








## ORIGINAL ARTICLE

# Development of a strategic approach for comprehensive detection of organophosphate pesticide metabolites in urine: Extrapolation of cadusafos and prothiofos metabolomics data of mice to humans

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## Abstract

**Objectives:** The comprehensive detection of environmental chemicals in biospecimens, an indispensable task in exposome research, is advancing. This study aimed to develop an exposomic approach to identify urinary metabolites of organophosphate (OP) pesticides, specifically cadusafos and prothiofos metabolites, as an example chemical group, using an original metabolome dataset generated from animal experiments.

**Methods:** Urine samples from 73 university students were analyzed using liquid chromatography–high-resolution mass spectrometry. The metabolome data, including the exact masses, retention time ( $t_R$ ), and tandem mass spectra obtained from the human samples, were compared with the existing reference databases and with our original metabolome dataset for cadusafos and prothiofos, which was produced from mice to whom two doses of these OPs were orally administered.

**Results:** Using the existing databases, one chromatographic peak was annotated as 2,4-dichlorophenol, which could be a prothiofos metabolite. Using our original dataset, one peak was annotated as a putative cadusafos metabolite and three peaks as putative prothiofos metabolites. Of these, all three peaks suggestive of prothiofos metabolites, 2,4-dichlorophenol, 3,4,5-trihydroxy-6-(2,4-dichlorophenoxy) oxane-2-carboxylic acid, and (2,4-dichlorophenyl) hydrogen sulfate were confirmed as authentic compounds by comparing their peak data with both the original dataset and peak data of the standard reagents. The putative cadusafos metabolite was identified as a level C compound (metabolite candidate with limited plausibility).

**Conclusions:** Our developed method successfully identified prothiofos metabolites that are usually not a target of biomonitoring studies. Our approach is extensively applicable to various environmental contaminants beyond OP pesticides.

## KEYWORDS

comprehensive analysis, high-resolution mass spectrometry, metabolite, organophosphate pesticide, urine

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## 1 | INTRODUCTION

The “exposome”, a term coined in 2005 to account for the unexplained risk factors underlying human diseases,<sup>1</sup> necessitates comprehensive analyses of life-course exposure. The biomonitoring approach is essential for exposome studies of environmental chemicals. Trials to monitor all unknown or suspected chemicals of concern in biospecimens, using the so-called comprehensive or untargeted analysis, have been conducted<sup>2,3</sup>; however, such an analytical approach is not adopted widely in epidemiological studies because existing technologies still cannot meet the requirements for measuring the exposome.<sup>4</sup> Therefore, conventional methods of targeted biomonitoring are prevalent in epidemiological studies of environmental chemicals.<sup>5–7</sup>

Characterizing unknown analytes remains a major challenge.<sup>8</sup> The methodology to identify and quantify low-abundance analytes is not yet fully developed.<sup>8</sup> Untargeted approaches rely heavily on library searches of tandem mass spectrometric (MS<sup>2</sup>) data for annotation with subsequent standard confirmation,<sup>8,9</sup> which is quite time-consuming. In addition, the major reference databases for metabolomics have largely focused on naturally occurring metabolites, and not exogenous chemical metabolites. Exposomic studies may thus benefit from a database specifically dedicated to chemicals with environmental exposure.<sup>8</sup>

Pesticides are a representative group of chemicals for which comprehensive analytical methods are required, and several methods have been developed using liquid chromatography combined with high-resolution mass spectrometry (LC-HRMS).<sup>10,11</sup> However, as described, limited information on pesticide metabolites is available in existing mass spectrometry reference databases. Therefore, it is challenging to identify the structure of these metabolites.

Our goal is to establish a comprehensive analysis method for human biomonitoring of environmental chemicals that can be applied to epidemiological studies. As the initial step in this direction, the objective of this study was to develop an exposomic approach to identify the organophosphate (OP) pesticide metabolome as an example chemical group, focusing on metabolites of cadusafos and prothiofos in human urine. In this study, we first show that the existing reference databases for pesticides are of limited effectiveness to identify exposure, and second, we developed a comprehensive detection method for OP metabolites by creating a reference dataset from in vivo experiments performed on pesticide-treated mice.

## 2 | MATERIALS AND METHODS

### 2.1 | Study design

Two approaches were used to annotate human urinary compounds with LC-HRMS: (a) the currently available reference

databases for pesticides and (b) our original mass information dataset created from animal experiments for in vivo generation of pesticide metabolites.

Regarding the first approach, the effectiveness of existing reference databases for searching OP compounds was examined by comparing the human urinary metabolome data with databases provided by the United States Environmental Protection Agency (US EPA). When building our original datasets of OP compounds and their metabolites that might be detected in Japan, we found only 52 OP compounds, including parents and their metabolites, in the existing databases.

Regarding the second approach, we created an original dataset of cadusafos and prothiofos as example chemicals, both highly ranked OP pesticides in terms of shipping volume in Japan in 2018. These pesticides were selected because (a) only one metabolite, 2,4-dichlorophenol, was listed in the existing databases, and (b) the parent compounds were not metabolized to the common urinary metabolites, dialkyl phosphates (DAPs), and human biomonitoring has not been conducted either in Japan or globally. Conventional biomonitoring of OP pesticides is usually conducted through the measurement of DAPs in epidemiological studies.<sup>12,13</sup> The major problem in using DAPs for exposure assessments is that OP pesticides with different magnitudes of neurotoxicity are metabolized and excreted in urine as the same DAPs. Another problem is that some OP pesticides are not metabolized to DAPs because of their chemical structures. Therefore, it is essential to develop a comprehensive analysis method for exposure assessment that can identify an overall picture of exposure to OP pesticides.

### 2.2 | Reagents and sample collection

#### 2.2.1 | Reagents

Cadusafos (*S, S*-di-*sec*-butyl *O*-ethyl phosphorodithioate, purity 99.8%), prothiofos [(2,4-dichlorophenoxy)-ethoxypropylsulfanyl-sulfanylidene- $\lambda$ 5-phosphane, purity 99.3%], 2,4-dichlorophenol (100  $\mu$ g/mL in MeOH), glyphosate (5.0 mg/mL in H<sub>2</sub>O), diazinon (purity 99.6%), benzoic acid (purity 99.5%), ultrapure water, acetonitrile, and formic acid of analytical grade were purchased from FUJIFILM Wako Pure Chemical Corporation. 4-Nitrophenol (purity 100%) was purchased from Sigma-Aldrich, and 4-nitro-*m*-cresol (purity 98.0%) was purchased from Tokyo Chemical Industry Co., Ltd. 3,4,5-Trihydroxy-6-(2,4-dichlorophenoxy) oxane-2-carboxylic acid (purity 99.9%), (2,4-dichlorophenyl) hydrogen sulfate (purity 99.9%), and 2-methylsulfonylbutane (purity 99.9%) were synthesized by Hayashi Pure Chemical Ind., Ltd. Pierce<sup>TM</sup> LTQ Velos electrospray ionization (ESI)-positive ion calibration solution and Pierce<sup>TM</sup> ESI-negative ion calibration solution were purchased from Thermo Fisher Scientific Co., Ltd.

