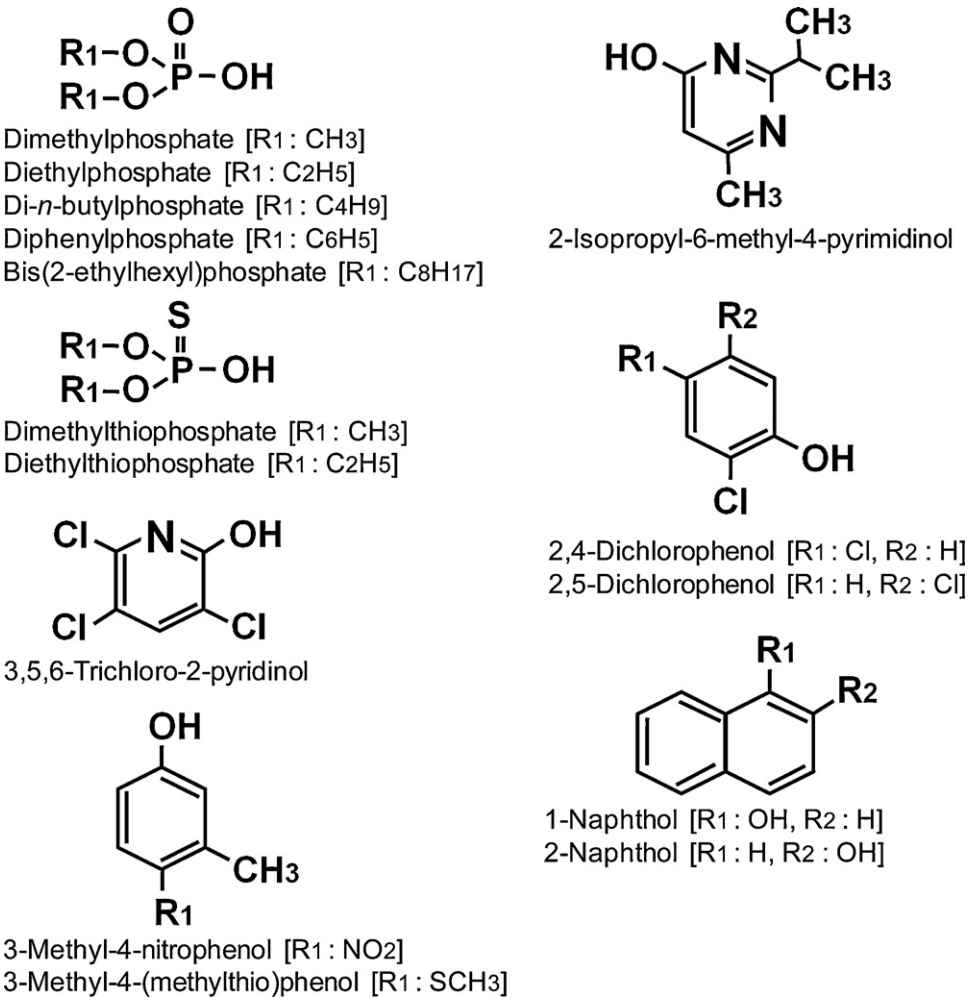


Fig. 1. Chemical structures of urinary metabolites targeted in the present study.

products. The target compounds for determination were their 15 urinary metabolites, for which standard materials could be obtained in Japan (Table 1 and Fig. 1).

Recently, many analytical methods have been reported for urinary dialkylphosphates of low concentrations for monitoring exposure to organophosphorus insecticides by using gas chromatography/mass spectrometry, though in the past, gas chromatography with flame photometric detection (GC-FPD) [38–50] or gas chromatography with nitrogen–phosphorus selective detection (GC-NPD) [44,51] were applied for their analysis. As GC-FPD and GC-NPD are for specially detecting only compounds containing phosphorus among the metabolites, other compounds such as the phenolic metabolites resulting from parent insecticides, cannot be detected. In previous analytical methods for the urinary metabolites of organophosphorus insecticides by gas chromatography/mass spectrometry, the dialkylphosphates were determined after derivatization (trimethylsilylation, benzylation, pentafluorobenzylation or chloropropylation) by gas chromatography combined with single quadrupole mass spectrometry (GC/MS) in the electron impact ionization mode (EI) (GC/MS(EI)) [19,25,52–56] or in the positive ion chemical ionization mode (CI) (GC/MS(CI)) [57], or by gas chromatography combined with tandem mass spectrometry (GC/MS/MS) in CI (GC/MS/MS(CI)) [21,23,57,58] or in the negative ion chemical ionization mode (NCI) (GC/MS/MS(NCI)) [20]. The instruments for GC/MS/MS, while offering highly sensitive analysis in comparison with GC/MS, are expensive and not readily available in many laboratories. GC/MS(EI) is more cost-effective and more common analytical methods than GC/MS(CI). In most previous studies on analytical methods for the dialkylphosphates resulting from organophosphorus insecticides by GC/MS(EI), the metabolites were determined after being transformed into their pentafluorobenzyl derivatives. However, in our preliminary examination by GC/MS(EI), most of the *tert*-butyldimethylsilyl derivatives of our target metabolites containing dialkylphosphates were found to have higher sensitivities than their pentafluorobenzyl derivatives. On the other hand, there was no report on the GC/MS methods for simultaneous determination of urinary phenolic metabolites and dialkylphosphates in the general population exposed to organophosphorus insecticides. Furthermore, there were hardly any reports on analytical methods using GC/MS to determine urinary dialkylphosphates metabolized from trialkylphosphates for flame retardants and plasticizers such as tris(2-ethylhexyl)phosphate.

