Online Solid Phase Extraction High-Performance Liquid Chromatography – Isotope Dilution – Tandem Mass Spectrometry Quantification of Organophosphate Pesticides, Synthetic Pyrethroids, and Selected Herbicide Metabolites in Human Urine

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Abstract

Analytical methods to quantify pesticide biomarkers in human population studies are critical for exposure assessment given the widespread use of pesticides for pest and weed control and their potential for affecting human health. We developed a method to quantify, in 0.2 mL of urine, concentrations of 10 pesticide biomarkers: four organophosphate insecticide metabolites (3,5,6-trichloro-2-pyridinol (TCPy), 2-isopropyl-6-methyl-4-pyrimidinol, para-nitrophenol, malathion dicarboxylic acid); five synthetic pyrethroid insecticide metabolites (4-fluoro-3-phenoxybenzoic acid, 3-phenoxybenzoic acid, *cis* and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA), *cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid); and the herbicide 2,4-dichlorophenoxyacetic acid.

The method is based on enzymatic hydrolysis of conjugated urinary metabolites, extraction and pre-concentration of the deconjugated metabolites using automated online solid-phase extraction, and separation and quantification using liquid chromatography-isotope dilution tandem mass spectrometry.

Depending on the analyte, method detection limits were 0.1-0.6 ng/mL; mean accuracy, calculated as spike recoveries, was 91-102%, and total precision, given as percent variation coefficient, was 5.9-11.5%. Percent differences associated with three freeze-thaw cycles, 24-hour benchtop storage, and short-term processed sample stability were < 14%.

Method suitability was assessed by recurring successful participation in external quality assessment schemes and by analyzing samples from subjects with suspected exposure to pesticides (n=40) or who self-reported consuming an organic diet (n=50). Interquartile ranges were considerably lower for people consuming an organic diet than for those potentially exposed for *cis*-DCCA (0.37 ng/mL vs 0.75 ng/mL), *trans*-DCCA (0.88 ng/mL vs 1.78 ng/mL) and TCPy (1.81 ng/mL vs 2.48 ng/mL). This method requires one-fifth of the sample used in our previous method and is suitable for assessing background exposures to select pesticides in large human populations and for studies with limited sample volumes.

Keywords

Metabolites; Pyrethroids; Organophosphate; Biomonitoring; LC-MS/MS; online SPE

Introduction

Pesticides, used by commercial applicators, farmers, and homeowners to kill or control pests, plant disease agents, and weeds, are a critical component of food security to support global population growth and for the control of human vector-borne diseases (Sharma et al., 2019). With the global population projected to reach 9.8 billion by 2050, the increasing food demand may result in a 3.7% pesticide use increase per year (UNEP, 2021). Despite their benefits, pesticides can display varying levels of toxicity even at low doses (Huen et al., 2012; Simaremare et al., 2019).

Organophosphates, which represent about 40% of all pesticides produced worldwide (Kaushal et al., 2021), are cost-effective in controlling a broad spectrum of pests in both agriculture and homes (Bhatt et al., 2021). Pyrethroids are widely applied in agricultural practices because of their relatively high effectiveness and low-toxicity properties. Pyrethroids are often applied to children for head lice and to pets for flea and tick control (Pitzer et al., 2021). Pyrethroids have been replacing organophosphates because of organophosphates' human toxicity concerns and the phasing out of some organophosphates from residential use (Bao et al., 2020). 2,4-Dichlorophenoxyacetic acid (2,4-D) is a low-cost herbicide used on broadleaf weeds (Robinson et al., 2012). According to the U.S. Environmental Protection Agency, 2,4-D was the fifth most used herbicide in agriculture (30–40 million pounds) and the most common in the home and garden market (7–9 million pounds) in the United States in 2012 (Atwood & Paisley-Jones, 2017). By 2019, the United States Geological Survey (USGS) agricultural pesticide use estimates, showed 2,4-D usage rose to 45 million pounds, making it the third most used herbicide after glyphosate and atrazine (USGS, 2023).

Pesticide exposure can occur through ingestion of contaminated food and hand-to-mouth contact with pesticide contaminated surfaces (Liu et al., 2021), as well as inhalation and dermal contact (Silva Pinto et al., 2020). After exposure, organophosphate and pyrethroid pesticides are rapidly metabolized and excreted in urine, with reported elimination half-lives of 2-41 hours (organophosphates) and 6.4 -16.5 hours (pyrethroids) (Chrustek et al., 2018; Nolan et al., 1984; Starr et al., 2008; Woollen et al., 1992). These urinary metabolites

are well-established pesticide exposure biomarkers (Chen & Cashman, 2013). In cases where multiple pesticides share a common metabolite, for example 3-phenoxybenzoic acid (3-PBA, a metabolite for several pyrethroids), the metabolite concentrations are considered a cumulative index of exposure for those pesticides (Starr et al., 2008).