## 2.2.2 | Study population and urine sampling

This part of the study using human urine was approved by the Ethics Committee of Nagoya City University Graduate School of Medical Sciences (approval no. 60-18-0021). The study was conducted in accordance with the Declaration of Helsinki and nationally valid regulations. We recruited 78 female students from a university located in Aichi prefecture, Japan, on the premise that they did not take any prescribed medicines. Among these, 73 students (mean age, 21.2 years) provided written informed consent, submitted first morning voids, and answered the questionnaire regarding foodstuffs ingested in the 2 days preceding the submitted voids. These urine samples were transported to our laboratory at 4°C and then stored at -80°C until analysis. The urine samples were collected between June 4 and July 12, 2018, and stored until December 2019 at -80°C.

## 2.3 | Comprehensive analysis using LC-HRMS

### 2.3.1 | Sample preparation

Sample preparation of pesticide metabolites followed the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method with minor modifications.<sup>14</sup> Briefly, a mixture of 1 mL urine sample, 10 µL internal standard chemicals diluted in acetonitrile, 4 mL ultrapure water, 10 mL acetonitrile, and the salt of the EN method QuEChERS kit (Agilent Technologies Japan, Ltd.) was vigorously shaken with a ceramic homogenizer and centrifuged at 3500 rpm (2356 g) for 10 minutes. The acetonitrile layer was transferred into a test tube and evaporated to dryness at 37°C under a stream of nitrogen. The dried residue was then dissolved in 200 µL ultrapure water, placed in a filter tube (Ultrafree-MC Centrifugal Filter 0.2 µm pore size, hydrophilic PTFE, 0.4 mL volume, non-sterile, Merck Ltd.), and centrifuged at 11 000 rpm (10 958 g) at 10°C for 3 minutes. The supernatant was transferred to a vial and analyzed using LC-HRMS.

### 2.3.2 | LC-HRMS settings

Chromatographic separation was performed using an UltiMate 3000 (Thermo Fisher Scientific Co., Ltd.) set with a Hypersil Gold C18 column (100 × 2.1 mm, 1.9 µm particles) (Thermo Fisher Scientific Co., Ltd.). The following LC operating conditions were used: total flow rate of the mobile phase, 0.2 mL/min; total run time including equilibration, 20 minutes. The initial mobile phase composition was 95% mobile phase A (0.1% formic acid in water) and 5% mobile phase B (0.1% formic acid in acetonitrile). After 1 minute, the percentage of mobile phase B was linearly increased to 95% within 9 minutes.

After that, mobile phase B was increased quickly to 100% and maintained for 5 minutes. The composition was returned to the initial conditions and equilibrated for 5 minutes. The injection volume was 5 µL. Eluted compounds were detected using a Q Exactive Focus Orbitrap (Thermo Fisher Scientific Co., Ltd.) equipped with an ESI source. Ionization settings for both positive and negative modes were selected in accordance with the method reported by Roca et al,<sup>14</sup> with minor modifications. The ion source parameters were as follows: 4.0 kV (positive mode) and 2.5 kV (negative mode); sheath gas flow rate, 45; auxiliary gas flow rate, 10; sweep gas flow rate, 2; auxiliary gas heater temperature, 400°C; capillary temperature, 250°C; S-lens RF level, 50. Switching for the fragmentation of ions with higher collision-induced dissociation cell was performed with N<sub>2</sub> (>99%) and three different collision energies (15, 30, and 45 eV). Calibration was performed at least once each week using Pierce<sup>TM</sup> LTQ Velos ESI Positive Ion Calibration Solution and Pierce<sup>TM</sup> ESI Negative Ion Calibration Solution.

The system was operated in full-scan mode ( $m/z$  120–1000) at a resolving power of 70 000 and in a data-dependent MS/MS mode to gain MS<sup>2</sup> fragment ions at a resolving power of 17 500.

### 2.3.3 | Candidate peak extraction

Candidate peaks were extracted from the raw data using Compound Discoverer 3.1 (Thermo Fisher Scientific Co., Ltd.), based on the HRMS peak of the exact mass of each of the [2M + H]<sup>+</sup>, [2M - H]<sup>-</sup>, [M + HCOO]<sup>-</sup>, [M + H]<sup>+</sup>, [M + K]<sup>+</sup>, [M + Na]<sup>+</sup>, [M + NH<sub>4</sub>]<sup>+</sup>, or [M - H]<sup>-</sup> ions, with a mass measurement error of ±5 ppm.

## 2.4 | Annotation of peak compounds using the existing pesticide reference databases

We compared the molecular formulas calculated from precursor isotopic ion spectra obtained from urine samples from 73 students with those in 10 free databases for pesticides provided by the US EPA (Pesticide Chemical Search Database,<sup>15</sup> Swiss Pesticides and Metabolites,<sup>16,17</sup> Pesticide Screening List for Luxembourg,<sup>18</sup> Natural Product Insecticides,<sup>19</sup> Office of Pesticide Programs Information Network,<sup>20</sup> PESTACTIVES,<sup>21</sup> List of Inert Ingredients Food and Nonfood Use [last updated October 25, 2019],<sup>22</sup> Pesticide Properties DataBase,<sup>23</sup> Swiss Pesticides and Transformation Products,<sup>24</sup> and TOXCAST\_PhaseI—EPA ToxCast Screening Library [Phase I subset]<sup>25</sup>). Although the databases contained pesticides other than OPs, such as neonicotinoid and pyrethroid insecticides, and several environmental chemicals, we confined annotations to the 52 OP compounds.

## 2.5 | Annotation strategy for identification of peaks detected in human urine

The key concept of this approach, that is, extrapolation of mouse metabolomics data to humans, was previously reported by Jamin et al.<sup>10</sup> To identify OP metabolites in human urine, we produced our original dataset for the urinary metabolites from an experiment in which two example OP pesticides, cadusafos and prothiofos, were administered to mice. The obtained information for exact masses, retention times ( $t_R$ ), and MS<sup>2</sup> spectra of the urinary components in the respective OP-administered mice were registered in the dataset. If standard reagents were available, their data were also added to the dataset. Finally, the human urinary metabolome data were compared with the original dataset. Subsequently, we classified the identification levels of each metabolite as described in Section 2.5.3.