In the present study, a method was developed for simultaneous analysis of 15 urinary metabolites, including several phenolic metabolites conducive for identifying the parent insecticides (Table 1), in the general population. Their *tert*-butyldimethylsilyl derivatives were observed by GC/MS(EI). The proposed method was applied to the analysis of several urine samples collected from health volunteers without occupational exposure.

2. Experimental

2.1. Chemicals

The purchase sources and purities of the standard materials for determination of the 15 targeted urinary metabolites are shown in Table 1. The standard materials were used without further purification. N-(*tert*-Butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) for transforming the metabolites into their *tert*-butyldimethylsilyl derivatives and isopropylphosphate (diisopropylphosphate: 68.3%) as an internal standard were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and sodium disulfite, which was of analytical-reagent grade, from Kanto Chemical (Tokyo, Japan). Other chemicals were obtained from Wako Pure Chemical (Osaka, Japan). Toluene and methylacetate were of a grade for analysis of dioxins and for environmental analysis, respectively. Acetonitrile was of a grade for analysis of pesticide residue and polychlorinated biphenyl. All other chemicals were of analytical-reagent grade. Isolute ENV+ (bulk; hydroxylated polystyrene-divinylbenzene copolymers), a sorbent for extracting metabolites from urine, was obtained from Biotage (Uppsala, Sweden).

2.2. Instruments

A Shimadzu (Kyoto, Japan) GCMS-QP2010 gas chromatograph mass spectrometer, combined with a Shimadzu AOC-20i auto injector, was controlled with a Fujitsu (Kanagawa, Japan) FM V-6766CL7c computer installed with GCMSsolution software for instrument control and data analysis. A Hitachi (Tokyo, Japan) Model Himac CF7D centrifuge was used. A wave rotor for agitation of samples and a heating block were purchased from Thermonics (Model WR-100, Tokyo, Japan) and Yamato Scientific (Model HF-21, Tokyo, Japan), respectively. A GL Sciences (Tokyo, Japan) Model Soldry SD905 was used to concentrate the sample and standard solutions.

All of the glassware for preparation of the samples and standard solutions used during the experiments were washed with deionized water and acetone and then dried at room temperature.

2.3. Mixed metabolite stock solution and internal standard solution

Each stock standard solution of 13 metabolites, except for dimethylthiophosphate and 2-isoproply-6-methyl-4-pyrimidinol, was prepared with 10.0 mg/ml of each compound by dissolving it in acetonitrile in 10 ml brown high-airtight vials (Kanto Kagaku, Tokyo, Japan). The solutions of dimethylthiophosphate and 2-isoproply-6-methyl-4-pyrimidinol were prepared by dissolving each compound in methanol and acetone, respectively, as for the above 13 metabolites. Aliquots of these stock standard solutions were combined, and the mixed metabolite stock solution of 15 compounds was prepared at 500 μg/ml by dilution with acetonitrile in another 10 ml brown high-airtight vial.

A solution of the internal standard, diisopropylphosphate, was prepared at 500 μg/ml by dissolving the compound in acetonitrile in a 2 ml brown high-airtight vial.

All of these solutions were stored at 4 °C in a refrigerator.

2.4. Sample preparation

Portions (5.0 ml) of spot urine samples obtained from subjects were decanted into 10 ml brown screw-capped tubes, and stored at −20 °C in a freezer until analysis.

The thawed urine sample was hydrolyzed with 0.5 ml of concentrated hydrochloric acid for 30 min at 100 °C after adding 3.0 μl of the internal standard solution. After cooling the hydrolysate to room temperature, 0.1 ml of 10 M sodium hydroxide solution and 4.0 g of ammonium sulfate were added, and the sample was agitated for 5 min using the wave rotor to sufficiently dissolve ammonium sulfate. Next, 100 mg of the sorbent Isolute ENV+ was added to the solution, and the sample was agitated for 10 min in order to allow the objective metabolites to become adsorbed onto the sorbent. After centrifugation (3000 rpm for 10 min), the aqueous phase of the sample was removed, and 4.0 ml of methylacetate was added to the residue. The sample was shaken with a stirring apparatus for 10 min to desorb the metabolites from the sorbent. The supernatant was decanted into another 10 ml brown screw-capped tube after centrifugation (3000 rpm for 10 min). Desorption of the metabolites from the sorbent was repeated by adding 2.0 ml

Table 2
Selected target ions and reference ions (*m/z*) for GC/MS analysis of metabolites.

Compound	Target	Reference
Dimethylphosphate	183	153
Dimethylthiophosphate	199	169
Diethylphosphate	211	155
Diethylthiophosphate	227	199
Di-*n*-butylphosphate	155	267
Diphenylphosphate	307	308
Bis(2-ethylhexyl)phosphate	155	213
3,5,6-Trichloro-2-pyridinol	254	258
3-Methyl-4-nitrophenol	267	210
3-Methyl-4-(methylthio)phenol	268	196
2-Isopropyl-6-methyl-4-pyrimidinol	209	210
2,4-Dichlorophenol	219	221
2,5-Dichlorophenol	221	219
1-Naphthol	258	201
2-Naphthol	201	258
Diisopropylphosphate (internal standard)	155	239