Reliable and accurate mass spectrometry methods for monitoring concentrations of pesticide metabolites are therefore necessary to assess exposure and evaluate potential adverse effects of pesticides on human health (Baker et al., 2000; Baker et al., 2004; Davis et al., 2013; Fišerová et al., 2021; Gao et al., 2022; Garí et al., 2018; Jagani et al., 2022; Li & Kannan, 2018; López-García et al., 2019; Olsson et al., 2004; Olsson et al., 2003; Roca et al., 2014). These methods, all based on HPLC separation, used various sample preparation techniques such as liquid-liquid extraction, offline or online solid phase extraction, and turbulent flow (Table 1) to quantify trace-concentrations of several pesticide metabolites in urine volumes ranging from 0.2 mL to 10 mL.

Improved instrument and column technology as well as the need to use smaller sample volumes, especially for studies involving children, are drivers for a constant reevaluation of biomonitoring methods. This paper describes an online solid phase extraction high-performance liquid chromatography-isotope dilution tandem mass spectrometry method for the quantification of 10 pesticide biomarkers (Figure 1) in only 0.2 mL of human urine: four organophosphate insecticide metabolites, five synthetic pyrethroid metabolites, and one herbicide.

Experimental

Reagents and chemicals

LC-MS grade methanol was purchased from Thermo Fisher Scientific (Pittsburg, PA). LC-MS grade water was obtained from TEDIA High Purity Solvents (Fairfield, OH). Glacial acetic acid, sodium acetate, and β-glucuronidase/sulfatase type H-1 from *Helix pomatia* were obtained from Sigma-Aldrich Co. (St. Louis, MO). All target analytes (Figure 1, Figure S1, Table S1) and their corresponding isotopically labelled internal standards (Table S2) were obtained from Cambridge Isotopes Laboratories (Andover, MA): 2-isopropyl-6-methyl-4-pyrimidinol (IMPy); 3,5,6-trichloro-2-pyridinol (TCPy); para-nitrophenol (PNP), 3-PBA, 4-fluoro-3-phenoxybenzoic acid (4FP), *cis-* and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA), *cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid [*cis*-DBCA]), and 2,4-D, except for malathion dicarboxylic acid (MDA) which was from LGC Standards USA (Manchester, NH). To select these target analytes, we considered potential pesticide exposures from agricultural and household products use by the general population, historical biomarkers data from the National Health and Nutrition Examination Survey (NHANES), and whether the chemical properties of the chosen analytes would be compatible with the experimental conditions of the method to ensure adequate method performance for all analytes.

Reagents, solvents, and standard materials were used without further purification.

Urine collection and preparation of quality control and proficiency testing materials

The urine used for quality control (QC) and proficiency testing (PT) materials was collected anonymously from both male and female adult volunteers, and was screened for the analytes of interest using the method described in Davis et al., 2013. Individual urines with the least amount of detected target analytes were pooled and used for the preparation of QC materials, as a blank for matrix-based calibration curve, and for method validation. The pool was stored at −20 °C until further use. The urine collection protocol used was reviewed and approved by the CDC's Human Subjects Institutional Review Board. A waiver of informed consent was requested under 45 CFR 46.116(d). No personal identifiable information was collected.

For preparation of QC materials and PT samples, the urine pool was thawed and homogenized at room temperature for four hours. Five separate 200 mL aliquots of the pool were obtained and spiked with target analytes to make a low-concentration QC (QCL), a high-concentration QC (QCH), and three method validation materials for an internal PT program. The pools were homogenized overnight at 4 °C then at room temperature for 4 hours, aliquoted and stored at −70 °C until further use. The levels spiked were selected based on distribution of the analytes of interest from NHANES (U.S.-CDC, 2023). QC and PT materials were prepared in duplicate and analyzed daily and characterized by repeated measurements during at least 20 days. The data were sent to a PT administrator within our institution to establish concentration means and acceptable limits for each of the materials. The QCs were then labeled for daily use and the PTs were sent to another CDC lab where they were relabeled (blinded). These samples were returned for storage in our laboratory at −70 °C until use.

Method validation

Preparation of standard solutions and spiking solution—Individual stock solutions for target analytes were prepared at a concentration of 100 μg/mL in methanol or used in the solvent they were received in. From the stocks, a solution containing 200 ng/mL concentration of all analytes was prepared. Eleven other solutions were prepared from this solution resulting in 12 working solutions ranging in concentrations from 0.10 -200 ng/mL. No more than three serial dilutions were done during the preparation of the standards. When 50 μL is spiked into a 200 μL sample size, these solutions create a 12-point calibration curve ranging from 0.025 ng/mL to 50 ng/mL.