### 2.5.1 | Urine collection from animal experiments to acquire metabolome data for cadusafos and prothiofos

This part of the study was conducted in accordance with the Japanese law concerning the protection and control of animals and the guidelines of Animal Care and Use in Nagoya City University Graduate School of Medical Sciences (approval No. H29M-37). Two 10-week-old male ICR mice were purchased from Japan SLC, Inc. The animal room temperature and relative humidity were maintained at 23°C–25°C and 40%–50%, respectively, with a 12 hours light/dark cycle (lights on from 9:00 AM to 9:00 PM). After a 1–2 week acclimation period, each mouse was administered vehicle only (corn oil) by gavage as a control on the first day. Thereafter, a quarter (second day) and half (fourth day) of the lethal dose 50 (LD<sub>50</sub>) of cadusafos or prothiofos (LD<sub>50</sub> = 68 and 940 mg/kg for cadusafos<sup>26</sup> and prothiofos,<sup>27</sup> respectively) were dissolved in corn oil and administered to the mice. They were allowed to rest on the third day. Immediately after the treatment, the mice were placed in metabolic cages and allowed ad libitum access to water and food (standard commercial diet, CE-2, CLEA Japan, Inc). Urine samples were collected 24 hours after administration and stored at –80°C until analysis. Samples were collected in February 2019 and stored

until July 2020 at –80°C. Urine samples diluted 10-fold with ultrapure water were analyzed using the same conditions as for human urine samples.

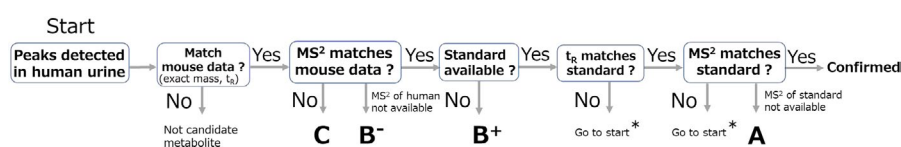
### 2.5.2 | Original dataset preparation of the in vivo metabolome of urine from OP-treated mice

First, metabolism information for pesticides (cadusafos<sup>28</sup> and prothiofos<sup>29</sup>) were acquired from previous reports, and each metabolite was listed. In addition, theoretical phase II metabolites of the listed metabolites were considered along with phase I metabolites. Next, chromatographic peaks detected in urine from OP-treated mice were selected and their peak information was used to construct our original metabolome dataset only when the peak areas from mice treated with both quarter and half LD<sub>50</sub> were larger than those in the vehicle-treated mouse. When the exact masses of detected peaks matched the listed metabolites and their MS<sup>2</sup> spectra were obtained, these data, along with  $t_R$ , were registered in mzVault™ 2.3 (Thermo Fisher Scientific Co., Ltd.) as “mouse data” without structure characterization and used for further analysis as an original dataset for the mass spectral database searches. Moreover, the mzVault best match score was calculated. This score indicates the degree of matching in mass-to-charge ratio ( $m/z$ ) and fragment ion intensities between the observed peak and the MS<sup>2</sup> fragment pattern registered in mzVault.

### 2.5.3 | Peaks comparison between human urine and mouse data

The exact masses,  $t_R$ , and MS<sup>2</sup> spectra (if available) obtained from human urine samples were compared with those of mouse data according to the workflow shown in Figure 1. If the corresponding standard reagent was available, we compared this information in human urine with that of the standard. The tolerances of the mass measurement error and difference in  $t_R$  were within ±5 ppm (±10 ppm for mzVault matching) and ±0.3 minutes, respectively. A formula estimated from the theoretical isotopic pattern was also considered.

Peaks in human urine that matched mouse data were categorized into four classes according to our original criteria of confidence levels (Table 1 and Figure 1) (confirmed



**FIGURE 1** Workflow for identifying peaks detected in human urine by comparing with “mouse data”. The identification levels in this figure are the same as those shown in Table 1. \*In this case, the peak detected from humans is not considered the same as the standard, so return to the start and reconsider the identification level

**TABLE 1** Criteria for the identification level of peaks detected in human urine

Level		Match with					
		Standard reagents			"Mouse data"		
		Exact mass	$t_R^a$	$MS^{2b}$	Exact mass	$t_R^a$	$MS^{2b}$
Confirmed	Confirmed metabolite	○	○	○	○	○	○
A	Probable metabolite	○	○	NA <sup>§</sup>	○	○	○
B	Possible metabolite						
+		NA <sup>¶</sup>	NA <sup>¶</sup>	NA <sup>¶</sup>	○	○	○
-		NA <sup>¶</sup>	NA <sup>¶</sup>	NA <sup>¶</sup>	○	○	NA <sup>#</sup>
C	Metabolite candidate with limited plausibility	NA <sup>¶</sup>	NA <sup>¶</sup>	NA <sup>¶</sup>	○	○	×

Notes: NA<sup>§</sup>, MS<sup>2</sup> spectral data of standard reagents were not available.

NA<sup>¶</sup>, standard reagent was not available.

NA<sup>#</sup>, MS<sup>2</sup> spectral data of human urine were not available.

<sup>a</sup> $t_R$ , retention time (error tolerance within  $\pm 0.3$  min).

<sup>b</sup>MS<sup>2</sup>, MS<sup>2</sup> spectra (error tolerance was within  $\pm 5$  ppm).

metabolite; probable metabolite [level A]; possible metabolite [level B]; metabolite candidate with limited plausibility [level C]) as per previous research.<sup>30,31</sup> First, if the exact masses and  $t_R$  of the peaks in human urine did not match those of the peaks in mouse data, they were judged as not metabolite candidates. If the exact masses and  $t_R$  of the peaks in human urine matched those of mouse data, but the MS<sup>2</sup> spectra did not match those of mouse data or the human MS<sup>2</sup> spectra data were unavailable, these were classified as level C or level B<sup>-</sup>, respectively. For the other peaks in human urine, if their MS<sup>2</sup> spectra matched those of mouse data and the standard reagents were unavailable, the urinary metabolite candidates were classified as level B<sup>+</sup>. When the standards were available and their  $t_R$  and MS<sup>2</sup> spectra matched those of human urinary peaks of the metabolite candidates, the candidates were identified as a confirmed metabolite. Meanwhile, the candidates were identified as the probable metabolites (level A) if MS<sup>2</sup> spectra of the standards were unavailable.