acetonitrile to the residual sorbent, and two extracts were combined. The mixed extract was concentrated to 0.5 ml by using the Soldry SD905 under a stream of nitrogen gas (flow rate, 150 ml/min per tube; temperature, 56 °C; purity of the gas, >99.999%). Two milliliters of acetonitrile was added to the concentrated solution, and similarly concentrated to 1.0 ml. This procedure was repeated once again to remove methylacetate and water. To the acetonitrile solution obtained (1.0 ml), 50 mg of sodium disulfite and 30 μl of MTBSTFA were added, and the solution was heated for 30 min at 90 °C to form *tert*-butyldimethylsilyl derivatives of the metabolites. After cooling the reaction solution to room temperature, it was centrifuged for 10 min at 3000 rpm. The supernatant was decanted into another graduated brown tube, and was concentrated and dried using Soldry SD905 (N_2 flow rate of 150 ml/min per tube at 56 °C). Toluene, 0.15 ml, was added to the residue, and the supernatant of the solution after centrifugation (3000 rpm for 10 min) was transferred to another 1.5 ml vial for the auto injector.

2.5. Pooled urine and standard urine solutions of metabolites

A mixture of urine obtained from several healthy volunteers was confirmed beforehand to contain only slight amounts or no detectable amount of the target metabolites. This was used as a pooled urine sample.

The mixed metabolite stock solution was diluted with acetonitrile on each day of analysis at concentrations of 0, 2.0, 5.0, 10, 20, 50, 100 and 200 μg/ml. A 5.0 μl portion of each solution was added to another 10 ml brown screw-capped tube containing 5.0 ml each of the pooled urine, and the working standard urine solutions of metabolites were treated in the same manner as above for the samples.

2.6. Gas chromatography/mass spectrometry

The analytical conditions for GC/MS were as follows: injection amount, 1.0 μl; injection mode into the gas chromatograph, splitless; sampling time, 2.0 min; capillary column, DB-5ms (30 m × 0.25 mm I.D., 0.25 μm film thickness, J&W Scientific, CA, USA); column oven temperature, 70 °C (2 min) – 10 °C/min – 280 °C; injection port temperature, 280 °C; carrier gas, helium; carrier gas pressure, 89.7 kPa; total flow rate, 20.0 ml/min; column flow rate, 1.38 ml/min; interface temperature, 300 °C; ionization method on the mass spectrometer, EI; ionization energy, 70 eV; ionization current, 60 μA; ion source temperature, 200 °C; analytical mode, selected ion monitoring (SIM). The ions selected for SIM quantification of the compounds are listed in Table 2.

2.7. Calibration

The metabolites in the urine samples were determined by the internal standard method. Their concentrations were calculated by interpolation from the linear least-squares regression line of the multi-level standard curve plot of peak-area ratio (area of metabolite/area of internal standard) *versus* the concentration of metabolites in the working standard urine solutions.

2.8. Recovery

Because authentic standards of *tert*-butyldimethylsilyl esters of the target metabolites were not available, the efficiency of the derivatizing reaction could not be determined, and therefore, the absolute recoveries of the metabolites during the complete sample preparation could not be evaluated. In this study, the recoveries from the hydrolysis of urine samples until immediately before the derivatization of the metabolites were examined as follows. The pooled urine samples (5.0 ml, $n = 5$) spiked with known amounts (200 μg/l as the urinary concentrations) of each compound were treated and determined according to the present method. On the other hand, the pooled urine samples non-spiked with the compounds were treated similarly until immediately before the derivatization with MTBSTFA, and the compounds spiked to 1 ml of the solutions (1000 μg/l as the concentrations in the solutions, $n = 5$) were determined after their derivatization. Both quantitative values for each compound were compared.

2.9. Quantification limits

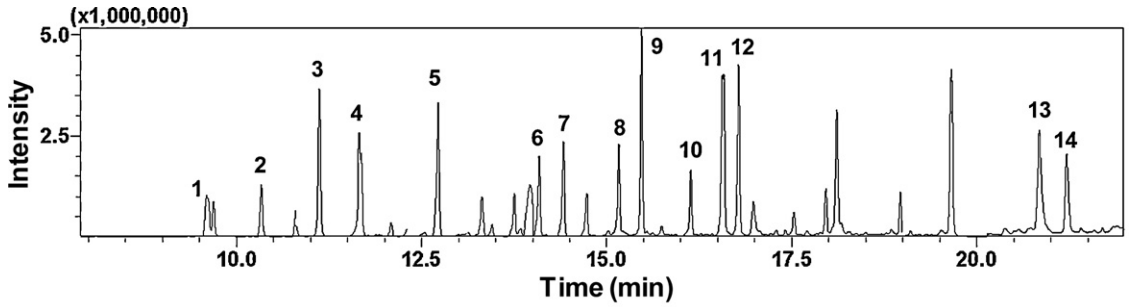
The concentrations of the quantification limits of the metabolites in urine samples were calculated from the quantitative values of the working standard urine solution of the minimum concentration needed to construct the calibration curves. The solution of 2.0 μg/ml of each metabolite concentration was prepared by diluting the mixed metabolite stock solution with acetonitrile. A 5.0 μl portion of the solution was added to 5.0 ml of the pooled urine sample ($n = 5$) and analyzed according to the present method. The standard deviations of the quantitative values were calculated for each metabolite, and the quantification limits were defined as being ten times the standard deviations [59].