Similarly, individual stock solutions for the isotopically labeled internal standards were prepared at a concentration of 100 μg/mL in methanol or used in the solvent they were received in (Table S2). A working solution containing all labelled compounds was prepared from the stock solutions at a concentration of 24 ng/mL (2,4-D, 4FP, 3-PBA, PNP), 48 ng/mL (*cis*-DBCA, TCPy, IMPy, MDA, *trans*-DCCA), and 96 ng/mL (*cis*-DCCA). This solution was spiked in all samples, procedural blanks, QCs and calibrants during sample preparation as described below. A solution of β-glucuronidase/sulfatase was prepared fresh prior to every plate preparation by gently dissolving 74.5 mg of β-glucuronidase/sulfatase in 10 mL of 0.36M sodium acetate buffer.

Samples and standards preparation—Sample preparation was conducted in 96 deep well plates. 50 μL of internal standard solution were spiked into all wells. 50 μL of each calibrant was spiked into the first row of the plate followed by a 150 μL spike of water into the calibrant wells. 200 μL of urine study samples and QCs were spiked into respective wells. For the procedural blank, 200 μL of water was spiked. 50 μL of the freshly prepared enzyme was spiked into each well. The plate was sealed with a pierceable silicon mat, gently swirled, and placed at 37 °C in an incubator overnight. The next day, the plate was retrieved from the incubator, and 100 μL of freshly prepared 10% acetic acid in water was added into each well to quench the enzyme. The contents of the plate were transferred using an eight-channel pipette into a Biotage ISOLUTE ®FILTER+ Plate 25 μm/0.2 μm (Biotage LLC, Charlotte, NC, USA) and filtered by positive pressure into a 96 deep well collection plate using a UCT 96 Well Plate Positive Pressure Extraction Manifold (United Chemical Technologies, Inc., Bristol, PA, USA). 300 μL was injected for analysis.

Online solid phase extraction (SPE) and chromatographic separation—Online cleanup and analytical separation were performed with a Thermo Scientific™ Dionex UltiMate™ 3000 Rapid Separation Dual HPLC system (Sunnyvale, CA, USA) which consists of a DGP-3600RS dual (right and left) gradient pumps, SRD-3600 Integrated Solvent and Degasser rack, a WPS3000TRS temperature-controlled autosampler, and a TCC-3200 column thermostat compartment with a 2-position six-port switching valve. The right pump was used exclusively for online SPE cleanup and is herein referred to as the SPE pump. The left pump was used exclusively for analytical separation and is herein referred to as the analytical pump. Pump conditions are described in Table S3.

A Chromolith Flash RP-18e monolithic (25 × 4.6 mm) column (EMD Chemicals, Gibbstown, NJ) was used as the SPE column and a Thermo Scientific™ Hypersil Gold aQ column (150 × 4.6 mm, 3 μm particle size), with guard column of similar packing, was used as the analytical column. For both, SPE and analytical separation, mobile phase A was 0.1% acetic acid in LC-MS grade water and mobile phase B was 100% methanol.

Using the SPE pump, a sample size of 300 μL was injected into the SPE column and washed isocratically with 1% mobile phase B at a flow rate of 2 mL per minute for 3.1 minutes (Figure 2_Step 1) to help eliminate salts and other matrix components. At 3.1 minutes, just before the analytes eluted from the SPE column, the flow was switched using the six-port switching valve and the analytical pump was used to back elute the sample from the SPE column onto the analytical column using 20% mobile phase B at a flow rate of 0.75 ml per minute for 3 minutes (Figure 2_Step 2). At 6.1 minutes, all the analytes had been fully transferred from the SPE onto the analytical column and chromatographic separation was performed using the analytical pump. Meanwhile, the SPE pump equilibrated the SPE column for the next injection (Figure 2_Step 3). The gradient for the separation in the analytical column began at 20% B and a flow rate of 0.75 mL/min at 6.1 minutes followed by an increase to 70% B at 8.0 minutes. The flow rate was dropped to 0.4 mL/min, maintaining 70% B for the next 10 minutes. At 20 minutes, the composition was changed to 100% B for column cleaning. From 23.5 to 27 minutes, the column was equilibrated to the initial conditions of 20% B at a flow rate of 0.75 mL/min.