Next, MS<sup>2</sup> spectra obtained by a fixed collision energy of 30 eV in humans were compared with those of mouse metabolome data and standard reagents, which were obtained at the same collision energy condition, using a different analysis software, Xcalibur Qual Browser (Thermo Scientific), to confirm the classifications.

## 3 | RESULTS

### 3.1 | Annotation of the human urinary compounds using the existing pesticide reference databases

In total, 43 991 peaks were detected in urine samples collected from 73 students. Of these, 5191 peaks (including

2804 peaks having fully matched molecular formula) were a hit with the molecular formula of compounds listed in 10 free reference databases provided by the US EPA. Based on the analyses, which focused on 52 OPs, of the 5191 peaks, 25 OP pesticides (including parent compounds and their metabolites) were suggested to have the same elemental composition based on the isotopic mass distribution. Examples of the suspected compounds are acephate, benzoic acid, diazinon, and its major metabolites 2-isopropyl-4-methyl-6-hydroxypirimidine, 2,4-dichlorophenol, glyphosate, 4-nitro-*m*-cresol, and 4-nitrophenol. Of these,  $t_R$  of only benzoic acid and 2,4-dichlorophenol matched those of the respective commercial standards, whereas the MS<sup>2</sup> spectra of all the putative OPs matched those of the standards (data not shown). Benzoic acid and 2,4-dichlorophenol were identified in three and 16 urine samples, respectively. DAPs were not identified in any of the samples.

### 3.2 | Annotation of human urinary compounds using our original dataset

#### 3.2.1 | Creating the original dataset of urinary OP metabolites (mouse data)

In the urine obtained from the cadusafos-treated mouse, the parent compound (cadusafos) and six putative metabolites, of which the exact masses matched those presented previously,<sup>28</sup> were detected in the following suspected chemical structures: butane-2-sulfonic acid, 2-methylsulfonylbutane, 3-methylsulfonylbutan-2-ol, butan-2-ylsulfanyl (ethoxy) phosphinic acid, bis (butan-2-ylsulfanyl) phosphinic acid, and 3,4,5-trihydroxy-6-[2-(methylsulfonyl)butanoxy]oxane-2-carboxylic acid (or (3,4,5-trihydroxy-6-[1-methyl-2-(meth

ylsulfanyl)propanoxy]oxane-2-carboxylic acid)). Of these, a standard reagent was available only for the parent compound, cadusafos. The  $t_R$  and  $MS^2$  spectra of cadusafos detected in mouse urine matched those of the standard reagent.

In the urine obtained from the prothiofos-treated mouse, 15 putative metabolites were detected for which the exact masses matched those presented previously.<sup>29</sup> The suspected compounds were: 2,4-dichloro-1-[ethoxy (propylsulfanyl) phosphoryl] oxybenzene (prothiofos oxon); 3,4,5-trihydroxy-6-(2,4-dichlorophenoxy) oxane-2-carboxylic acid; ethoxy-dihydroxy-sulfanylidene- $\lambda$ 5-phosphane; ethyl dihydrogen phosphate; ethoxy-hydroxy-isopropylsulfanyl-sulfanylidene- $\lambda$ 5-phosphane; propane-2-ylsulfanyl(ethoxy)phosphinic acid; 2,4-dichlorophenol; 3,5-dichlorobenzene-1,2-diol; (2,4-dichlorophenyl) hydrogen sulfate; (2,4-dichlorophenoxy)-ethoxy-sulfanyl-sulfanylidene- $\lambda$ 5-phosphane; 2,4-dichloro-1-[ethoxy(sulfanyl)phosphoryl] oxybenzene; (2,4-dichlorophenyl) ethyl hydrogen phosphate; 2,4-dichloro-1-[ethoxy (methylsulfanyl) phosphoryl] oxybenzene; 3,4,5-trihydroxy-6-(2-hydroxy-4,6-dichlorophenoxy) oxane-2-carboxylic acid (or 3,4,5-trihydroxy-6-(2-hydroxy-3,5-dichlorophenoxy) oxane-2-carboxylic acid); and (2,4-dichloro-6-hydroxyphenyl) hydrogen sulfate (or (3,5-dichloro-6-hydroxy) phenyl hydrogen sulfate). Of these, standard reagents were available for prothiofos oxon, (2,4-dichlorophenyl) hydrogen sulfate, 2,4-dichlorophenol, and 3,4,5-trihydroxy-6-(2,4-dichlorophenoxy) oxane-2-carboxylic acid, of which the  $t_R$  and  $MS^2$  spectra matched those of the respective signal peaks detected in mouse urine.

### 3.2.2 | Identification of peak compounds detected in human urine by comparison of the peak data with our original dataset (mouse data)

A summary of the identified peaks detected in human urine at levels higher than level C is shown in Table 2. Of these, three peak compounds were attributed to prothiofos metabolites and identity confirmed (2,4-dichlorophenol [Figure 2], 3,4,5-trihydroxy-6-(2,4-dichlorophenoxy) oxane-2-carboxylic acid [Figure 3], and (2,4-dichlorophenyl) hydrogen sulfate [Figure 4]) by comparing with mouse data and with data obtained from the standard reagent. One peak compound was attributed to the cadusafos metabolite and identified as level C because the  $MS^2$  spectra did not match that of mouse data. The mzVault best match scores, which indicate the extent of matching (0%-100%) between the best fragmentation scan results for the compounds in human urine and the spectra registered in mouse data in mzVault, were 62.8%, 97.0%, and 85.6% for 2,4-dichlorophenol, 3,4,5-trihydroxy-6-(2,4-dichlorophenoxy) oxane-2-carboxylic acid, and (2,4-dichlorophenyl) hydrogen sulfate, respectively.

Regarding the peaks identified as confirmed compounds, three human urine samples showed peaks corresponding to 2,4-dichlorophenol at concentrations of 10-15 ppb. 3,4,5-Trihydroxy-6-(2,4-dichlorophenoxy) oxane-2-carboxylic acid at concentrations of 7-100 ppb and (2,4-dichlorophenyl) hydrogen sulfate at below 10 ppb were identified in five and four human urine samples, respectively.

For suspected 2-methylsulfanylbutane as a putative cadusafos metabolite, the exact mass and  $t_R$  but not  $MS^2$  spectra of the peaks from 63 human samples matched those of mouse data, and the  $t_R$  and  $MS^2$  spectra from human and mouse urine samples were inconsistent with those of the standard reagent. Therefore, the peak compounds detected in the samples were presumably structural isomers of 2-methylsulfanylbutane. That of the mouse urine may be a possible unknown metabolite of cadusafos because of its dose-dependent peak height increase.

