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Pharmacokinetics, Metabolite Measurement, and Biomarker Identification of Dermal Exposure to Permethrin Using Accelerator Mass Spectrometry

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The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

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ABSTRACT

Impregnating military uniforms and outdoor clothing with the insecticide permethrin is an approach to reduce exposure to insect borne diseases and to repel pests and disease vectors such as mosquitos and sandflies, but the practice exposes wearers to prolonged dermal exposure to the pesticide. Key metabolite(s) from a low dose dermal exposure of permethrin were identified using accelerator mass spectrometry. Metabolite standards were synthesized and a high performance liquide chromatography (HPLC) elution protocol to separate individual metabolites in urine was developed. Six human subjects were exposed dermally on the forearm to 25 mg of permethrin containing 1.0 μCi of ^{14}C for 8 h. Blood, saliva and urine samples were taken for 7d. Absorption/elimination rates and metabolite concentrations varied by individual. Average absorption was 0.2% of the dose. Serum concentrations rose until 12–24 h postdermal application then rapidly declined reaching predose levels by 72 h. Maximum saliva excretion occurred 6 h postdosing. The maximum urinary excretion rate occurred during 12–24 h; average elimination half-life was 56 h. 3-Phenoxybenzyl alcohol glucuronide was the most abundant metabolite identified when analyzing elution fractions, but most of the radioactivity was in still more polar fractions suggesting extensive degradative metabolism and for which there were no standards. Analyses of archived urine samples with the ultra performance liquid chromatography-accelerator mass spectrometry-mass spectrometry (UPLC-AMS-MS) system isolated a distinct polar metabolite but it was much diminished from the previous analyses a decade earlier.

Key words: permethrin; dermal exposure; ADME; pharmacokinetics.

Synthetic pyrethroids are among the most widely used insecticides in the United States and worldwide due to their effectiveness in controlling and repelling insect pests and other arthropod vectors of disease and relatively low mammalian toxicity of pyrethroids (Casida and Durkin, 2013; Casida and Quistad, 1998). As residential use of organophosphates declined due to neurotoxicity concerns, synthetic pyrethroids replaced them in many home and garden applications (Casida and Bryant, 2017; Casida

and Durkin, 2013). Among synthetic pyrethroids, permethrin is widely used in commercial agriculture, consumer home and garden products, home termite treatment and prevention, indoor aerosol bombs and foggers, over-the-counter lice treatments, insect repellent on mosquito nets in regions with malaria, and insect repellent in consumer outdoor clothing and military uniforms (Casida and Durkin, 2013; Chrustek *et al.*,2018). Occupational exposure to permethrin through oral, inhalation, and dermal routes is widespread among agricultural workers (Barr *et al.*, 2010; Ferland *et al.*, 2015; Maule *et al.*, 2019; Llewellyn *et al.*, 1996; Rossbach *et al.*, 2014; Trunnelle *et al.*, 2014) and flight attendants (Wei *et al.*, 2013). The dominant exposure route to the general population is probably pesticide residue on fruits and vegetables (Darney *et al.*, 2018; Juraske *et al.*, 2009; Morgan 2012). People who wear permethrin treated clothes to repel insects during outdoor activities such as hiking, fishing, golf, or deployed military experience prolonged or chronic dermal exposures.

Although pyrethroids possess lower mammalian toxicity than most other classes of insecticides, they still pose health risks for people routinely exposed to them. Permethrin disrupts voltage-gated sodium ion channels, crosses the blood-brain barrier (Bradberry *et al.*, 2005; Mortuza *et al.*, 2019; Soderlund, 2012) and retains neurotoxicity in mammals (Soderlund, 2012). Permethrin exposures have also been shown to suppress the immune system (Joshi *et al.*, 2019; Repetto and Baliga, 1997) and trigger developmental toxicity (Mortuza *et al.*, 2019; Shafer *et al.*, 2005; Shelton *et al.*, 2014). Additionally, there are reports of exposure to permethrin causing endocrine disruption in animal models (Brander *et al.*, 2016; Leemans *et al.*, 2019; Tu *et al.*, 2016).

Permethrin readily enters circulation when ingested orally or in dermal contact, gets metabolized and/or conjugated in the liver, and is excreted mostly in urine (Ahn *et al.*, 2006, 2007; Chrustek *et al.*, 2018; Ratelle *et al.*, 2015; Tomalik-Scharte *et al.*, 2005; Wei *et al.*, 2013). Absorption across the skin is attenuated and slow compared with ingestion (Côté *et al.*, 2014). At least 60–70% of orally ingested permethrin enters circulation while topical application results in <2% of the applied dose being absorbed (National Research Council, 1994; Tomalik-Scharte *et al.*, 2005). The relatively low level of dermal absorption and desire to examine saliva and urine for metabolites produced from an occupationally and environmentally relevant dermal exposure to permethrin required the use of a highly sensitive analytical method. Accelerator mass spectrometry (AMS) is ideally suited to trace low level xenobiotics in humans to complete absorption, distribution, metabolism, and excretion (ADME) studies (Buchholz *et al.*, 1999b; Harrell *et al.*, 2019; Madeen *et al.*, 2016; Spracklin *et al.*, 2020). The extraordinarily high sensitivity of AMS for ^{14}C labeled materials is based on its detection method of counting individual atoms of the isotope as the free carbon rather than waiting for radioactive decay. AMS precisely quantifies the elevation in $^{14}C/C$ above the low natural background abundance in the biosphere ($^{14}C/C = 1.2 \times 10^{-12}$) and is approximately 100 000× more sensitive than liquid scintillation counting (Gilman *et al.*, 1998; Vogel and Love, 2005). AMS was utilized to examine the pharmacokinetics of an occupational or environmental dermal exposure to permethrin. The goals of this human dermal permethrin exposure study were to determine the optimal time frame for analyses, to assess the suitability of noninvasively acquired urine and saliva samples for biomarker identification, and to determine biomarkers of dermal exposure amenable to conventional immunoassay measurement.

MATERIALS AND METHODS

Human Subjects

Informed consent was obtained from all human subjects according to the Declaration of Helsinki. The human subject protocol was reviewed and approved by the Internal Review Boards (IRB) of University of California Davis, Lawrence Livermore National Laboratory, the U.S. Department of Defense, the California Department of Pesticide Regulation, and the Veteran's Administration at Sacramento, California and is registered as Metabolism of the insecticide permethrin on ClinicalTrials.gov with Identifier: NCT00572884. Subjects recruited for this study were healthy (self-report) adult men and premenopausal women. Individuals reported that they did not consume alcohol beyond a rare drink during a social event, use cigarettes, or drugs, or maintained any unusual exercise or dietary habits for the last 3 years. They were not under the care of a physician for a disease and had not been subjects in a radioactive drug research study. Women reported that they were not pregnant or breastfeeding and a urine pregnancy test was conducted just prior to beginning the study. No one reported any use of permethrin in the previous 6 months from common home pesticide products or to their knowledge, commercial application to their residence, nor did any of the subjects report that they were pest control operators. Six subjects were recruited to participate. Some generalized demographics are shown in Table 1.