Mass spectrometric quantification—We used a Thermo Scientific™ TSQ Altis™ triple-stage quadrupole mass spectrometer (San Jose, CA, USA) equipped with a Heated Electrospray Ionization Source (HESI). The settings on the HESI source were: positive spray voltage 3500 V, negative spray voltage 2500 V, sheath gas flow rate 50 arbitrary units (a.u.), auxiliary gas flow rate 10 a.u., sweep gas flow rate 1 a.u., ion transfer tube temperature of 325 °C, and vaporizer temperature of 350 °C. Nitrogen was used as the sheath, sweep, and auxiliary gas. Argon was used as the collision gas at 1.0 mTorr. The ion source probe position was optimized to maximize instrument sensitivity by setting the probe at different positions and establishing the total ion count for all analytes. The mass spectrometer operated in the Selected Reaction Monitoring (SRM) mode (Table 2). Analytes, and their corresponding isotopically labeled internal standards (_L), were identified by their retention time, precursor ion, two product ions, one for quantification (_Q) and one for confirmation(_C). Positive and negative polarity switching was used to accomplish detection of analytes in the different modes. Cycle time was set at 0.7 seconds. Q1 and Q3 resolution (FWHM) were set at 0.7 amu.

Daily Operation and Quality Control Procedures—Each analytical run comprised 76 study samples, two procedural blanks (include all reagents in solvent, not urine), two instrument checks, two QCL, two QCH and twelve calibrants. The procedural blanks are critical to establish the integrity of the sample preparation process and to identify solvent or other reagents contamination that would compromise the validity of the measured concentrations. Instrument checks contained all analytes at a concentration of 0.3 ng/mL and underwent the same procedure as study samples, QCs, procedural blanks and standards. The instrument checks were used to confirm acceptable chromatographic retention time and shape, and mass spectrometry sensitivity before the start of an analytical run.

The analytical run data were integrated using Xcalibur 4.1 software (Thermo Scientific, San Jose, CA) and transferred to a database where results were checked against previously established criteria. Samples with analyte results outside acceptable ranges were re-extracted and reanalyzed. Samples with analyte concentrations above the calibration curve were re-extracted with a smaller sample volume. Duplicate QCL and QCH data were measured and statistically analyzed applying modified Westgard QC rules (Caudill et al., 2008) using SAS (SAS Institute Inc., Cary, NC) to ensure statistical control of the analytical run.

To monitor method performance, twice a year the PT administrator randomly choses five samples from the characterized internal PT pools for analysis. The data are evaluated by the PT administrator and the laboratory should receive an 80% or better score to pass the challenge. The internal PT samples contain all method analytes, including IMPy and MDA for which there are no external quality assurance programs. In addition, we participate in the German External Quality Assessment Scheme (GEQUAS) which includes 3-PBA, 4FP, cis-DCCA, PNP, TCPy, trans- and cis-DCCA, and cis-DBCA (http://www.gequas.de/), and in the External Quality Assessment Scheme for Organic substances in Urine (OSEQAS) for 2,4-D (https://www.inspq.qc.ca/en/ctq/eqas/oqesas/description).

Results and discussion

Online SPE sorbent selection

Automation of sample preparation in large biomonitoring studies reduces manual work and minimizes analyst-specific influence on test results (Christler et al., 2020). Online extraction followed by chromatographic separation enables production of consistent data and elimination of repetitive physical tasks (Viglino et al., 2008). The reduced sample manipulation and elimination of offline sample concentration can improve method performance (Wang et al., 2022).

For SPE retention, we tested various types of C4, C8, C18, Chromolith® Flash RP-18 and Chromolith® High Resolution RP-18 columns. We chose the Chromolith™ Flash RP-18e monolithic (25mm x 4.6mm, 2μm particle size) column *(Millipore Sigma, Burlington, MA, USA)* for its balance between analyte retention (that could vary considerably because of the wide pKa range for the target analytes included in the method), matrix elimination, and chromatographic peak resolution.

Analytical column and solvent selection.

We tested multiple phases for analytical separation including various forms of C18, phenyl-hexyl and pentafluorophenylpropyl. We chose Hypersil GOLD™ aQ C18 Polar Endcapped HPLC column (150mm x 4.6mm, 3 μm particle size) with Hypersil GOLD™ aQ C18 guard cartridge (10mm x 4mm, 3 μm), both from *Thermo Fisher Scientific (Waltham, MA, USA)* for their separation efficiency, including baseline separation of *cis*- and *trans*-DCCA (Figure 3).

Both methanol and acetonitrile and 50:50 mixture of both solvents were evaluated for separation efficiency. We selected methanol because of its effective separation of the *cis*-DCCA/*trans*-DCCA isomers. We evaluated acetic acid and formic acid as mobile phase A modifiers. Because we observed a reduced signal when using formic acid, we chose acetic acid as modifier.