2.10. Precision and accuracy

The precision and accuracy of the method were demonstrated by repeated analysis of the urine samples spiked with each authentic standard. Portions of 5.0 μl of the solution, which was diluted to the concentration of 200 μg/ml from the mixed metabolite stock solution, were spiked to 5.0 ml of the pooled urine samples ($n = 5$, 200 μg/l as urinary concentrations), and they were analyzed according to the present method. The precision of this method was evaluated by the relative standard deviations (RSD) in studies with replicate assays ($n = 5$), and the accuracy of the method was evaluated based on the error of the assayed samples relative to their spiked concentrations (RE). Furthermore, the urine samples (5.0 ml) spiked with 5.0 μl aliquots of the other solutions, which were diluted to the concentrations of 20 and 2.0 μg/ml from the mixed metabolite stock solution, were examined similarly (urinary concentrations: 20 and 2.0 μg/l each of metabolites, respectively).

2.11. Storage stabilities of metabolites in urine samples

About 50 μl of the mixed metabolite stock solution was added to 150 ml of the pooled urine (*ca.* 170 μg/l as urinary concentrations).



No.	Compound	t_R (min)	No.	Compound	t_R (min)
1	Dimethylphosphate	9.59	8	3,5,6-Trichloro-2-pyridinol	15.16
2	Dimethylthiophosphate	10.33	9	Di-*n*-butylphosphate	15.46
3	Diethylphosphate	11.12	10	3-Methyl-4-(methylthio)phenol	16.13
4	Diisopropylphosphate	11.66	11	1-Naphthol	16.55
	Diethylthiophosphate	11.68		3-Methyl-4-nitrophenol	16.58
5	2-Isopropyl-6-methyl-4-pyrimidinol	12.72	12	2-Naphthol	16.78
6	2,5-Dichlorophenol	14.09	13	Diphenylphosphate	20.81
7	2,4-Dichlorophenol	14.42	14	Bis(2-ethylhexyl)phosphate	21.17

Fig. 2. Chromatogram of standard urine solution (each compound; 200 μg/l).

The urine solution was transferred in 5 ml portions into 27 brown screw-capped tubes. The metabolites in three tubes were analyzed immediately after preparation. The average concentrations of each compound determined for the tubes were considered to be those of the metabolites at time zero. The remaining tubes were stored at −20 °C in the freezer until analysis. The contents of three samples each were analyzed periodically.

2.12. Application

Spot urine samples from five healthy nonsmoking subjects without occupational exposure (16–48 years old) were collected, and the target metabolites in the samples were determined by the proposed method.

3. Results and discussion

3.1. Chromatography

The chromatogram of the standard urine solution containing the 15 target metabolites (concentrations of each metabolite: 200 μg/l) determined by the present method is shown in Fig. 2. All metabolites were resolved within 22 min. In the chromatogram, the peaks of diisopropylphosphate (internal standard) and diethylthiophosphate, and 1-naphthol and 3-methyl-4-nitrophenol overlapped with each other (peak no. 4 and 11 in Fig. 2), namely, their separation was insufficient. However, the target ions and the reference ions of these compounds shown in Table 2 did not exist or only trace amounts appeared as fragments of mass spectrum peaks that interfered with the determination. Therefore, the present chromatographic method by SIM is satisfactory for the separate determination of all metabolites without their affecting each other.

3.2. Calibration

The slopes and intercepts of the calibration curves obtained by measurement of the standard urine solutions are shown in Table 3. For all compounds, the values of the correlation coefficient of the normal linear regression lines were better than 0.9990 ($n = 8$), that is, these calibration curves were linear over the concentration range 0–200 μg/l in the urine.

3.3. Recovery

As shown in Table 3, the recoveries for many metabolites during the sample preparation, namely, hydrolysis of conjugates of the metabolites, adsorption to the sorbent Isolute ENV+, desorption from the sorbent by methylacetate and acetonitrile, and concentration of the extraction solution, were more than 80%, indicating adequate recoveries in spite of the long preparation processes. On the other hand, the recoveries for dimethylphosphate (50%) and bis(2-ethylhexyl)phosphate (54%) were low compared to others. Therefore, to accurately quantify the urinary metabolites containing these compounds, it was necessary to construct calibration curves using the pooled urine spiked with authentic standard materials of the metabolites, and treated in the same manner as the samples.

The recoveries of urinary dimethylphosphate were low, similar to findings in previous studies using liquid–liquid extraction technique (recoveries; 57–71%) [25,52,56], solid-phase extraction (56–67%) [55,58], or azeotropy with the appropriate solvent (32–45%) [21], though they were higher with analytical methods using the lyophilization technique which required a long time and special instruments (85–95%) [23,26]. The low recovery of dimethylphosphate was presumed to be due to its high polarity.

Table 3
Calibration graphs, quantification limits and recoveries of urinary metabolites in the present analytical method.

Compound	Calibration graph			Quantification limit	Recovery[b]
	Slope	Intercept	r[a]	(μg/l)	(%)
Dimethylphosphate	0.0033	0.0018	1.0000	1.4	50
Dimethylthiophosphate	0.0028	−0.0002	0.9998	0.8	76
Diethylphosphate	0.0021	0.0003	0.9998	1.2	103
Diethylthiophosphate	0.0037	0.0111	0.9991	1.6	92
Di-*n*-butylphosphate	0.0080	−0.0055	0.9992	2.3	100
Diphenylphosphate	0.0049	−0.0024	0.9992	2.6	82
Bis(2-ethylhexyl)phosphate	0.0025	0.0099	0.9990	3.1	54
3,5,6-Trichloro-2-pyridinol	0.0035	0.0099	0.9990	2.4	102
3-Methyl-4-nitrophenol	0.00070	−0.0004	0.9998	3.7	104
3-Methyl-4-(methylthio)phenol	0.0016	0.0008	0.9998	2.9	81
2-Isopropyl-6-methyl-4-pyrimidinol	0.0078	−0.0066	0.9995	1.8	84
2,4-Dichlorophenol	0.0028	0.0009	0.9997	2.7	101
2,5-Dichlorophenol	0.0021	0.0002	0.9996	2.1	99
1-Naphthol	0.0016	0.0023	0.9993	3.8	84
2-Naphthol	0.0044	0.0048	0.9991	3.7	92

[a] Correlation coefficient (n = 8).
[b] Values are means for five samples.