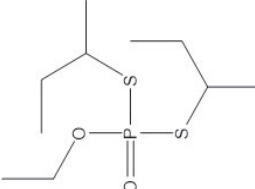
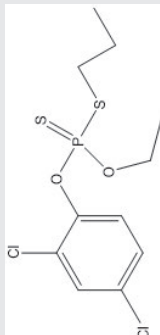
## 4 | DISCUSSION

In this study, we developed an exposomic approach to comprehensively detect metabolites of OP pesticides in human urine collected from the general population. Searches against 10 reference databases for pesticides resulted in the detection of benzoic acid and 2,4-dichlorophenol; however, such searches were theoretically considered to be of limited effectiveness for the purpose of comprehensive detection of OP exposure because the number of registered metabolites of major OPs is limited. Although these databases cover pesticides other than OPs, such as pyrethroid and neonicotinoid insecticides, herbicides, bactericides, and rodenticides, a new database for biomonitoring of pesticides is indispensable.

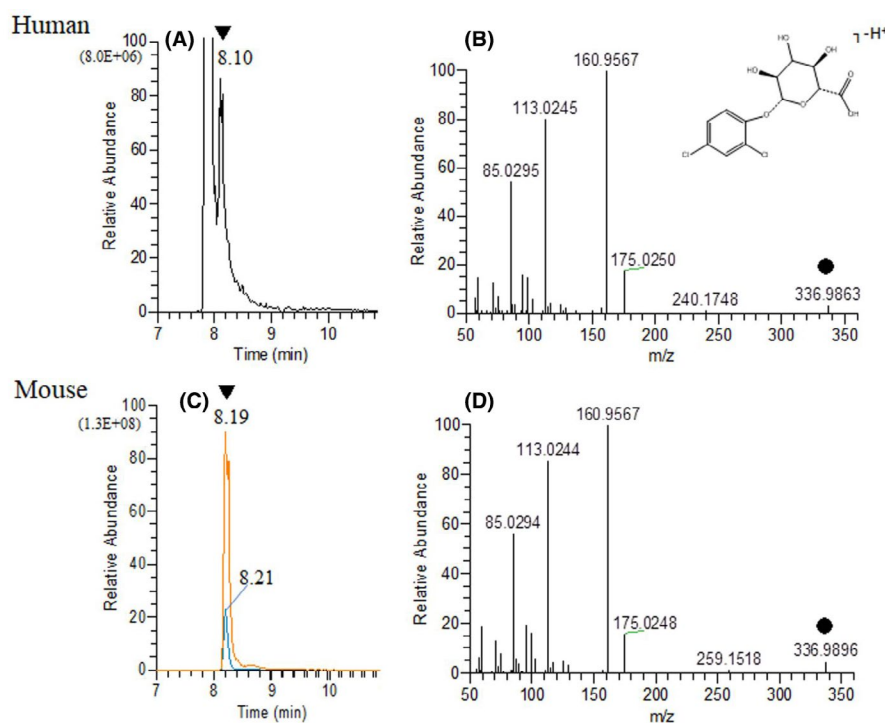
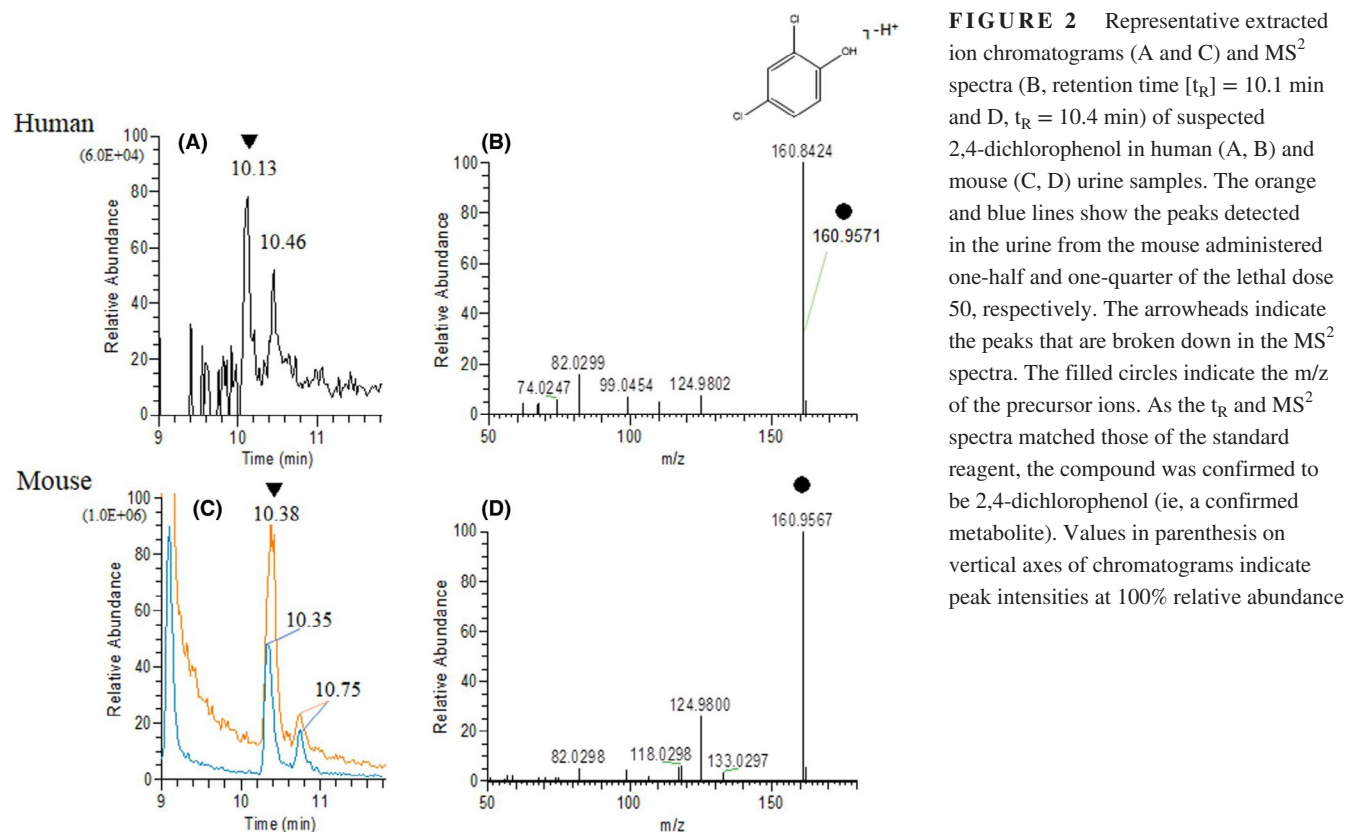
Using our original dataset, we succeeded in identifying three prothiofos metabolites, which we could not find except for 2,4-dichlorophenol in the existing reference databases and have not previously been a target of human biomonitoring. Hence, our developed approach for the creation of datasets from animal experiments, including exact mass,  $t_R$ , and  $MS^2$  spectra, should be effective in assessing human exposure not only to OPs but also to other chemicals for which reference standards are unavailable.