Accuracy

The accuracy of the method was determined by analyzing two different urine materials with target analytes at zero and three other concentrations spanning the method linear range. Each concentration was prepared in triplicate and analyzed on two different days. This resulted in 12 measurements for each of the concentrations which were averaged for the recovery calculations. Recovery of the spiked analytes was calculated as [(final concentration – initial concentration)/spiked concentration]; recovery was 91-102% (Table 3).

Precision

Precision was determined by calculating the relative standard deviation (RSD) of repeat measurements of quality control materials at two concentrations, performed in duplicate in ten different analytical runs. Total precision ranged from 5.9% to 11.5% (Table 3). Within-run precision ranged from 3.0% - 11.3%, between-run precision ranged from 2.3%-10.3% %

(Table S4). These values were within the 15% RSD recommended by FDA for bioanalytical method validation (U.S.-FDA, 2018).

Analytical Sensitivity

Limits of detection (LODs) ranged from 0.1 to 0.6 ng/mL, depending on the analyte (Table 3). LODs were calculated as $3S_0$, where S_0 is the estimated standard deviation (SD) at zero concentration and is determined by linear regression analysis of the absolute standard deviation (SD) versus concentration of the 4 lowest calibrators of the calibration curve using the Taylor method (Taylor, 1987). Twenty replicates of each calibrator were used for the calculation.

Analytical Specificity

The use of isotopically labeled internal standard analogs created a reliable reference for peak identification and integration. Further, we included a quantitation (Q) and a confirmation (C) ion for each analyte. Calculated concentrations using Q and C ions were compared to verify that the right metabolite was measured. Differences above 20% between the Q and C ion concentrations, suggest presence of interfering species; such results are not reported. Our data evaluation also include checks to ensure that retention times for Q and C ions match, and that these retention times match those of the isotopically labeled internal standards.

Freeze-Thaw, Bench Top and Processed Sample Stability

Freeze-thaw stability was determined by comparing analyte concentrations in the high- and low-concentration quality control materials before and after three subsequent freeze-thaw cycles. For each quality control material, three replicate samples were analyzed initially and then three replicate samples were analyzed after three freeze-thaw cycles. The samples were retrieved from the −70 °C freezer, thawed at room temperature for three hours and frozen again at −70 °C overnight. This process was repeated two more times and at the end the samples were analyzed as any other sample. Bench-top stability was assessed by comparing analyte concentrations also in the two quality control materials prepared in triplicate before and after they were left on the bench at room temperature for one day. Processed sample stability was assessed by comparing processed samples left in the autosampler at room temperature for 24 hours. Percent differences associated with three freeze-thaw cycles, 24-hour benchtop storage, and short-term processed sample stability were less than 14% (Table S5).

Matrix effects

Matrix effects were investigated by comparing calibration curves made in urine versus those made in water. Both urine matrix and water-based calibration curves were prepared in triplicate and analyzed on the same run. The differences in the resultant slopes weighted at 1/X were averaged; percent differences are shown in Table S6. No significant differences were observed in the matrix versus water-based curves; therefore, a solvent-based curve was chosen.

Method application

We further confirmed the adequacy of the method for exposure assessment of pesticides by successful participation in proficiency testing programs (see Table S7) and by analyzing 90 non-spiked urine samples, purchased from BioreclamationIVT (Hicksville, NY). The company had IRB approval to collect urine and obtained informed consent from donors who were verbally asked whether they had been in contact with pesticides within the last 24 h of specimen donation (n = 40) or whether they had been on an organic diet (n = 50). No personal identifiers were provided to CDC. We detected pesticide metabolites in most of the 90 samples used for this application (Table 4), suggesting widespread exposure to the parent compounds whose metabolites are included in the method. Median concentrations are comparable for both subject groups and these concentrations are comparable to those of the U.S. general population (U.S.-CDC, 2022, 2023). On the other hand, interquartile ranges were lower for people consuming an organic diet than for people potentially exposed for *cis*-DCCA (0.37 ng/mL vs 0.75 ng/mL), *trans*-DCCA (0.88 ng/mL vs 1.8 ng/mL) and TCPy (1.8 ng/mL vs 2.5 ng/mL). These results suggest that diet contributes to pesticide exposure, as reported before (Liu et al., 2021). Because we did not have any other information from the donors, we cannot determine whether these differences may also relate to non-dietary exposures such as inhalation and dermal contact (Silva Pinto et al., 2020) from non-dietary sources and use of pesticides.