Exposure. Prior to exposure, the treatment area on the forearm was cleaned with an alcohol wipe to remove any lotions or surface skin oils. Each subject was administered dermally, 25 mg of permethrin containing 1.0 μCi of ^{14}C. The ^{14}C mass was just 2 pg. Subjects 10, 12, 14, and 20 received a dosing solution consisting of an equal mixture of permethrin that was labeled in the cyclopropane ring or the phenoxybenzyl ring (see Figure 1). Subject 16 received permethrin labeled only in the phenoxybenzyl ring only and Subject 18 received permethrin labeled only in the cyclopropane ring. The dosing solution was prepared in isopropyl alcohol and applied onto an approximately 50-cm^2 area of 1 forearm. The treated area was monitored with a pancake probe Geiger counter then covered with gauze and remained in place for 8 h. At the end of 8 h, the area was swabbed several times with soap- and then water-saturated gauzes to remove surface material. Between washes, the area was monitored with a pancake probe Geiger counter until the count rate did not change. The Geiger counter provided a crude estimate of the counts applied and remaining as the probe was not large enough to cover the entire treated area at once. The probe was moved over the surface and count rates recorded at 3 different parts of the treatment area. The count method was relatively crude and could not accurately determine the amount absorbed. The amount of activity measured on the skin and in the gauze cover combined accounted for about one-half of the activity, but the wet soapy gauze could not be accurately measured with a Geiger counter due the low energy of the emitted ^{14}C β-particle. The gauze absorbed permethrin and soapy water and could not be quantitatively extracted to measure recovered permethrin. Tape stripping the skin repeatedly would have likely recovered more permethrin, but measurement of ^{14}C in adhesive is difficult and tape stripping also adversely damages the integrity of the skin. During IRB review, however, it was determined that maintaining the protective layers of the skin was more important than removing residual permethrin. Total ^{14}C in urine determined by AMS indicates that the absorbed dose was closer to 0.2% of the

Table 1. Subject Demographics

Gender	2 male (subjects 10 and 14), 4 female (subjects 12, 16, 18, and 20)
Race	3 White (2 male and 1 female), 2 Hispanic (2 female), 1 African American/Asian (female)
Age (average)	35 years (range 22–48 years)
Weight (average)	87 kg (range 69.5–118.2 kg)
Height (average)	172 cm (range 163–180 cm)
BMI	29.2*a* (range 23.4–39.5)

[a]BMIs of 18.5–24.9 are classed as normal; BMIs of 25–29.9 are classes as overweight (BMI Calculator: http://www.nhlbisupport.com/bmi/bmicalc.htm).

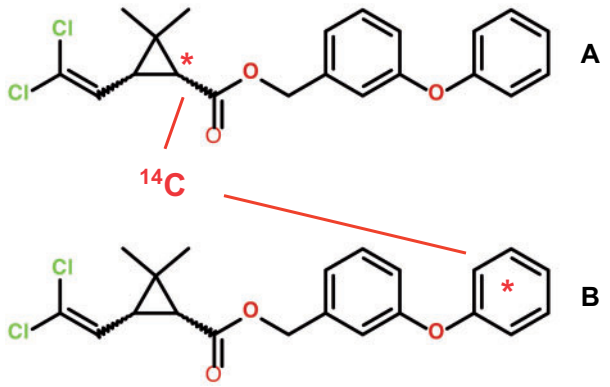


Figure 1. *cis-/trans*-Permethrin with ^{14}C label (*) in the (A) cyclopropane-4-^{14}C ring or (B) [phenoxy-U-^{14}C]benzyl ring.

applied dose (described below). No adverse effects were reported during the 1-week period of the study.

Sample collection. A 24-h urine sample was collected from each subject the day before treatment. On treatment day, a predose blood and saliva sample were taken, and then the radiolabeled permethrin was applied to the forearm and covered with a gauze patch. Blood (sampled from the untreated arm), and saliva samples were taken at 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, and 168 h postapplication. Urine samples were collected from 0–6, 6–12, 12–24, 24–48, 48–72, 72–96, 96–120, 120–144, and 144–168 h postapplication. Blood samples were centrifuged to separate the serum. The serum was removed to a separate tube and both fractions were stored at −80°C until analysis. The saliva collection device was centrifuged to extract the saliva from the cotton collection swab. The separated saliva was then stored at −80°C until analysis. The volume of the saliva and urine samples at each time point was recorded. Five 50-ml aliquots of urine were taken and stored at −80°C until analysis. The remainder of the urine was discarded. Saliva, urine and serum samples were assessed for ^{14}C activity by liquid scintillation counting before processing for AMS (Buchholz *et al.*, 2000). Because of the low amount of radioactivity administered and the even lower amount absorbed, as expected, there were no counts detected above background with a background of 11.4 cpm on the full ^{14}C window (4–156 keV).

Analyze blood, saliva, and urine: Carbon analysis. The graphite preparation method used for these samples (Ognibene *et al.*, 2003) works best when samples contain 0.5–1.0 mg of total carbon. Aliquots of saliva and urine samples were submitted for carbon analysis. For measurement, volume of 5.0 µl was added to a silica support (Chromsorb W/AW 80% 100 mesh; Carlo Erba/Fisons Instruments, Valencia, California) and analyzed using a Carlo Erba model 1500 NCS (Valencia, California) elemental analyzer (Gilman *et al.*, 1998). Extensive experience has shown that the carbon content of plasma samples is consistent and averages 4.2% so serum samples were not analyzed for carbon content (Buchholz *et al.*, 1999a; Vogel and Love, 2005).

Tissue AMS analyses. Based on the carbon content, aliquots of each sample were prepared for AMS measurement. Tissue sample volumes were adjusted to include approximately1 mg carbon and processed using standard procedures (Buchholz *et al.*, 1999a; Ognibene *et al.*, 2003). Graphite samples were packed into aluminum sample holders, and carbon isotope ratios were measured on the 1 MV National Electrostatics Corporation (Middleton, Wisconsin) AMS system at LLNL On this system, typical AMS measurement times were 3 min/sample, with a counting precision of 1.4–2.0% and a SD among 3–7 measurements of 1–3% (Zhao *et al.*, 2014).

HPLC method to separate permethrin metabolites. A high performance liquid chromatography (HPLC) separation method was developed for both the separation of metabolites prior to analysis of samples by AMS. Since the Liquid chromatography-mass spectrometry (LC/MS) required a microbore LC to keep the volumes very low, we chose to use these conditions for the urine analysis by AMS as well. The initial optimized system used a Magic 2002 microbore HPLC (Michrom BioResources, Inc., Folsom, California) equipped with an Onyx monolithic C_{18} (5 × 4.6 mm) precolumn and an Onyx monolithic C_{18} (3.0 × 100, 2 µ particle size, 130 Å pore size) column in tandem with a Magic C_{18} (1.0 × 150 mm, 3 µ particle size, 100 Å pore size) column. The mobile phase was a gradient mixture of double distilled water adjusted with trifluoroacetic acid (TFA) to pH 2.2 (Fraction Solvent A) and 90% acetonitrile in water (v/v) adjusted to pH 2.2 with TFA (Fraction Solvent B) at a flow rate of 50 µl/min at room temperature. The gradient is detailed in Figure 2A. Standards were prepared in 4% acetonitrile in 0.1 M sodium phosphate, pH 7.5 and were detected with a UV/visible detector at either 230 or 210 nm. The urine samples were prepared by mixing 40 µl of the standard mixture with 960 µl of filtered urine. An aliquot of 50 µl was injected onto the column for analysis. Figure 3 is an example UV chromatogram for a urine sample collected between 0 and 6 h during dosing that was spiked with standards (Table 2). The standards are baseline resolved and there is good resolution between *cis-* and *trans*-isomers of dichlorovinylchrysanthemic acids (DCVA, peaks 5 and 7), DCVA-glycine (peaks 2 and 3), and permethrin (peaks 9 and 10). Peaks are well separated so that fractions collected for AMS should yield distinct peaks. We have found previously that although UV resolution of the peaks was at baseline, resolution by AMS showed that the peaks were much broader (Buchholz *et al.*, 1999b).