3.4. Quantification limits

The quantification limits of the 15 urinary metabolites were 0.8–3.8 μg/l (Table 3). The concentrations for dimethylphosphate, dimethylthiophosphate, diethylphosphate and diethylthiophosphate were 0.8–1.6 μg/l in urine. Detection limits rather than quantification limits were discussed in many previous studies on their analytical methods in urine samples. In the present study, the detection limits of the metabolites in the urine samples were defined as being three times [59] the standard deviations that were obtained in the examinations for the quantification limits (Section 2.9). The concentrations of the detection limits of the 4 metabolites were calculated to be 0.2–0.5 μg/l in urine. These values were lower or the same as those reported by many researchers who analyzed their pentafluorobenzyl-derivatives by GC/MS(EI) [19,24,25,55,60] although Ueyama et al. [56] recently reported lower detection limits (0.05–0.15 μg/l) of the metabolites on revision of their previously reported method [25]. In addition, the detection limits obtained were markedly lower than those for their chloropropyl-derivatives determined by GC/MS(CI) (13–644 μg/l) [57] or for their benzyl-derivatives determined by GC/MS(EI) (3–6 μg/l) [53]. The values of the detection limits for diphenylphosphate (0.8 μg/l) and di-*n*-butylphosphate (0.7 μg/l) approximately agreed with those obtained using expensive equipment, GC/MS/MS [32,33].

3.5. Precision and accuracy

The precision and accuracy of the present method for the determination of the urinary metabolites are shown in Table 4. The RSD and RE values of many metabolites tended to increase at low concentrations of the compounds. In urine samples with concentrations of 20 and 200 μg/l of the compounds, the RSD and RE values for all 15 metabolites were <10% and <11%, respectively, which indicate good reproducibility and accuracy. On the other hand, in the study using samples with 2.0 μg/l, these values for many compounds, except for dimethylphosphate, dimethylthiophosphate, diethylphosphate and diethylthiophosphate, were more than 10%. These findings were considered to be due to the quantification limits of the compounds (Table 3). The concentrations of the quantification limits of the metabolites, except for above 4 compounds and 2-isopropyl-6-methyl-4-pyrimidinol, were higher than 2.0 μg/l.

3.6. Storage stability

The stabilities of the metabolites in urine at −20 °C were examined, and the results are shown in Table 5. For all of the metabolites, no degradation with time was observed over one month. The results show that urine samples collected from subjects can be stored for

Table 4
Precision and accuracy of the present analytical method.

Compound	200 μg/l[a]		20 μg/l[a]		2.0 μg/l[a]	
	RSD[b]	RE[c]	RSD[b]	RE[c]	RSD[b]	RE[c]
Dimethylphosphate	0.9	1.4	4.2	5.2	6.9	10.4
Dimethylthiophosphate	3.4	5.2	1.4	7.1	3.8	6.2
Diethylphosphate	2.3	4.0	7.7	8.2	5.8	5.3
Diethylthiophosphate	3.6	0.5	4.4	9.3	7.8	5.1
Di-*n*-butylphosphate	2.2	2.0	5.5	10.4	11.4	10.7
Diphenylphosphate	2.3	3.9	8.7	7.4	12.9	14.6
Bis(2-ethylhexyl)phosphate	6.4	4.9	9.6	4.6	15.5	13.7
3,5,6-Trichloro-2-pyridinol	6.7	6.8	5.1	7.9	12.1	12.1
3-Methyl-4-nitrophenol	4.0	2.9	3.9	10.4	18.6	14.1
3-Methyl-4-(methylthio)phenol	3.1	4.4	9.2	2.2	14.6	10.1
2-Isopropyl-6-methyl-4-pyrimidinol	1.8	2.3	6.9	8.0	9.1	17.1
2,4-Dichlorophenol	6.3	0.2	5.3	3.1	13.5	10.1
2,5-Dichlorophenol	5.8	1.9	8.6	10.1	10.3	12.0
1-Naphthol	6.4	3.9	8.7	4.0	18.8	10.3
2-Naphthol	6.4	0.9	8.1	5.0	18.3	9.8

[a] Concentrations of metabolites spiked into pooled urine samples.
[b] RSD values in studies with assays of five spiked samples (%).
[c] Error of assayed samples relative to their spiked concentrations (%).

Table 5
Stabilities of metabolites in urine at −20 °C.