Taking into consideration the limited size of the dataset and the unavailability of creatinine measurements, we recommend caution in drawing conclusions regarding the extent of pesticide exposures among the two participant groups. Of note, accounting for urinary dilution (e.g., use of creatinine-corrected biomarker concentrations) is a common practice when evaluating pesticide exposures in epidemiologic investigations that rely on biomonitoring for exposure assessment (Barr et al., 2005; O'Brien et al., 2017; Weaver et al., 2016; Yeh et al., 2015).

Strengths and limitations

SPE is used widely for the extraction of pesticides from urine matrices (Gao et al., 2022) and this approach was used in our previous methods (Baker et al., 2004; Davis et al., 2013; Olsson et al., 2004; Olsson et al., 2003). SPE allows for cleaner analyte extracts and is relatively easy to automate compared to treatments like liquid-liquid extraction (Liang et al., 2019; López-García et al., 2019). A notable advantage of the online SPE method presented here is the minimal sample loss during cleanup and the potential for improved recovery because all sample injected is subsequently analyzed in the mass spectrometer (López-García et al., 2019). However, injecting the sample in full does not allow for a reinjection.

Monolithic online SPE columns have large pore sizes to enable high flow rates during sample cleanup and back elution onto analytical columns. These high flow rates somewhat limit the choice of analytical columns to prevent high back pressure. Back elution of analytes from the online SPE on to the analytical column can reduce separation efficiency and increase analysis time. In our case, the combination of the Chromolith™ Flash RP-18e, a monolithic online SPE column with a Hypersil GOLD™ aQ C18 Polar endcapped HPLC

analytical column and a Hypersil GOLD™ aQ C18 guard cartridge provided optimal separation. The choice of columns should be carefully considered should one opt to include additional analytes in the method.

The reduced sample manipulation typical of online SPE-LC-MS/MS methods resulted in higher selectivity and precision when compared to our previous methods. Additionally, the current method uses a much smaller volume of urine (200 μL) than the 1 and 5 mL volumes required in some of our previous offline SPE-mass spectrometry methods (Baker et al., 2004; Davis et al., 2013) and the 10 mL of urine used for our liquid-liquid extraction method (Baker et al., 2000). Injection of large volume urine samples introduces considerable amounts of matrix into the mass spectrometer, necessitating regular instrument cleaning and maintenance. Therefore, smaller sample volumes reduce instrument maintenance and facilitates the performance of studies where access to sufficient urine volume may be limited (e.g. young children).

Conclusion

We validated a sensitive, accurate, precise, and high throughput method for quantifying multiple pesticides biomarkers in 200 μL human urine using online SPE and isotope dilution HPLC-MS/MS. Manual sample preparation was limited to spiking, addition of internal standard solution and filtration, which significantly reduced analyst-specific influence on test results. The relatively high sensitivity of the current mass spectrometric system together with an online sample cleanup approach were critical aspects of reducing sample volume in this method to investigate pesticide exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

Atwood D, & Paisley-Jones C (2017). Pesticides Industry Sales and Usage, 2008 – 2012 Market Estimates. https://www.epa.gov/sites/production/files/2017-01/documents/pesticides-industry-sales-usage-2016_0.pdf

Baker SE, Barr DB, Driskell WJ, Beeson MD, & Needham LL (2000). Quantification of selected pesticide metabolites in human urine using isotope dilution high-performance liquid chromatography/tandem mass spectrometry. J Expo Anal Environ Epidemiol, 10(6 Pt 2), 789–798. 10.1038/sj.jea.7500123 [PubMed: 11138671]

Baker SE, Olsson AO, & Barr DB (2004). Isotope dilution high-performance liquid chromatography-tandem mass spectrometry method for quantifying urinary metabolites of synthetic pyrethroid insecticides. Arch Environ Contam Toxicol, 46(3), 281–288. 10.1007/s00244-003-3044-3 [PubMed: 15195798]

Bao W, Liu B, Simonsen DW, & Lehmler HJ (2020). Association Between Exposure to Pyrethroid Insecticides and Risk of All-Cause and Cause-Specific Mortality in the General US Adult Population. JAMA Intern Med, 180(3), 367–374. 10.1001/jamainternmed.2019.6019 [PubMed: 31886824]

Barr DB, Wilder LC, Caudill SP, Gonzalez AJ, Needham LL, & Pirkle JL (2005). Urinary creatinine concentrations in the U.S. population: implications for urinary biologic monitoring measurements. Environ Health Perspect, 113(2), 192–200. 10.1289/ehp.7337 [PubMed: 15687057]

Bhatt P, Zhou X, Huang Y, Zhang W, & Chen S (2021). Characterization of the role of esterases in the biodegradation of organophosphate, carbamate, and pyrethroid pesticides. J Hazard Mater, 411, 125026. 10.1016/j.jhazmat.2020.125026 [PubMed: 33461010]

Caudill SP, Schleicher RL, & Pirkle JL (2008). Multi-rule quality control for the age-related eye disease study. Stat Med, 27(20), 4094–4106. 10.1002/sim.3222 [PubMed: 18344178]

Chen S, & Cashman JR (2013). Chapter Six - Organophosphate Exposure: Detection and Remediation. In (Vol. Volume 7, pp. 207–233). Advances in Molecular Toxicology: Elsevier.