AMS analyses of HPLC fractions. For AMS analyses, we collected 2 min elution fractions from 40 to 180 min, but collected 5 min fractions in the first 40 min as none of our standards eluted in that period of time. Carbon carrier, 1.2 mg of glycerol tributyrate (ICN Pharmaceuticals, Inc. Costa Mesa, California) was added to each HPLC fraction to produce individual graphite samples for AMS analyses (Buchholz *et al.*,1999b). Graphite samples were again packed into aluminum sample holders, and carbon isotope ratios were measured on the 1 MV National Electrostatics Corporation AMS system at LLNL. Typical AMS measurement

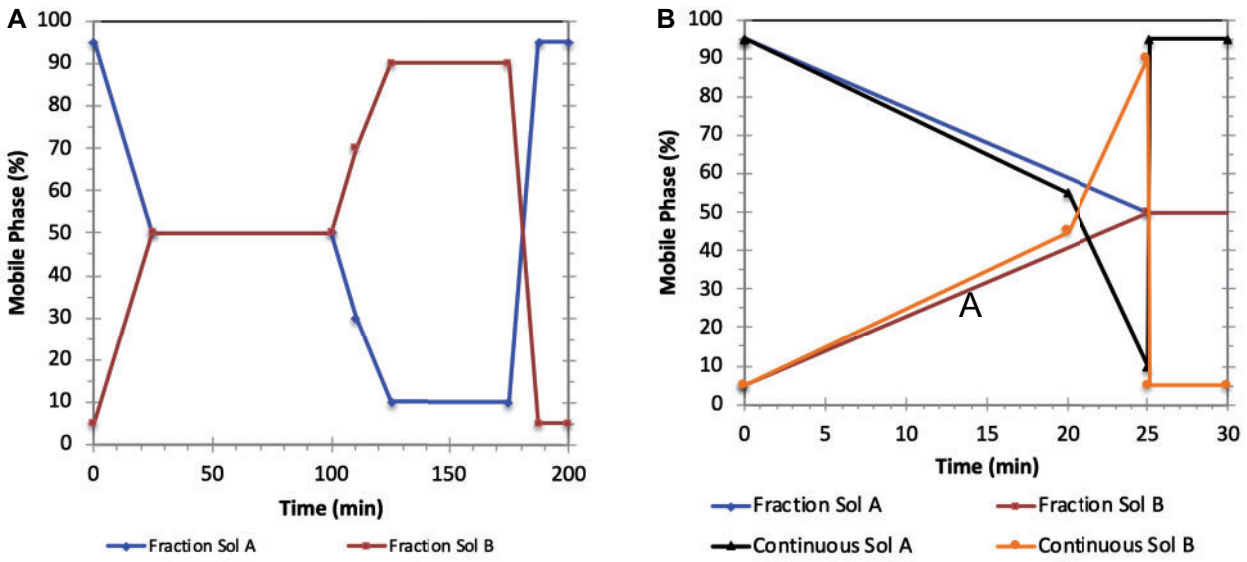


Figure 2. HPLC elution conditions for permethrin metabolite separation. A, Elution conditions for collection of fractions for conventional individual graphite accelerator mass spectrometry (AMS) samples. B, Elution conditions for the continuous moving wire liquid sample interface of the UPLC-MS-AMS system compared with the corresponding times of the fraction collection procedure.

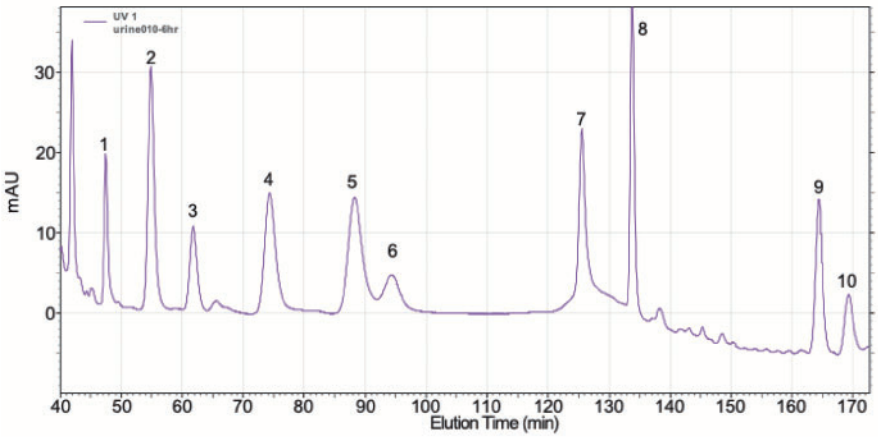


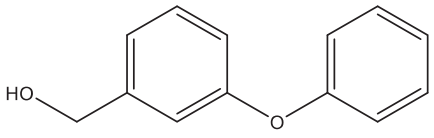
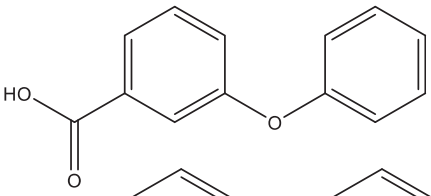
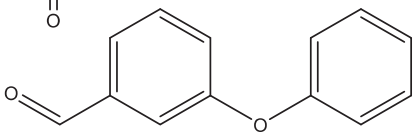
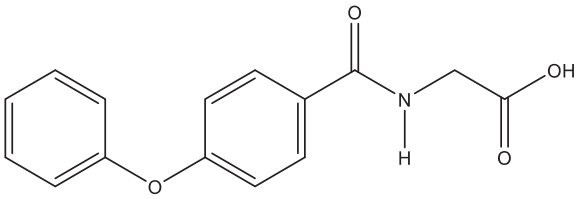
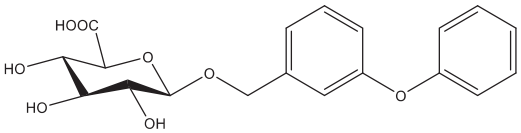
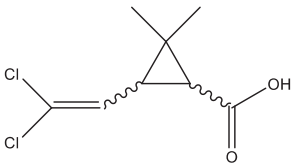
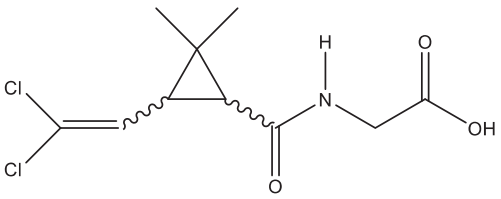
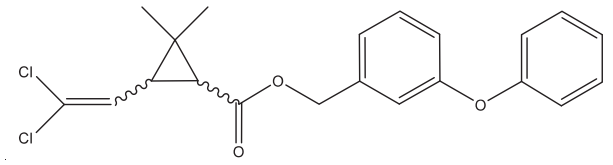
Figure 3. HPLC separation of the metabolites of permethrin in a spiked urine sample. Peaks for each standard were: (1) Glucuronide conjugate of 3-phenoxybenzyl alcohol (3-PBAlc); (2) *trans*-DCVA-glycine/3-phenoxybenzoic acid (3PBA)-glycine; (3) *cis*-DCVA-glycine; (4) 3-PBAlc; (5) *trans*-DCVA; (6) 3PBA; (7) *cis*-DCVA; (8) 3-phenoxybenzaldehyde; (9) *trans*-permethrin; (10). *cis*-permethrin and were detected by UV absorbance.

times were 3–5 min/sample, with a counting precision of 1.4–3.0% and a standard deviation among 3–7 measurements of 1–4%.

Permethrin metabolite separation revisited. Large ^{14}C peaks early in the elution spectra corresponding to polar metabolites were not identified using fraction collection. No standards eluted before 48 min and as much as 75% of ^{14}C eluted earlier. Development of a liquid-sample continuous-flow ion source interface presented a new opportunity to reanalyze the archived urine samples. HPLC eluent is deposited directly upon a moving wire, dried, combusted to CO_2, and directly injected into a gas-accepting AMS ion source (Thomas *et al.*, 2011; Madeen *et al.*, 2016). Elution conditions were designed to mimic the initial 20 min of the 180 min protocol used previously since about 45% of the of the ^{14}C associated with unidentified permethrin metabolites eluted over that time (Figure 2B).