Compound	Storage time (days)							
	1	2	4	7	11	16	22	30
Dimethylphosphate	95 (2.4)	97 (1.4)	95 (3.0)	92 (7.3)	95 (0.48)	87 (6.7)	101 (4.5)	91 (5.4)
Dimethylthiophosphate	102 (1.7)	97 (3.6)	105 (3.7)	98 (8.2)	107 (4.7)	93 (8.3)	94 (3.4)	110 (6.7)
Diethylphosphate	97 (2.0)	96 (2.7)	110 (3.2)	93 (5.1)	107 (8.8)	92 (6.1)	104 (5.2)	93 (4.8)
Diethylthiophosphate	98 (2.6)	94 (3.1)	108 (3.7)	100 (8.0)	105 (7.8)	87 (7.8)	93 (1.2)	107 (8.2)
Di-*n*-butylphosphate	98 (2.1)	98 (3.7)	106 (4.2)	96 (9.5)	93 (5.9)	94 (5.9)	104 (2.7)	96 (9.7)
Diphenylphosphate	102 (7.4)	89 (4.4)	109 (5.6)	110 (5.3)	97 (7.2)	91 (6.4)	109 (8.0)	103 (3.6)
Bis(2-ethylhexyl)phosphate	113 (4.8)	118 (7.4)	116 (6.8)	112 (6.6)	100 (6.1)	106 (6.2)	109 (7.1)	106 (6.0)
3,5,6-Trichloro-2-pyridinol	97 (3.5)	104 (5.8)	110 (1.5)	108 (5.5)	92 (5.8)	109 (5.6)	115 (2.6)	92 (1.7)
3-Methyl-4-nitrophenol	95 (3.8)	91 (1.7)	107 (4.8)	94 (5.5)	89 (3.8)	88 (5.7)	95 (7.9)	85 (2.5)
3-Methyl-4-(methylthio)phenol	105 (3.4)	99 (5.3)	110 (3.7)	105 (8.4)	87 (4.2)	97 (9.3)	96 (3.2)	90 (5.6)
2-Isopropyl-6-methyl-4-pyrimidinol	93 (1.2)	98 (3.9)	107 (2.5)	99 (9.3)	104 (2.1)	93 (8.5)	111 (1.4)	109 (2.1)
2,4-Dichlorophenol	87 (2.4)	103 (8.1)	97 (0.87)	112 (7.7)	113 (7.2)	98 (5.3)	91 (5.5)	103 (5.2)
2,5-Dichlorophenol	100 (3.5)	97 (5.6)	111 (4.6)	100 (6.3)	105 (5.1)	87 (6.0)	89 (4.4)	89 (5.2)
1-Naphthol	100 (2.8)	103 (3.2)	105 (2.4)	96 (7.4)	95 (0.67)	91 (5.2)	96 (0.69)	96 (1.1)
2-Naphthol	102 (5.1)	114 (4.9)	110 (4.6)	114 (4.7)	119 (7.7)	111 (4.0)	118 (5.3)	118 (1.6)

Concentration of each compound just before storage (time: 0) was set at 100% (each compound: *ca.* 170 μg/l). The values are means for three samples. SD is in parentheses ($n = 3$).

Table 6
Urinary concentrations (μg/l) of metabolites in five healthy nonsmoking subjects without occupational exposure.

Compound	Subject				
	A	B	C	D	E
Dimethylphosphate	2.1	1.2	2.5	1.7	1.9
Dimethylthiophosphate	3.2	4.2	5.0	3.0	2.3
Diethylphosphate	2.0	6.5	3.2	1.6	2.6
Diethylthiophosphate	1.7	–	3.4	<QL	<QL
Di-*n*-butylphosphate	–	–	–	<QL	<QL
Diphenylphosphate	9.8	–	–	–	–
Bis(2-ethylhexyl)phosphate	–	–	–	–	–
3,5,6-Trichloro-2-pyridinol	–	–	17	–	–
3-Methyl-4-nitrophenol	–	–	–	–	–
3-Methyl-4-(methylthio)phenol	–	–	–	–	–
2-Isopropyl-6-methyl-4-pyrimidinol	2.2	–	–	–	3.3
2,4-Dichlorophenol	–	–	–	–	–
2,5-Dichlorophenol	7.0	–	11	3.9	172
1-Naphthol	–	4.4	–	–	–
2-Naphthol	–	<QL	–	–	–

–: not detected.
<QL: The concentrations of the detectable metabolites were less than their quantification limits shown in Table 3.

up to one month at −20 °C protected from light in a freezer until analysis.

3.7. Application

The results of determination of the urinary metabolites in the five subjects are shown in Table 6. Urinary dimethylthiophosphate or diethylthiophosphate, which was one of the indexes for monitoring exposure to organophosphorus insecticides, were found in the samples of all subjects. 3,5,6-Trichloro-2-pyridinol was detected in the urine sample of subject C, and 2-isopropyl-6-methyl-4-pyrimidinol in those of subjects A and E. Therefore, subject C and subjects A and E were presumed to be exposed to chlorpyrifos and diazinon, respectively. The urinary concentrations of diethylthiophosphate, which was one of the major urinary metabolites of chlorpyrifos and diazinon, in subjects A and C were higher than those in other subjects. As just described, the determination of phenolic metabolites such as 3,5,6-trichloro-2-pyridinol and 2-isopropyl-6-methyl-4-pyrimidinol could help to identify the parent compounds exposed.

Diphenylphosphate and di-*n*-butylphosphate, of which urinary excretion indicated the possibility of exposure to the corresponding trialkylphosphates for flame retardants and plasticizers, were detected in several subjects. The urinary 2,5-dichlorophenol concentration in subject E was markedly higher than those for the others, and both 1-naphthol and 2-naphthol were found in the urine of subject B. Therefore, it seemed highly possible that *p*-dichlorobenzene and naphthalene, respectively, were employed as moth repellents or toilet bowl deodorants in their households.