Similar to the present study, Jamin et al<sup>10</sup> orally administered pesticides to rats to create a comparison dataset. They attempted a three-step structure confirmation of hypothesized metabolites, that is, matching of the exact mass and isotopic pattern, observation of characteristic  $MS^n$  fragmentation patterns of hypothesized Phase II conjugated metabolites, and confirmation of the authentic standard. Although none of the standards for metabolites putatively characterized were available except for a fenitrothion metabolite, they showed the application of the characterized data to the statistical data analysis for an exposomic epidemiological study. In the

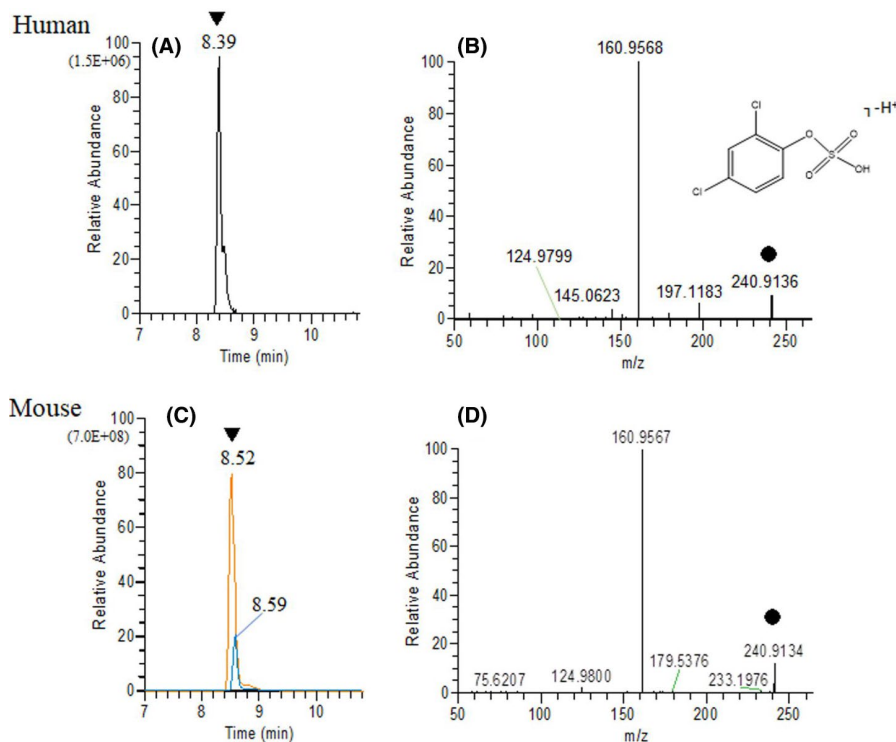
TABLE 2 Peaks detected in human urine

Pesticide	Chemical structure	Expected metabolite	Formula	Ionization mode	$t_R^a$ (min)		Exact mass		Identification level	
					Mouse	Human	Predicted	Mouse		Human
Cadusafos		2-methylsulfonylbutane <sup>b</sup>	$C_5H_{12}O_2S$	ESI+	3.3	3.1-3.3	136.05580	136.05588	136.05574-136.05606	C
Prothiofos		3,4,5-trihydroxy-6-(2,4-dichlorophenoxy) oxane-2-carboxylic acid 2,4-dichlorophenol (2,4-dichlorophenyl) hydrogen sulfate	$C_{12}H_{12}Cl_2O_7$ $C_6H_4Cl_2O$ $C_6H_4Cl_2O_4S$	ESI- ESI- ESI-	8.2 10.4 8.5	8.1 10.2-10.3 8.4-8.5	337.99601	337.99683	337.99715-337.99730	Confirmed Confirmed Confirmed

<sup>a</sup> $t_R$ , retention time.<sup>b</sup>The  $t_R$  and MS<sup>2</sup> spectra obtained from mouse and human samples did not match their respective data from the standard reagent.







**FIGURE 4** Representative extracted ion chromatograms (A and C) and MS<sup>2</sup> spectra (B, retention time [ $t_R$ ] = 8.4 min and D,  $t_R$  = 8.5 min) of suspected (2,4-dichlorophenyl) hydrogen sulfate in human (A, B) and mouse (C, D) urine. The orange and blue lines show the peak detected in the urine from the mouse administered a half and a quarter of the lethal dose 50, respectively. The arrowheads indicate the peaks that are broken down in the MS<sup>2</sup> spectra. The filled circles indicate the  $m/z$  of the precursor ions. As the  $t_R$  and MS<sup>2</sup> spectra matched those of the standard reagent, the compound was confirmed to be (2,4-dichlorophenyl) hydrogen sulfate (ie, a confirmed metabolite). Values in parenthesis on vertical axes of chromatograms indicate peak intensities at 100% relative abundance

present study, three metabolites of prothiofos were detected in both human and mouse urine at high identification levels (confirmed metabolites).

Prothiofos is widely used in Japan, with 64 tons used in 2018 (National Institute for Environmental Studies, Japan).<sup>32</sup> In Japan, it is applied to various crops, including soybean, potato, and onion.<sup>33</sup> We used a questionnaire to survey meals taken during the 2 days preceding sampling, which showed that most participants had eaten foodstuffs for which prothiofos use was approved (data not shown; this information was blinded to the measurer of the urinary metabolites).

As prothiofos metabolites were synthesized as required by our study, most of the peaks could not be compared with information obtained from standard reagents, because they were not commercially available. Similar to the present study, López et al<sup>11</sup> verified the detection of urinary metabolites in humans only for the OP metabolites for which standard reagents were available.