Christler A, Felföldi E, Mosor M, Sauer D, Walch N, Dürauer A, & Jungbauer A (2020). Semi-automation of process analytics reduces operator effect. Bioprocess Biosyst Eng, 43(5), 753–764. 10.1007/s00449-019-02254-y [PubMed: 31813007]

Chrustek A, Hoły ska-Iwan I, Dziembowska I, Bogusiewicz J, Wróblewski M, Cwynar A, & Olszewska-Słonina D (2018). Current Research on the Safety of Pyrethroids Used as Insecticides. Medicina (Kaunas), 54(4). 10.3390/medicina54040061

Davis MD, Wade EL, Restrepo PR, Roman-Esteva W, Bravo R, Kuklenyik P, & Calafat AM (2013). Semi-automated solid phase extraction method for the mass spectrometric quantification of 12 specific metabolites of organophosphorus pesticides, synthetic pyrethroids, and select herbicides in human urine. J Chromatogr B Analyt Technol Biomed Life Sci, 929, 18–26. 10.1016/j.jchromb.2013.04.005

Fišerová PS, Kohoutek J, Degrendele C, Dalvie MA, & Klánová J (2021). New sample preparation method to analyse 15 specific and non-specific pesticide metabolites in human urine using LC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci, 1166, 122542. 10.1016/j.jchromb.2021.122542

Gao B, Poma G, Malarvannan G, Dumitrascu C, Bastiaensen M, Wang M, & Covaci A (2022). Development of an analytical method based on solid-phase extraction and LC-MS/MS for the monitoring of current-use pesticides and their metabolites in human urine. J Environ Sci (China), 111, 153–163. 10.1016/j.jes.2021.03.029 [PubMed: 34949345]

Garí M, González-Quinteiro Y, Bravo N, & Grimalt JO (2018). Analysis of metabolites of organophosphate and pyrethroid pesticides in human urine from urban and agricultural populations (Catalonia and Galicia). Sci Total Environ, 622-623, 526–533. 10.1016/j.scitotenv.2017.11.355 [PubMed: 29220776]

Huen K, Bradman A, Harley K, Yousefi P, Boyd Barr D, Eskenazi B, & Holland N (2012). Organophosphate pesticide levels in blood and urine of women and newborns living in an agricultural community. Environ Res, 117, 8–16. 10.1016/j.envres.2012.05.005 [PubMed: 22683313]

Jagani R, Pulivarthi D, Patel D, Wright RJ, Wright RO, Arora M, … Andra SS (2022). Validated single urinary assay designed for exposomic multi-class biomarkers of common environmental exposures. Anal Bioanal Chem, 414(19), 5943–5966. 10.1007/s00216-022-04159-4 [PubMed: 35754089]

Kaushal J, Khatri M, & Arya SK (2021). A treatise on Organophosphate pesticide pollution: Current strategies and advancements in their environmental degradation and elimination. Ecotoxicol Environ Saf, 207, 111483. 10.1016/j.ecoenv.2020.111483 [PubMed: 33120277]

Li AJ, & Kannan K (2018). Urinary concentrations and profiles of organophosphate and pyrethroid pesticide metabolites and phenoxyacid herbicides in populations in eight countries. Environ Int, 121(Pt 2), 1148–1154. 10.1016/j.envint.2018.10.033 [PubMed: 30808487]

Liang D, Liu W, Raza R, Bai Y, & Liu H (2019). Applications of solid-phase micro-extraction with mass spectrometry in pesticide analysis. J Sep Sci, 42(1), 330–341. 10.1002/jssc.201800804 [PubMed: 30444314]

Liu H, Campana AM, Wang Y, Kannan K, Liu M, Zhu H, … Ghassabian A (2021). Organophosphate pesticide exposure: Demographic and dietary predictors in an urban pregnancy cohort. Environ Pollut, 283, 116920. 10.1016/j.envpol.2021.116920 [PubMed: 33839620]