LC-MS measurements were made with a Waters Xevo G2-XS QTOF mass spectrometer (Waters Corporation, Milford, Massachusetts) coupled with a Waters Acquity H Class chromatography system. Permethrin and metabolites were separated on a Waters Acquity UPLC HSS C18 1.8 µm 2.1 × 100 mm column. Mobile phases were water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The separation method was as follows: initial, 5% B; 0.01 min, 5% B; 20 min, 45% B; 25 min, 90% B; 25.1 min, 5% B; and 30 min, 5% B. The flow rate was 400 µl/min and total analysis time was 30 min. Injection volume was 10 µl. The column compartment thermostat was set at 30°C. Detection was accomplished by MS with the electrospray ion source operating in negative ion, resolution mode. Data acquisition was performed using MassLynx Version 4.1 data acquisition software (Waters Corp.), with MS^e data-independent centroid acquisition and leucine enkephalin lockmass correction. The

Table 2. Structures of Permethrin Metabolites

Name	Structure
3-PBAlc	
3-Phenoxybenzoic acid	
3-Phenoxybenzaldehyde	
3-PBA-glycine	
Glucuronide conjugate of 3-PBAlc	
cis-/trans-Dichlorovinylchrysanthemic acid or Dichlorovinylcyclopropane carboxylic acid (DCVA)	
cis-/trans-DCVA glycine	
cis-/trans-permethrin	

adjustable flow splitter was set to deliver 100 μl/min to the AMS liquid sample interface and 300 μl/min to the QTOF mass spectrometer.

AMS analyses of HPLC continuous flow. All liquid-sample continuous-flow ion source AMS measurements utilized the National Electrostatics Corporation 250 kV Single Stage AMS with the

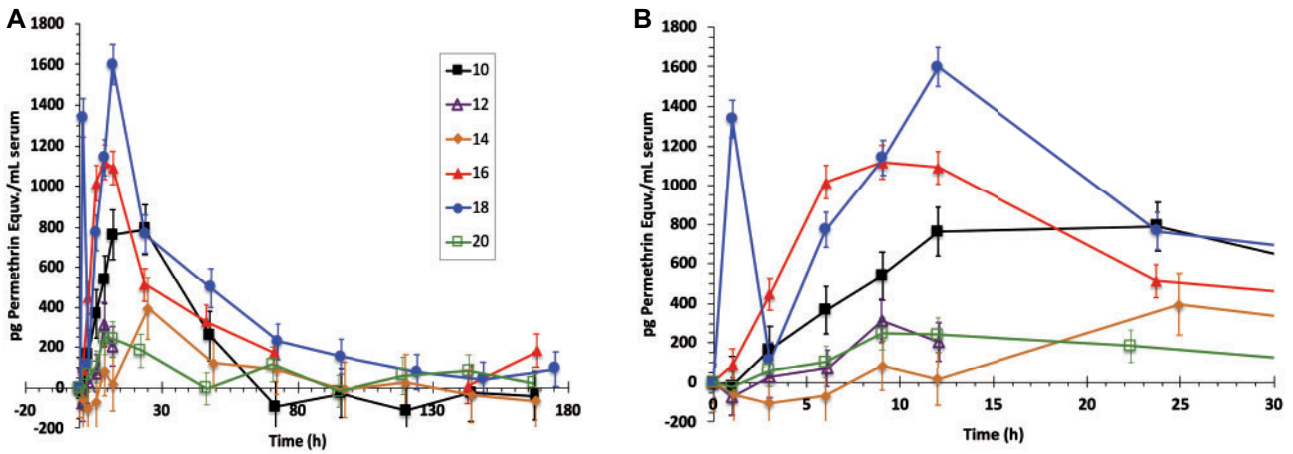


Figure 4. Time course of the absorption and elimination of permethrin in the serum. (A) full time course; (B) 0–24 h.

Table 3. Relationship of BMI to Peak Serum Levels of Permethrin

Subject	BMI	[Permethrin] at peak serum level (pg/ml serum)
010	27.9	814.9
012	29.8	464.8
014	39.5	765.2
016	23.4	1239.2
018	28.5	1765.4
020	26.3	483.9

moving wire interface system at the Center for AMS at LLNL (Ognibene *et al.*, 2019). General operating conditions were optimized for measurement of $^{14}C/C$ using solid graphite standards. Aqueous solutions of isotopic standards (IAEA C-6) were measured intermittently throughout the analysis to monitor the ionization and counting efficiency of the AMS.

Statistics. AMS isotope counting obeys Poisson statistics. All reported concentration uncertainties reflect the conversion of ^{14}C counts and counting uncertainty to permethrin equivalents using the specific activity of the dose material. Averages and standard deviations were calculated using Microsoft Excel software.

Results

The concentration of dosed permethrin in whole serum, saliva, and urine is expressed as permethrin equivalents (parent + metabolites)/ml since an elevated ^{14}C signal measured was due solely to the dosed permethrin as shown previously (Vogel and Love, 2005).

Serum

The concentration of circulating permethrin equivalents in serum are represented in Figure 4. The amount of permethrin absorbed from the skin varied widely by individual. Permethrin is a lipophilic compound and one might anticipate that absorption and distribution may be related to body fat stores. However, there is no apparent trend to the amount absorbed and the body mass index (BMI, a measure of obesity, Table 3). The highest absorption was seen for subjects 16 and 18 (both female), whereas absorption was more similar for subjects 10, 12, 14 and 20 (10 and 14 were males and 12 and 20 were females). The serum concentration of ^{14}C permethrin equivalents climbed steadily over 12 h (the treatment was removed at 8 h), leveling between 12 and 24 h (Figure 4), and then showing a rapid elimination phase. By 72 h, the blood concentrations of ^{14}C permethrin equivalents had returned to predose levels. Interestingly, one would anticipate that the predose levels of ^{14}C would be zero, but each subject showed some "basal" level of ^{14}C above anticipated background for an unexposed subject. One cannot presume that these predose sample values were spurious because the 7-day values for each subject were similar to the respective predose values.

Saliva

Saliva samples were taken to determine if permethrin is excreted in the saliva, and if so, to assess saliva as a potential biomonitoring fluid for permethrin since it can be obtained noninvasively. The excreted ^{14}C permethrin equivalents in the saliva vary widely among subjects (Figure 5). Although the trends are not as obvious as with serum or urine, there is a distinct increase following dosing. The peak excretion time varies by individual and occurs between 6 and 24 h. Subjects 16 and 18 show the highest absorbed concentrations (Figure 4). Subject 18 also shows the highest saliva elimination. The correlation cannot be made with subject 16 as critical samples are missing between 9 and 48 h (trend line is indicated only for the collected samples). Subjects were allowed to eat and drink at their discretion during the study. The amount of ^{14}C label found in saliva may be more related to the abundance of saliva (*ie*, those subjects that ate or drank more, excreted more saliva, and consequently, more ^{14}C label may appear in the samples), relative to the time that the samples were taken, but this cannot be verified. For subjects 14 and 18, we know that the nonocclusive cover came loose sometime in the 8-h dosing period. Therefore, it is also possible that the high levels seen for these 2 subjects is related to hand-to-mouth activity, where they attempted to adjust the cover, contaminating a hand that was later used for eating.

Urine

The total amount of ^{14}C-permethrin equivalents excreted in urine at each time point is shown in Figure 6A for 5 subjects who completed multiple days of the study. The higher levels of permethrin that was excreted correlates loosely to the levels absorbed for each individual, with the exception of subject 16