4. Conclusions

To evaluate exposure to organophosphorus compounds (insecticides, and trialkylphosphates used as flame retardants and plasticizers) and moth repellents in Japanese general population, a simultaneous analytical method was developed for their 15 urinary metabolites, including several phenolic metabolites which help identification of the parent compounds. The urine sample collected was hydrolyzed with concentrated hydrochloric acid, and a saturated amount of ammonium sulfate was added to the hydrolysate, and then the metabolites were extracted using the sorbent Isolute ENV+ (hydroxylated polystyrene-divinylbenzene copolymers). The metabolites were desorbed with methylacetate and acetonitrile from the sorbent, concentrated, and after being transformed into their *tert*-butyldimethylsilyl derivatives, were analyzed by GC/MS(EI). The metabolites in the final analytical solution were prepared to 33 times the concentrations in the urine sample. The calibration curves were constructed by preparing and analyzing the standard urine solutions spiked with authentic materials of the metabolites in the same manner as the samples. The metabolites displayed linear calibration curves over the urinary concentration range of 0–200 μg/l, and could be determined accurately and precisely at the concentrations studied though the precision and accuracy of the values measured for several metabolites were slightly poor at low concentrations. The quantification limits of the metabolites were 0.8–4 μg/l in urine. In addition, the urine samples collected could be stored for up to one month at −20 °C in a freezer until analysis.