Currently, as there are limited data on pesticide metabolites in the existing reference databases, it is not practical to use them for the detection of pesticide metabolites in human urine. To resolve this, we orally administered dosages of two OP pesticides to mice, thereby creating an original metabolite dataset of these two OP pesticides for comparison and

increased the credibility of the peak compounds as metabolites by confirming the dose-dependency of the peaks. One may argue that the metabolites detected in human urine were not necessarily detected in pesticide-treated mice because of species differences in enzyme abundance and activity, such as carboxylesterase and cytochrome P450.<sup>34,35</sup> However, from an ethical perspective, it is practically impossible to administer sufficient concentrations of OP pesticides to humans or to primates that might minimize kinetic differences with humans. Nonetheless, we believe that the present *in vivo* generation system of the metabolites should work not only for cadusafos and prothiofos, but also for other environmental chemicals, as high-dose administration would produce the minimum necessary amount of the relevant metabolites. This trial warrants continuation, and we plan to extend the created dataset to cover all the major OP pesticides used in Japan.

There are some limitations to the present approach. First, DAPs were not detected in the present study samples. Exposomic untargeted approaches can only measure chemicals that are isolated in extraction processes.<sup>8</sup> In addition, untargeted analyses of xenobiotics presenting at low levels are significant challenges. Therefore, hybrid approaches, that is, the use of both targeted and untargeted approaches, are recommended for exposomic biomonitoring.<sup>8</sup> Second, structural

characterization through in vivo generation of metabolites and the following standard confirmation does not necessarily result in the identification of their parent compounds. In the present study, the confirmed prothiofos metabolites, other than 3,4,5-trihydroxy-6-(2,4-dichlorophenoxy) oxane-2-carboxylic acid, were also reported to be the metabolites of 2-(2,4-dichlorophenoxy) acetic acid (2,4-D), a phenoxy herbicide.<sup>36-38</sup> Although the possibility that the parent compound was 2,4-D was less likely (see Supporting Information for this confirmation), another possibility is that the parent was 2,4-dichlorophenol. The World Health Organization reported the detection of 2,4-dichlorophenol from tap water in the chlorination process.<sup>39</sup> Likewise, the possibility that the detected metabolites were derived from other parent compounds or have the same structures as the intermediate metabolites of the chemicals of interest cannot be ruled out in some cases.

In conclusion, we successfully identified urinary compounds suggesting the exposure to prothiofos, which has not previously been a target pesticide in biomonitoring studies in humans, by developing a comprehensive analysis method using an original extensive dataset produced through animal experiments. Although authentic analytical standards are not required for the discovery of new and relevant exposure biomarkers, and are necessary only when a new biomarker is identified and needs to be validated in exposomic epidemiological studies,<sup>8</sup> our approach is promising as being applicable not only to other OP pesticides, but also to various environmental contaminants.

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## DISCLOSURES

**Approval of the research protocol:** This study was approved by the Ethics Committee of the Nagoya City University Graduate School of Medical Sciences (approval no. 60-18-0021). The present study was conducted in accordance with the Declaration of Helsinki and nationally valid regulations. **Informed consent:** Written informed consent was obtained from all participants in this study. **Registry and the registration no. of the study/trial:** N/A. **Animal studies:** This study was conducted in accordance with Japanese law concerning the protection and control of animals and the guidelines of Animal Care and Use in Nagoya City University Graduate

School of Medical Sciences (approval No. H29M-37) for laboratory animal use and care. **Conflict of interest:** The authors declare no conflict of interest.


## AUTHOR CONTRIBUTIONS

KN and NO investigated and analyzed the data and wrote the first draft of the manuscript. TN and NCM supported this investigation. TT and MT validated and supervised the data. YI, HS, and MK conceptualized this study, acquired funding, and supervised the study. All the authors interpreted the data, contributed to the revised manuscript, and agreed with the final version and the findings.

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## REFERENCES

1. Wild CP. Complementing the genome with an “exposome”: the outstanding challenge of environmental exposure measurement in molecular epidemiology. *Cancer Epidemiol Biomarkers Prev.* 2005;14(8):1847-1850.
2. Newton SR, McMahan RL, Sobus JR, et al. Suspect screening and non-targeted analysis of drinking water using point-of-use filters. *Environ Pollut.* 2018;234:297-306.
3. Phillips KA, Yau A, Favela KA, et al. Suspect screening analysis of chemicals in consumer products. *Environ Sci Technol.* 2018;52(5):3125-3135.
4. Cernansky R. A blend of old and new: biomonitoring methods to study the exposome. *Environ Health Perspect.* 2017;125(4):A74.
5. Donauer S, Altaye M, Xu Y, et al. An observational study to evaluate associations between low-level gestational exposure to organophosphate pesticides and cognition during early childhood. *Am J Epidemiol.* 2016;184(5):410-418.
6. Khoury C, Werry K, Haines D, Walker M, Malowany M. Human biomonitoring reference values for some non-persistent chemicals in blood and urine derived from the Canadian Health Measures Survey 2009–2013. *Int J Hyg Environ Health.* 2018;221(4):684-696.
7. Oya N, Ito Y, Ebara T, et al. Exposure levels of organophosphate pesticides in Japanese diapered children: Contributions of exposure-related behaviors and mothers' considerations of food selection and preparation. *Environ Int.* 2020;134:105294.
8. Dennis KK, Marder E, Balshaw DM, et al. Biomonitoring in the era of the exposome. *Environ Health Perspect.* 2017;125(4):502-510.
9. Tautenhahn R, Cho K, Uritboonthai W, Zhu Z, Patti GJ, Siuzdak G. An accelerated workflow for untargeted metabolomics using the METLIN database. *Nat Biotechnol.* 2012;30(9):826-828.