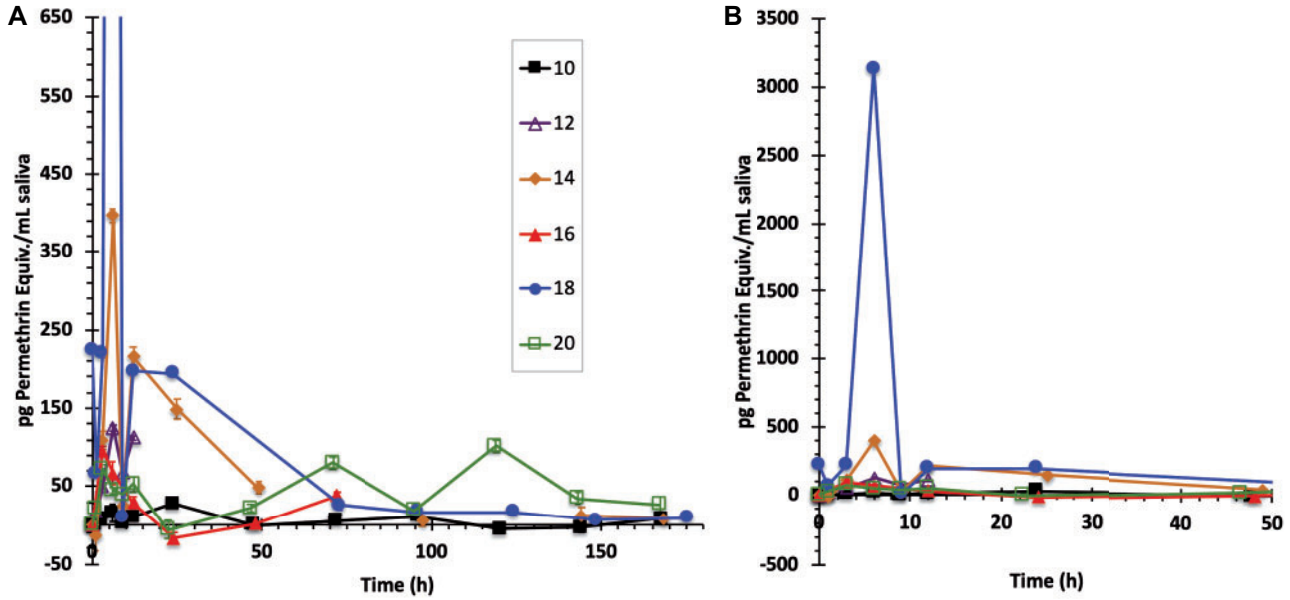


Figure 5. Excretion of ^{14}C permethrin equivalents in saliva.

who had relatively high absorption, but excretion in urine was low. It is possible that this subject retained the labeled permethrin/permethrin metabolites, or they were excreted by alternate routes (feces, sweat) or urine collections were incomplete.

The elimination rate of ^{14}C permethrin equivalents in the urine increased through the first 24 h, reached maxima in the 12–24 h period and then declined rapidly, correlating to the availability of permethrin in the serum (Figure 6B). The elimination half-life was 33.8, 38.9, 72.9, 67.9, and 68.6 h for subjects 10, 14, 16, 18, and 20, respectively. The shorter half-lives were found in male subjects, whereas the longer ones were found in female subjects. The permethrin equivalents eliminated in the first 24 h averaged 7.6 μg/l for the 5 subjects with complete 24-h urine collections (range 2.8–17.1 μg/l).

Analyze Urine Samples for Metabolite Profile

The LC method developed for potential LC/MS was used to separate metabolites in the urine samples. As mentioned above, urine samples were spiked with unlabeled standards so that peaks could be identified for collection of fractions. Due to the long elution time (180 min), we collected 2 min fractions from 40 to 180 min, but collected 5 min fractions in the first 40 min as none of our standards eluted in that period of time. Figure 7A is the UV chromatogram for the 12–24 h urine sample of subject 10, whereas Figure 7B is the AMS measurements of the corresponding fractions.

Some radioactivity is associated with peaks 2, 7, and 8 (Figure 7) that correspond to the glucuronide of 3-phenoxybenzyl alcohol (3-PBAlc), *cis*-DCVA and 3-phenoxybenzaldehyde, respectively. No parent compound was detected. Surprisingly, a large percentage of the radioactivity chromatographed was found in the first 40 min of the run and did not correspond to the known standards. The peak at about 18 min corresponds to about 45.4% of the chromatographed ^{14}C. The broad peak between 22 and 45 min constitutes an additional 31.7% of the ^{14}C, whereas the peak centered around 52 min

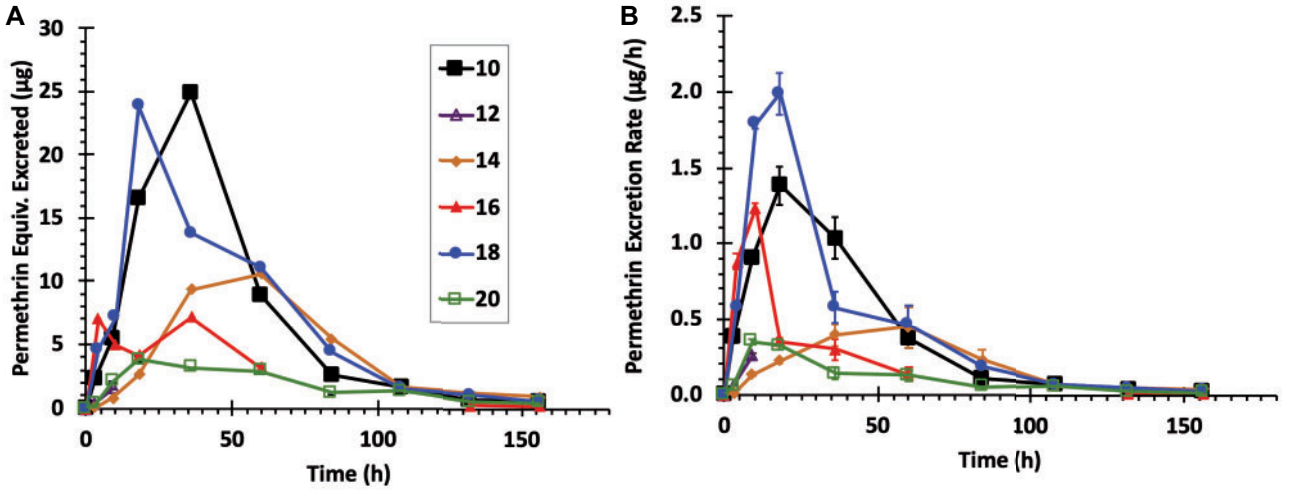


Figure 6. Excretion of ^{14}C permethrin equivalents in urine. A, Total mass of excreted permethrin equivalents in the urine at each collection. B, Permethrin equivalents elimination rate in urine. Subject 12 dropped out of the study just prior to the 24-h time point.

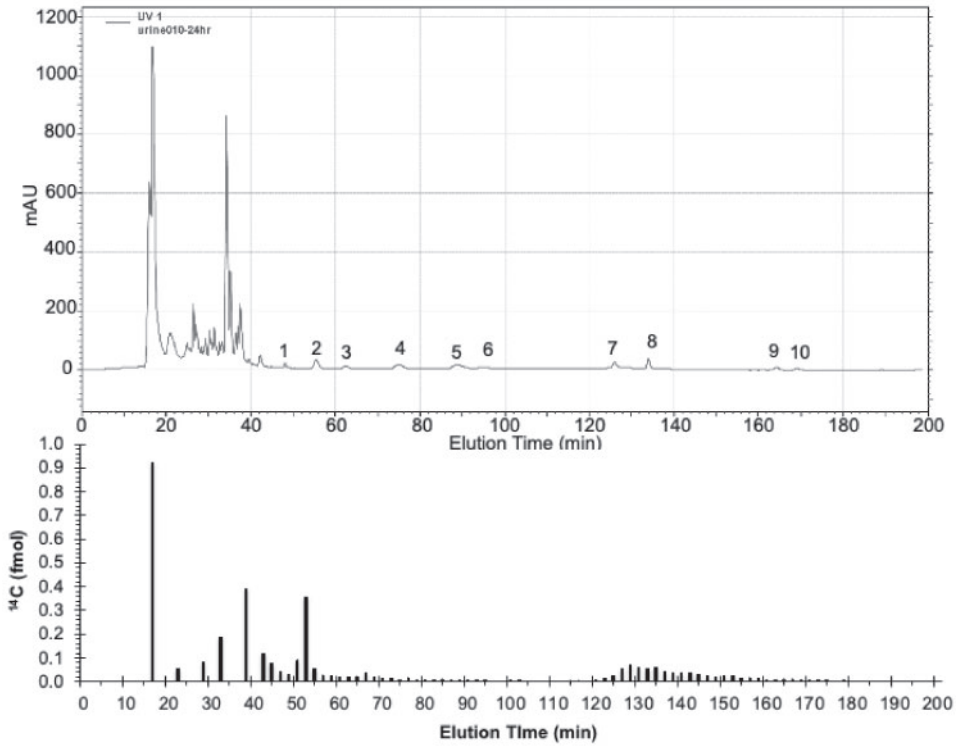


Figure 7. A, HPLC chromatogram of metabolites detected by UV. (1) glucuronide of 3- phenoxybenzyl alcohol; (2) *trans*-DCVA-glycine + 3-phenoxybenzoic acid (3PBA)-glycine; (3) *cis*-DCVA-glycine; (4) 3- phenoxybenzyl alcohol; (5) *trans*-DCVA; (6) 3PBA; (7) *cis*-DCVA; (8) 3-phenoxybenzaldehyde; (9) *trans*-permethrin; (10) *cis*-permethrin. B, Corresponding accelerator mass spectrometry measurement of collected fractions from the same HPLC run. The values are plotted at the center point of the collection time period.