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References

[1] Y. Liu, L. Zhu, X. Shen, Environ. Sci. Technol. 35 (2001) 840.
[2] T. Yoshida, I. Matsunaga, Bull. Osaka Pref. Inst. Publ. Health 39 (2001) 49.
[3] P.C. Hartmann, D. Burgi, W. Giger, Chemosphere 57 (2004) 781.
[4] C. Lu, G. Kedan, J. Fisker-Andersen, J.C. Kissel, R.A. Fenske, Environ. Res. 96 (2004) 283.
[5] A. Marklund, B. Andersson, P. Haglund, J. Environ. Monit. 7 (2005) 814.
[6] G. Bouvier, O. Blanchard, I. Momas, N. Seta, Sci. Total Environ. 366 (2006) 74.
[7] T. Ohura, T. Amagai, Y. Senga, M. Fusaya, Sci. Total Environ. 366 (2006) 485.
[8] T. Yoshida, I. Matsunaga, K. Tomioka, S. Kumagai, Indoor Built Environ. 15 (2006) 425.
[9] I. Saito, A. Onuki, H. Seto, Indoor Air 17 (2007) 28.
[10] T. Yoshida, I. Matsunaga, H. Oda, Y. Miyake, S. Sasaki, Y. Ohya, S. Miyamoto, Y. Hirota, J. Soc. Indoor Environ. Jpn. 9 (2007) 83.
[11] C. Bergh, R. Torgrip, G. Emenius, C. Ostman, Indoor Air 21 (2011) 67.
[12] R.J. Weir, Organic Phosphate, in: G.D. Clayton, F.E. Clayton (Eds.), Patty's Industrial Hygiene and Toxicology, 4th ed., vol. II, Part A Toxicology, John Wiley & Sons, New York, 1993.
[13] The Chemical Daily, 14906 no Kagaku Shouhin, Tokyo, 2006.
[14] International Agency for Research on Cancer (IARC), Occupational Exposures in Insecticide Application, and Some Pesticides, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 53, IARC, Lyon, 1991, p. 267.
[15] International Agency for Research on Cancer (IARC), Some Chemicals that Cause Tumours of the Kidney or Urinary Bladder in Rodents and Some Other Substances, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 73, IARC, Lyon, 1999, p. 223.
[16] International Agency for Research on Cancer (IARC), Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 82, IARC, Lyon, 2002, p. 367.
[17] W.J. Murray, C.A. Franklin, in: B. Ballantyne, T.C. Marrs (Eds.), Clinical and Experimental Toxicology of Organophosphates and Carbamates, Butterworth–Heinemann, Oxford, England, 1992, p. 430.
[18] C. Aprea, G. Sciarra, D. Orsi, P. Boccalon, P. Sartorelli, E. Sartorelli, Sci. Total Environ. 177 (1996) 37.
[19] J. Hardt, J. Angerer, J. Anal. Toxicol. 24 (2000) 678.
[20] A.N. Oglobline, H. Elimelakh, B. Tattam, R. Geyer, G.E. O'Donnell, G. Holder, Analyst 126 (2001) 1037.
[21] R. Bravo, W.J. Driskell, R.D. Whitehead Jr., L.L. Needham, D.B. Barr, J. Anal. Toxicol. 26 (2002) 245.
[22] D.B. Barr, R. Bravo, G. Weerasekera, L.M. Caltabiano, R.D. Whitehead Jr., A.O. Olsson, S.P. Caudill, S.E. Schober, J.L. Prikle, E.J. Sampson, R.J. Jackson, L.L. Needham, Environ. Health Perspect. 112 (2004) 186.
[23] R. Bravo, L.M. Caltabiano, G. Weerasekera, R.D. Whitehead, C. Fernandez, L.L. Needhan, A. Bradman, D.B. Barr, J. Expos. Anal. Environ. Epidemiol. 14 (2004) 249.
[24] U. Heudorf, J. Angerer, H. Drexler, Int. Arch. Occup. Environ. Health 77 (2004) 67.
[25] J. Ueyama, I. Saito, M. Kamijima, T. Nakajima, M. Gotoh, T. Suzuki, E. Shibata, T. Kondo, K. Takagi, K. Miyamoto, J. Takamatsu, T. Hasegawa, K. Takagi, J. Chromatogr. B 832 (2006) 58.
[26] X. Ye, F.H. Pierik, R. Hauser, S. Duty, J. Angerer, M.M. Park, A. Burdorf, A. Hofman, V.W.V. Jaddoe, J.P. Mackenbach, E.A.P. Steegers, H. Tiemeier, M.P. Longnecker, Environ. Res. 108 (2008) 260.
[27] S. Yucra, M. Gasco, J. Rubio, G.F. Gonzales, Environ. Health 7 (2008) 59.
[28] C. Wu, P. Liu, L. Zheng, J. Chen, Z. Zhou, J. Chromatogr. B 878 (2010) 2575.
[29] R.S. Murphy, F.W. Kutz, S.C. Strassman, Environ. Health Perspect. 48 (1983) 81.
[30] K. Sasaki, T. Suzuki, M. Takeda, M. Uchiyama, Bull. Environ. Contam. Toxicol. 33 (1984) 281.
[31] H. Kurebayashi, A. Tanaka, T. Yamaha, Toxicol. Appl. Pharmacol. 77 (1985) 395.
[32] B.K. Schindler, K. Forster, J. Angerer, J. Chromatogr. B 877 (2009) 375.
[33] B.K. Schindler, K. Forster, J. Angerer, Anal. Bioanal. Chem. 395 (2009) 1167.
[34] L.D. Pagnotto, J.E. Walkley, Am. Ind. Hyg. Assoc. J. 26 (1965) 137.
[35] J.V. Mackell, F. Rieders, H. Brieger, E.L. Bauer, Pediatrics 7 (1951) 722.
[36] The Society for the Research of Household Insect Pests, Japan, Kaoku Gaichu Jiten, Inoueshoin, Tokyo, 1995.
[37] S. Uemura, H. Kawamura, M. Tsuji, Nouyaku Dokusei no Jiten, Third Revision, Sanseido, Tokyo, 2006.
[38] T. Shafik, D.E. Bradway, H.F. Enos, A.R. Yobs, J. Agric. Food Chem. 21 (1973) 625.
[39] D. Blair, H.R. Roderick, J. Agric. Food Chem. 24 (1976) 1221.
[40] E.M. Lores, D.E. Bradway, J. Agric. Food Chem. 25 (1977) 75.
[41] C.G. Daughton, A.M. Cook, M. Alexander, Anal. Chem. 51 (1979) 1949.
[42] J.B. Knaak, K.T. Maddy, S. Khalifa, Bull. Environ. Contam. Toxicol. 21 (1979) 375.
[43] D.Y. Takade, J.M. Reynolds, J.H. Nelson, J. Agric. Food Chem. 27 (1979) 746.
[44] D.E. Bradway, R. Moseman, R. May, Bull. Environ. Contam. Toxicol. 26 (1981) 520.
[45] S.J. Reid, R.R. Watts, J. Anal. Toxicol. 5 (1981) 126.
[46] R.A. Fenske, J.T. Leffingwell, J. Agric. Food Chem. 37 (1989) 995.
[47] B.P. Nutley, J. Cocker, Pestic. Sci. 38 (1993) 315.
[48] C. Aprea, G. Sciarra, L. Lunghini, J. Anal. Toxicol. 20 (1996) 559.
[49] T.F. Moate, C. Lu, R.A. Fenske, R.M.A. Hahne, D.A. Kalman, J. Anal. Toxicol. 23 (1999) 230.
[50] W.C. Lin, C.H. Kuei, H.C. Wu, C.C. Yang, H.Y. Chang, J. Anal. Toxicol. 26 (2002) 176.
[51] B. Muan, J.U. Skare, J. Agric. Food Chem. 37 (1989) 1081.
[52] M. Katagi, H. Tsuchihashi, S. Hanada, H. Jinmori, K. Otsuki, Jpn. J. Toxicol. Environ. Health 39 (1993) 459.
[53] N. Kupfermann, A. Schmoldt, H. Steinhart, J. Anal. Toxicol. 28 (2004) 242.
[54] F.A. Tarbah, B. Kardel, S. Pier, O. Temme, T. Daldrup, J. Anal. Toxicol. 28 (2004) 198.
[55] G.K.H.D. Alwis, L.L. Needham, D.B. Barr, J. Anal. Toxicol. 32 (2008) 721.
[56] J. Ueyama, M. Kamijima, T. Kondo, K. Takagi, E. Shibata, T. Hasegawa, S. Wakusawa, T. Taki, M. Gotoh, I. Saito, J. Chromatogr. B 878 (2010) 1257.
[57] G. Weerasekera, K.D. Smith, L.L. Needham, D.B. Barr, J. Anal. Toxicol. 32 (2008) 106.
[58] G.K.H.D. Alwis, L.L. Needham, D.B. Barr, J. Chromatogr. B 843 (2006) 34.
[59] Pharmaceutical and Food Safety Bureau of the Ministry of Health, Labour and Welfare, A Notice (IYAKU-828), Japanese Government, Tokyo, Japan, 2001.
[60] C-H. Lee, M. Kamijima, H. Kim, E. Shibata, J. Ueyama, T. Suzuki, K. Takagi, I. Saito, M. Gotoh, H. Hibi, H. Naito, T. Nakajima, Int. Arch. Occup. Environ. Health 80 (2007) 217.