10. Jamin EL, Bonvallot N, Tremblay-Franco M, et al. Untargeted profiling of pesticide metabolites by LC-HRMS: an exposomics tool for human exposure evaluation. *Anal Bioanal Chem*. 2014;406(4):1149-1161.
11. López A, Dualde P, Yusà V, Coscollà C. Retrospective analysis of pesticide metabolites in urine using liquid chromatography coupled to high-resolution mass spectrometry. *Talanta*. 2016;160:547-555.
12. Rohlman DS, Anger WK, Lein PJ. Correlating neurobehavioral performance with biomarkers of organophosphorous pesticide exposure. *Neurotoxicology*. 2011;32(2):268-276.
13. Koureas M, Tsakalof A, Tsatsakis A, Hadjichristodoulou C. Systematic review of biomonitoring studies to determine the association between exposure to organophosphorus and pyrethroid insecticides and human health outcomes. *Toxicol Lett*. 2012;210(2):155-168.
14. Roca M, Leon N, Pastor A, Yusà V. Comprehensive analytical strategy for biomonitoring of pesticides in urine by liquid chromatography-orbitrap high resolution mass spectrometry. *J Chromatogr A*. 2014;1374:66-76.
15. United States Environmental Protection Agency. EPA: Pesticide Chemical Search Database. [https://comptox.epa.gov/dashboard/chemical\\_lists/EPAPCS](https://comptox.epa.gov/dashboard/chemical_lists/EPAPCS). Accessed October 2, 2020.
16. United States Environmental Protection Agency. NORMAN: Swiss Pesticides and Metabolites from Keifer et al. 2019. [https://comptox.epa.gov/dashboard/chemical\\_lists/SWISSPEST19](https://comptox.epa.gov/dashboard/chemical_lists/SWISSPEST19). Accessed October 2, 2020.
17. Kiefer K, Müller A, Singer H, Hollender J. New relevant pesticide transformation products in groundwater detected using target and suspect screening for agricultural and urban micropollutants with LC-HRMS. *Water Res*. 2019;165:114972.
18. United States Environmental Protection Agency. Pesticide Screening List for Luxembourg. [https://comptox.epa.gov/dashboard/chemical\\_lists/LUXPEST](https://comptox.epa.gov/dashboard/chemical_lists/LUXPEST). Accessed October 2, 2020.
19. United States Environmental Protection Agency. Natural Product Insecticides. [https://comptox.epa.gov/dashboard/chemical\\_lists/NPINSECT](https://comptox.epa.gov/dashboard/chemical_lists/NPINSECT). Accessed October 2, 2020.
20. United States Environmental Protection Agency. CATEGORY Office of Pesticide Programs Information Network. [https://comptox.epa.gov/dashboard/chemical\\_lists/OPPIN](https://comptox.epa.gov/dashboard/chemical_lists/OPPIN). Accessed October 2, 2020.
21. United States Environmental Protection Agency. PESTACTIVES. [https://comptox.epa.gov/dashboard/chemical\\_lists/PESTACTIVES](https://comptox.epa.gov/dashboard/chemical_lists/PESTACTIVES). Accessed October 2, 2020.
22. United States Environmental Protection Agency. EPA: List of Inert Ingredients Food and Nonfood Use. Updated 10/25/2019. [https://comptox.epa.gov/dashboard/chemical\\_lists/PESTINERTS](https://comptox.epa.gov/dashboard/chemical_lists/PESTINERTS). Accessed October 2, 2020.
23. United States Environmental Protection Agency. PPDB: Pesticide Properties DataBase. [https://comptox.epa.gov/dashboard/chemical\\_lists/PPDB](https://comptox.epa.gov/dashboard/chemical_lists/PPDB). Accessed October 2, 2020.
24. United States Environmental Protection Agency. Swiss Pesticides and Transformation Products. [https://comptox.epa.gov/dashboard/chemical\\_lists/SWISSPEST](https://comptox.epa.gov/dashboard/chemical_lists/SWISSPEST). Accessed October 2, 2020.
25. United States Environmental Protection Agency. TOXCAST\_PhaseI - EPA ToxCast Screening Library (Phase I subset). [https://comptox.epa.gov/dashboard/chemical\\_lists/TOXCAST\\_PHASEI](https://comptox.epa.gov/dashboard/chemical_lists/TOXCAST_PHASEI). Accessed October 2, 2020.
26. Food Safety Commission of Japan. Pesticides evaluation report of cadusafos. <https://www.fsc.go.jp/hyouka/hy/hy-hyouka-170629-cadusafos.pdf>. Accessed October 5, 2020. [in Japanese].
27. Food Safety Commission of Japan. Pesticides evaluation report of prothiofos. [https://www.fsc.go.jp/iken-bosyu/iken-kekka/kekka.data/pc5\\_no\\_prothiofos\\_300725.pdf](https://www.fsc.go.jp/iken-bosyu/iken-kekka/kekka.data/pc5_no_prothiofos_300725.pdf). Accessed October 5, 2020. [in Japanese].
28. Donovan W. Metabolic Profile of Cadusafos in Plants and Rats. [http://www.fao.org/fileadmin/templates/agphome/documents/Pests\\_Pesticides/JMPR/Evaluation10/Cadusafos.pdf](http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation10/Cadusafos.pdf). Accessed November 24, 2020.
29. Roberts LC, Roberts TR, Hutson DW, et al. *Metabolic Pathways of Agrochemicals: Part 2: Insecticides and Fungicides*. London: The Royal Society of Chemistry; 1999.
30. Sumner LW, Amberg A, Barrett D, et al. Proposed minimum reporting standards for chemical analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics*. 2007;3(3):211-221.
31. Schymanski EL, Jeon J, Gulde R, et al. Identifying small molecules via high resolution mass spectrometry: communicating confidence. *Environ Sci Technol*. 2014;48(4):2097-2098.
32. National Institute for Environmental Studies. Database of chemical compounds Webkis-Plus. <https://www.nies.go.jp/kisplus/>. Accessed February 16, 2021. [in Japanese].
33. The Health Labor and Welfare Ministry Japan. Prothiofos. <https://www.mhlw.go.jp/content/11120000/000532911.pdf>. Accessed September 9, 2020. [in Japanese].
34. Martignoni M, Groothuis GM, de Kanter R. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opin Drug Metab Toxicol*. 2006;2(6):875-894.
35. Bahar FG, Ohura K, Ogihara T, Imai T. Species difference of esterase expression and hydrolase activity in plasma. *J Pharm Sci*. 2012;101(10):3979-3988.
36. Ministry of the Environment Japan. 2,4-dichlorophenol. <https://www.env.go.jp/chemi/report/h22-01/pdf/chpt1/1-2-2-04.pdf>. Accessed February 9, 2021. [in Japanese].
37. van Ravenzwaay B, Hardwick TD, Needham D, Pethen S, Lappin GJ. Comparative metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) in rat and dog. *Xenobiotica*. 2003;33(8):805-821.
38. United States Environmental Protection Agency. 2,4-D Human Health Risk Assessment for. Registration Review. 2016. [https://www.24d.org/Studies/PDF/24D\\_EPA\\_Human\\_Health\\_Risk\\_Assmnt\\_2017.pdf](https://www.24d.org/Studies/PDF/24D_EPA_Human_Health_Risk_Assmnt_2017.pdf). Accessed September 9, 2020.
39. World Health Organization. Chlorophenols in Drinking-water. [https://www.who.int/water\\_sanitation\\_health/dwq/chemicals/chlorophenols.pdf](https://www.who.int/water_sanitation_health/dwq/chemicals/chlorophenols.pdf). Accessed February 9, 2021.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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