(putatively the glucuronide of 3-PBAlc) made up 10.2%. The broad peak between 122 and 150 min was made up of about 11.2% of the ^{14}C.

One subject each was administered permethrin containing a label in only the phenoxy group or the cyclopropane group. At about 20 and 40 min, peaks appear for all 3 subjects suggesting that the compounds eluting at those times are mixtures of very polar metabolites (Figure 8). At about 55 min, there is a peak for subject 18 administered the cyclopropane label and this corresponds to the *trans*-DCVA as the coeluting 3-phenoxybenzoic acid (3PBA)-glycine is not labeled. However, one would expect subject 10 who was administered a mixed label to also have a peak at that elution time, which does not appear to be the case. Subject 10 shows a distinct peak around 48–50 min that is tentatively identified as the glucuronide of 3PBA. Subject 16, who received permethrin labeled in the phenoxy group also shows a peak at that time, although much smaller. All 3 subjects show a broad band around 128 min that could be associated with *cis*-DCVA and the 3-phenoxybenzaldehyde. Generally, we did not expect the presence of 3-phenoxybenzaldehyde as it is rapidly converted to 3PBA. Alternatively, the broad peak that elutes just before the parent compound could be the hydroxylated parent compounds.

Further confirmation of the metabolite identities is needed. One approach would be to hydrolyze the urine prior the HPLC/AMS analysis to determine if the distribution of radioactivity changes to less polar peaks. Further work on the HPLC separation system could be conducted to determine how many compounds may be present in the early eluting peaks. MS can be used for specific identification of the peaks following concentration and purification of the samples to provide adequate mass.

Preliminary analysis of urine samples using the immunoassays for 3PBA showed that acid hydrolysis results in the appearance of 3PBA suggesting that conjugates of 3PBA are present (Ahn *et al.*, 2011; Kim *et al.*, 2007; Shan *et al.*, 2004). The AMS data suggest that 3PBA-glycine may be one conjugate, but another may be 3PBA-glucuronide for which we did not have a standard. The 3PBA found comprised only a portion of the total permethrin equivalents found by AMS (Figs. 8 and 9).

Discussion

To reliably estimate internal dosimetry from a spot saliva sample requires good understanding of the pharmacokinetics of the chemical and the relationship between chemical concentration in the saliva and blood (Timchalk *et al.*, 2004). This has been demonstrated for the herbicide atrazine (Denovan *et al.*, 2000). The elevated ^{14}C signal observed in this study suggests saliva may be a viable biomonitoring media, but further studies are needed with better control of subject activity and observed variability.

Assuming nearly all absorbed permethrin is excreted in urine, is not sequestered in other compartments and is complete by the end of 7 days, the estimated % of the dose absorbed was 0.25, 0.12, 0.27, and 0.06 for subjects 10, 14, 18, and 20 who completed all urine collections. Similar levels (0.35–0.52%) were found by Tomalik-Scharte *et al.* (2005) where subjects were exposed to permethrin in a head shampoo and by Woollen *et al.* (1992; 0.85–1.2%) where subjects were exposed to 31 mg cypermethrin to an 800 cm^2 area of skin for 8 h.

Approximately 0.1% of the applied dermal dose of 25 mg cypermethrin was excreted within 72 h as the urinary

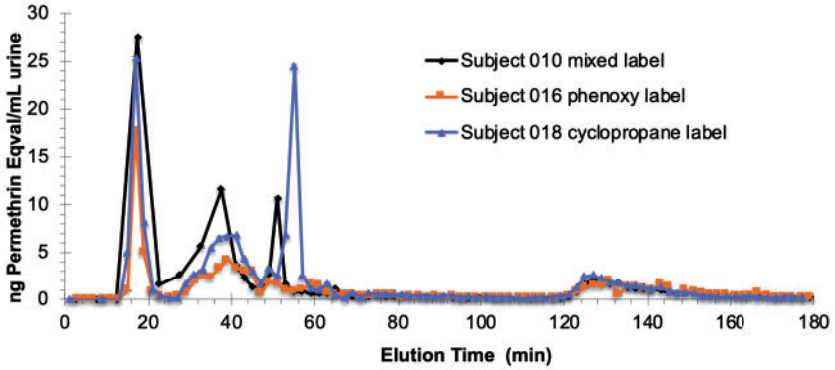


Figure 8. Representation of accelerator mass spectrometry analysis of HPLC fractions for subjects treated with a mixed label (black diamond), the cyclopropane only label (blue triangle) and the phenoxy label only (red square). Error bars are smaller than the symbols in nearly all cases.

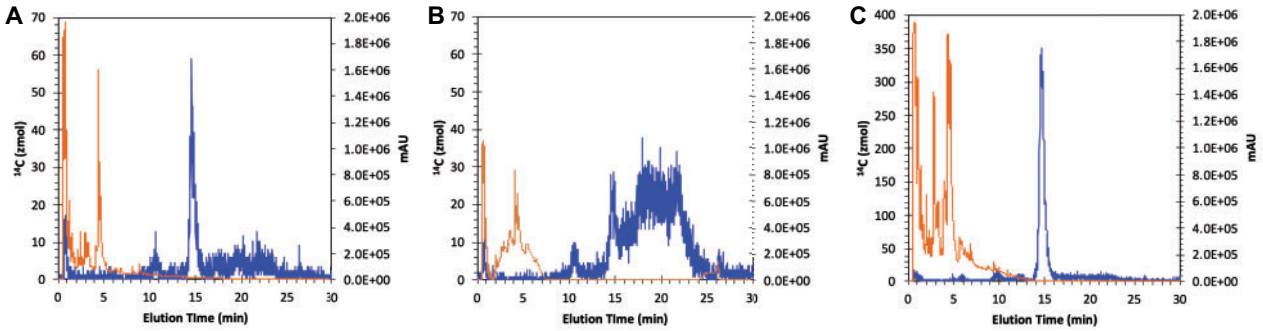


Figure 9. Continuous-flow moving-wire HPLC Traces of urine samples for (A) mixed label, (B) phenoxy label, and (C) cyclopropane label. Each panel graphs the ^{14}C signal in zmol (blue trace) and UV absorbance with the orange trace. The UV absorbance uses the same vertical scale on the right axis but the ^{14}C for the cyclopropane label was about 5× the other samples.

cyclopropanecarboxylic acid (Eadsforth *et al.*, 1988). No conclusions can be drawn from such urinary excretion data as to the concentration of cypermethrin and its metabolites in the skin or other organs, or the possibility of other routes of metabolism or excretion (Eadsforth *et al.*, 1988). Woollen *et al.* (1992) described maximum urinary excretion rates to occur between 12 and 36 h postdosing for dermally applied cypermethrin, very similar to observations for permethrin in this study. The elimination half-lives in this study are in close agreement with Tomalik-Scharte *et al.* (2005) who determined maximal urinary excretion at 13.9 h and elimination half-life of 32.7 h from male subjects dosed with 215 mg of permethrin in alcohol to the hair and head. However, elimination half-lives are much longer than determined by Woollen *et al.* (1992) for cypermethrin (13.0 ± 5.1 h) following a dermal exposure. Although not explicitly confirmed with the data in this study, the shorter elimination half-life of cypermethrin is probably due to the relative resistance to metabolism of its secondary hindered ester compared with permethrin (Hutson *et al.*, 1981; Shono *et al.*, 1979; Soderlund and Casida, 1977). The range of permethrin equivalents recovered in urine during the first 24 h were within the range reported by Leng *et al.* (1997) for pest control operators in Europe with daily occupational exposure for 1 week (<0.5 and 277 μg/l) and demonstrates that our dose exposure was similar to typical occupational exposure scenarios. Our data support literature conclusions that parent permethrin in saliva and urine are not adequate indicators of permethrin exposure but that the glucuronides, other conjugates and aglycones dichlorovinyl chrysanthemic acid and *meta*-phenoxybenzoic acid are adequate indicators of short-term human exposure to permethrin. Because of dramatic differences in absorption and excretion rates as well as metabolite distribution the saliva and urinary levels are only semi quantitative estimates of previous levels of permethrin exposure. The use UPLC followed by parallel AMS and molecular MS is ideal for identifying metabolites and conjugates in metabolism studies. Although the archived samples in this study degraded over a decade in storage and were not amenable to parallel AMS and molecular MS, the technique is capable of identifying metabolites without a priori knowledge of all metabolites. The ^{14}C-label unequivocally ties an eluted molecule to the dosing compound and the molecular MS provides possible molecules derived from the starting compound. The unidentified polar metabolites and conjugates of permethrin could be identified if this exposure study were repeated.

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DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

Ahn, K. C., Gee, S. J., Kim, H., Aronov, P. A., Vega, H., Krieger, R. I., and Hammock, B. D. (2011). Immunochemical analysis of 3-phenoxybenzoic acid, a biomarker of forestry worker exposure to pyrethroid insecticides. *Anal. Bioanal. Chem.* **401**, 1285–1293.

Ahn, K. C., Lohstroh, P., Gee, S. J., Gee, N. A., Lasley, B., and Hammock, B. D. (2007). High-throughput automated luminescent magnetic particle-based immunoassay to monitor human exposure to pyrethroid insecticides. *Anal. Chem.* **79**, 8883–8890.

Ahn, K. C., Ma, S., Tsai, H., Gee, S. J., and Hammock, B. D. (2006). An immunoassay for a urinary metabolite as a biomarker of human exposure to the pyrethroid insecticide permethrin. *Anal. Bioanal. Chem.* **384**, 713–722.

Barr, D. B., Olsson, A. O., Wong, L. Y., Udunka, S., Baker, S. E., Whitehead, R. D., Magsumbol, M. S., Williams, B. L., and Needham, L. L. (2010). Urinary concentrations of metabolites of pyrethroid insecticides in the general U.S. population: National Health and Nutrition Examination Survey 1999–2002. *Environ. Health Perspect.* **118**, 742–748.

Bradberry, S. M., Cage, S. A., Proudfoot, A. T., and Vale, J. A. (2005). Poisoning due to pyrethroids. *Toxicol. Rev.* **24**, 93–106.

Brander, S. M., Gabler, M. K., Fowler, N. L., Connon, R. E., and Schlenk, D. (2016). Pyrethroid pesticides as endocrine disruptors: Molecular mechanisms in vertebrates with a focus on fishes. *Environ. Sci. Technol.* **50**, 8977–8992.

Buchholz, B. A., Arjomand, A., Dueker, S. R., Schneider, P. D., Clifford, A. J., and Vogel, J. S. (1999a). Intrinsic erythrocyte labeling and attomole pharmacokinetic tracing of ^{14}C-folic acid with accelerator mass spectrometry. *Anal. Biochem.* **269**, 348–352.

Buchholz, B. A., Freeman, S. P. H. T., Haack, K. W., and Vogel, J. S. (2000). Tips and traps in the biological ^{14}C AMS prep lab. *Nucl. Instr. Meth. B* **172**, 404–408.

Buchholz, B. A., Fultz, E., Haack, K. W., Vogel, J. S., Gilman, S. D., Gee, S., Hammock, B. D., Hui, X., Wester, R. C., and Maibach, H. I. (1999b). HPLC-AMS measurement of atrazine metabolites in human urine after dermal exposure. *Anal. Chem.* **71**, 3519–3525.

Casida, J. E., and Bryant, R. J. (2017). The ABCs of pesticide toxicology: Amounts, biology, and chemistry. *Toxicol. Res.* **6**, 755–763.

Casida, J. E., and Durkin, K. A. (2013). Neuroactive insecticides: Targets, selectivity, resistance, and secondary effects. *Annu. Rev. Entomol.* **58**, 99–117.

Casida, J. E., and Quistad, G. B. (1998). Golden age of insecticide research: Past, present, or future? *Annu. Rev. Entomol.* **43**, 1–16.

Chrustek, A., Hołyńska-Iwan, I., Dziembowska, I., Bogusiewicz, J., Wróblewski, M., Cwynar, A., and Olszewska-Słonina, D. (2018). Current research on the safety of pyrethroids used as insecticides. *Medicina (Kaunas)* **54**, 61.

Côté, J., Bonvalot, Y., Carrier, G., Lapointe, C., Fuhr, U., Tomalik-Scharte, D., Wachall, B., and Bouchard, M. (2014). A novel toxicokinetic modeling of cypermethrin and permethrin and their metabolits in humans for dose reconstruction from biomarker data. *PLoS One* **9**, e88517.

Darney, K., Bodin, L., Bouchard, M., Cote, J., Volatier, J. L., and Desvignes, V. (2018). Aggregate exposure of the adult French population to pyrethroids. *Toxicol. Appl. Pharm.* **351**, 21–31.

Denovan, L. A., Lu, C., Hines, C. J., and Fenske, R. A. (2000). Saliva biomonitoring of atrazine exposure among herbicide applicators. *Int. Arch. Occup. Environ. Health* **73**, 457–462.

Eadsforth, C. V., Bragt, P. C., and Van Sittert, N. J. (1988). Human dose-excretion studies with pyrethroid insecticides cypermethrin and alphacypermethrin: Relevance for biological monitoring. *Xenobiotica* **18**, 603–614.

Ferland, S., Côté, J., Ratelle, M., Thuot, R., and Bouchard, M. (2015). Detailed urinary excretion time courses of biomarkers of exposure to permethrin and estimated exposure in workers of a corn production farm in Quebec, Canada. *Ann. Occup. Hyg.* **59**, 1152–1167.

Gilman, S. D., Gee, S. J., Hammock, B. D., Vogel, J. S., Haack, K., Buchholz, B. A., Freeman, S. P., Wester, R. C., Hui, X., and Maibach, H. I. (1998). Analytical performance of accelerator mass spectrometry and liquid scintillation counting for detection of 14C-labeled atrazine metabolites in human urine. *Anal. Chem.***70**, 3463–3469.

Harrell, A. W., Wilson, R., Man, Y. L., Riddell, K., Jarvis, E., Young, G., Chambers, R., Crossman, L., Georgiou, A., Pereira, A., *et al.* (2019). An innovative approach to characterize clinical ADME and pharmacokinetics of the inhaled drug nemiralisib using an intravenous microtracer combined with an inhaled dose and an oral radiolabel dose in healthy male subjects. *Drug Metab. Dispos.* **47**, 1457–1468.

Hutson, D. H., Gaughan, L. C., and Casida, J. E. (1981). Metabolism of the *cis*- and *trans*-isomers of cypermethrin in mice. *Pestic. Sci.* **12**, 385–398.

Joshi, U., Pearson, A., Evans, J. E., Langlois, H., Saltiel, N., Ojo, J., Klimas, N., Sullivan, K., Keegan, A. P., Oberlin, S., *et al.* (2019). A permethrin metabolite is associated with adaptive immune responses in Gulf War Illness. *Brain Behav. Immun.* **81**, 545–559.

Juraske, R., Mutel, C. L., Stoessel, F., and Hellweg, S. (2009). Life cycle human toxicity assessment of pesticides: Comparing fruit and vegetable diets in Switzerland and the United States. *Chemosphere* **77**, 939–945.

Kim, H. J., Ahn, K. C., Ma, S. J., Gee, S. J., and Hammock, B. D. (2007). Development of sensitive immunoassays for the detection of the glucuronide conjugate of 3-phenoxybenzyl alcohol, a putative human urinary biomarker for pyrethroid exposure. *J. Agric. Food Chem* **55**, 3750–3757.

Leemans, M., Couderq, S., Demeneix, B., and Fini, J. B. (2019). Pesticides with potential thyroid hormone-disrupting effects: A review of recent data. *Front. Endocrinol. (Lausanne)* **10**, 743.

Leng, G., Kühn, K., and Idel, H. (1997). Biological monitoring of pyrethroids in blood and pyrethroid metabolites in urine: Applications and limitations. *Sci. Total Environ.* **199**, 173–181.

Llewellyn, D. M., Brazier, A., Brown, R., Cocker, J., Evans, M. L., Hampton, J., Nutley, B. P., and White, J. (1996). Occupational exposure to permethrin during its use as a public hygiene insecticide. *Ann. Occup. Hyg.* **40**, 499–509.

Madeen, E. P., Ognibene, T. J., Corley, R. A., McQuistan, T. J., Henderson, M. C., Baird, W. M., Bench, G., Turteltaub, K. W., and Williams, D. E. (2016). Human microdosing with carcinogenic polycyclic aromatic hydrocarbons: *In vivo* pharmacokinetics of dibenzo[*def,p*]chrysene and metabolites by UPLC accelerator mass spectrometry. *Chem. Res. Toxicol.* **29**, 1641–1650.

Maule, A. L., Scarpaci, M. M., and Proctor, S. P. (2019). Urinary concentrations of permethrin metabolites in US Army personnel in comparison with the US adult population, occupationally exposed cohorts, and other general populations. *Int. J. Hyg. Environ. Health* **222**, 355–363.

Morgan, M. K. (2012). Children's exposures to pyrethroid insecticides at home: A review of data collected in published exposure measurement studies conducted in the United States. *Int. J. Environ. Res. Public Health* **9**, 2964–2985.

Mortuza, T. B., Edwards, G. L., White, C. A., Patel, V., Cummings, B. S., and Bruckner, J. V. (2019). Age dependency of blood-brain barrier [enetration by *cis*- and *trans*-permethrin in the rat. *Drug Metab. Dispos.* **47**, 234–237.

National Research Council. (1994). *Health Effects of Permethrin-Impregnated Army Battle-Dress Uniforms*. The National Academies Press, Washington, DC.

Ognibene, T. J., Bench, G., Vogel, J. S., Peaslee, G. F., and Murov, S. (2003). A high-throughput method for the conversion of CO_2 obtained from biochemical samples to graphite in septa-sealed vials for quantification of ^{14}C via accelerator mass spectrometry. *Anal. Chem.* **75**, 2192–2196.

Ognibene, T. J., Haack, K. W., Bench, G., and Turteltaub, K. W. (2019). Trials and tribulations in the first three years in operation of the SSAMS for biomedical ^{14}C-AMS at LLNL. *Nucl. Instr. Meth. B* **438**, 166–171.

Ratelle, M., Côté, J., and Bouchard, M. (2015). Toxicokinetics of permethrin biomarkers of exposure in orally exposed volunteers. *Toxicol. Lett.* **232**, 369–735.

Repetto, R., and Baliga, S. S. (1997). Pesticides and immunosuppression: The risks to public health. *Health Policy Plan* **12**, 97–106.

Rossbach, B., Niemietz, A., Kegel, P., and Letzel, S. (2014). Uptake and elimination of permethrin related to the use of permethrin treated clothing for forestry workers. *Tox. Lett.* **231**, 147–153.

Shafer, T. J., Meyer, D. A., and Crofton, K. M. (2005). Developmental neurotoxicity of pyrethroid insecticides: Critical review and future research needs. *Environ. Health Perspect.* **113**, 123–136.

Shan, G., Huang, H., Stoutamire, D. W., Gee, S. J., Leng, G., and Hammock, B. D. (2004). A sensitive class specific immunoassay for the detection of pyrethroid metabolites in human urine. *Chem. Res. Toxicol.* **17**, 218–225.

Shelton, J. F., Geraghty, E. M., Tancredi, D. J., Delwiche, L. D., Schmidt, R. J., Ritz, B., Hansen, R. L., and Hertz-Picciotto, I. (2014). Neurodevelopmental disorders and prenatal residential proximity to agricultural pesticides: The CHARGE study. *Environ. Health Perspect.* **122**, 1103–1109.

Shono, T., Ohsawa, K., and Casida, J. E. (1979). Metabolism of *trans*- and *cis*-permethrin, *trans*- and *cis*-cypermethrin, and decamethrin by microsomal enzymes. *J. Agric. Food Chem.* **27**, 316–325.

Soderlund, D. M. (2012). Molecular mechanisms of pyrethroid insecticide neurotoxicity: Recent advances. *Arch. Toxicol.* **86**, 165–181.

Soderlund, D. M., and Casida, J. E. (1977). Effects of pyrethroid Structure on rates of hydrolysis and oxidation by mouse liver microsomal enzymes. *Pestic Biochem. Physiol.* **7**, 391–401.

Spracklin, D. K., Chen, D., Bergman, A. J., Callegari, E., and Obach, R. S. (2020). Mini-review: Comprehensive drug disposition knowledge generated in the modern human radiolabeled ADME study. *CPT Pharmacometrics Syst. Pharmacol.* **9**, 428–434.

Thomas, A. T., Ognibene, T., Daley, P., Turteltaub, K., Radousky, H., and Bench, G. (2011). Ultrahigh efficiency moving wire combustion interface for online coupling of high performance liquid chromatography (HPLC). *Anal. Chem.* **83**, 9413–9417.

Timchalk, C., Poet, T. S., Kousba, A. A., Campbell, J. A., and Lin, Y. (2004). Noninvasive biomonitoring approaches to determine dosimetry and risk following acute chemical exposure: Analysis of lead or organophosphate insecticide in saliva. *J. Toxicol. Environ. Health A* **67**, 635–650.

Tomalik-Scharte, D., Lazar, A., Meins, J., Bastian, B., Ihrig, M., Wachall, B., Jetter, A., Tantchev-Poór, I., Mahrle, G., and Fuhr, U. (2005). Dermal absorption of permethrin following topical administration. *Eur. J. Clin. Pharmacol.* **61**, 399–404.

Trunnelle, K. J., Bennett, D. H., Ahn, K. C., Schenker, M. B., Tancredi, D. J., Gee, S. J., Stoecklin-Marois, M. T., and Hammock, B. D. (2014). Concentrations of the urinary pyrethroid metabolite 3-phenoxybenzoic acid in farm worker families in the MICASA study. *Environ. Res.* **131**, 153–159.

Tu, W., Xu, C., Jin, Y., Lu, B., Lin, C., Wu, Y., and Liu, W. (2016). Permethrin is a potential thyroid-disrupting chemical: In vivo and in silico evidence. *Aquat. Toxicol.* **175**, 39–46.

Vogel, J. S., and Love, A. H. (2005). Quantitating isotopic molecular labels with accelerator mass spectrometry. *Methods Enzymol.* **402**, 402–422.

Wei, B., Isukapalli, S. S., and Weisel, C. P. (2013). Studying permethrin exposure in flight attendants using a physiologically based pharmacokinetic model. *J. Expo. Sci. Environ. Epidemiol.* **23**, 416–427.

Woollen, B. H., Marsh, J. R., Laird, W. J. D., and Lesser, J. E. (1992). The metabolism of cypermethrin in man: Differences in urinary metabolite profiles following oral and dermal administration. *Xenobiotica* **22**, 983–991.

Zhao, C., Hwang, S. H., Buchholz, B. A., Carpenter, T. S., Lightstone, F. C., Lightstone, F., Yang, J., Hammock, B. D., and Casida, J. E. (2014). $GABA_A$ receptor target of tetramethylenedisulfotetramine. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 8607–8612.