López-García M, Romero-González R, & Garrido Frenich A (2019). Monitoring of organophosphate and pyrethroid metabolites in human urine samples by an automated method (TurboFlow™) coupled to ultra-high performance liquid chromatography-Orbitrap mass spectrometry. J Pharm Biomed Anal, 173, 31–39. 10.1016/j.jpba.2019.05.018 [PubMed: 31108421]
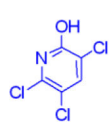
Nolan RJ, Rick DL, Freshour NL, & Saunders JH (1984). Chlorpyrifos: pharmacokinetics in human volunteers. Toxicol Appl Pharmacol, 73(1), 8–15. 10.1016/0041-008x(84)90046-2 [PubMed: 6200956]

O'Brien KM, Upson K, & Buckley JP (2017). Lipid and Creatinine Adjustment to Evaluate Health Effects of Environmental Exposures. Curr Environ Health Rep, 4(1), 44–50. 10.1007/s40572-017-0122-7 [PubMed: 28097619]

Olsson AO, Baker SE, Nguyen JV, Romanoff LC, Udunka SO, Walker RD, … Barr DB (2004). A liquid chromatography--tandem mass spectrometry multiresidue method for quantification of specific metabolites of organophosphorus pesticides, synthetic pyrethroids, selected herbicides, and deet in human urine. Anal Chem, 76(9), 2453–2461. 10.1021/ac0355404 [PubMed: 15117183]

Olsson AO, Nguyen JV, Sadowski MA, & Barr DB (2003). A liquid chromatography/electrospray ionization-tandem mass spectrometry method for quantification of specific organophosphorus pesticide biomarkers in human urine. Anal Bioanal Chem, 376(6), 808–815. 10.1007/s00216-003-1978-y [PubMed: 12811448]

Pitzer EM, Williams MT, & Vorhees CV (2021). Effects of pyrethroids on brain development and behavior: Deltamethrin. Neurotoxicol Teratol, 87, 106983. 10.1016/j.ntt.2021.106983 [PubMed: 33848594]

Robinson AP, Simpson DM, & Johnson WG (2012). Summer Annual Weed Control with 2,4-D and Glyphosate. Weed Technology, 26(4), 657–660, 654.

Roca M, Leon N, Pastor A, & Yusà V (2014). Comprehensive analytical strategy for biomonitoring of pesticides in urine by liquid chromatography–orbitrap high resolution masss pectrometry. J Chromatogr A, 1374, 66–76. 10.1016/j.chroma.2014.11.010 [PubMed: 25499061]

Sharma A, Kumar V, Shahzad B, Tanveer M, Sidhu GPS, Handa N, … Thukral AK (2019). Worldwide pesticide usage and its impacts on ecosystem. SN Applied Sciences, 1(11), 1446. 10.1007/s42452-019-1485-1

Silva Pinto BG, Marques Soares TK, Azevedo Linhares M, & Castilhos Ghisi N (2020). Occupational exposure to pesticides: Genetic danger to farmworkers and manufacturing workers - A meta-analytical review. Sci Total Environ, 748, 141382. 10.1016/j.scitotenv.2020.141382 [PubMed: 32818891]

Simaremare SRS, Hung CC, Hsieh CJ, & Yiin LM (2019). Relationship between Organophosphate and Pyrethroid Insecticides in Blood and Their Metabolites in Urine: A Pilot Study. Int J Environ Res Public Health, 17(1). 10.3390/ijerph17010034

Starr J, Graham S, Stout D, Andrews K, & Nishioka M (2008). Pyrethroid pesticides and their metabolites in vacuum cleaner dust collected from homes and day-care centers. Environ Res, 108(3), 271–279. 10.1016/j.envres.2008.07.022 [PubMed: 18790476]

Taylor JK (1987). Quality assurance of chemical measurements. Lewis Publishers, Chelsea, MI.

U.S.-CDC. (2023). Centers for Disease Control and Prevention, U.S. Department of Health and Human Services. National Report on Human Exposure to Environmental Chemicals. Updated April 2023. Retrieved August 14th, 2023 from https://www.cdc.gov/exposurereport/

U.S.-FDA. (2018). Bioanalytical Method Validation Guidance for Industry. Center for Drug Evaluation and Research (CDER). Retrieved August 16th, 2022, from https://www.fda.gov/media/70858/download. (Accessed 12 August 2022).

UNEP. (2021). Environmental and Health Impacts of Pesticides and Fertilizers and Ways of Minimizing Them. Envisioning a chemical-safe world. In U. E. Programme (Ed.), (pp. 24). https://wedocs.unep.org/xmlui/bitstream/handle/20.500.11822/34463/JSUNEPPF.pdf?sequence=13: United Nations Environmental Programme.

USGS. (2023). Estimated Annual Agricultural Pesticide Use. United States Geological Survey. Retrieved August 7th, from

Viglino L, Aboulfadl K, Mahvelat AD, Prévost M, & Sauvé S (2008). On-line solid phase extraction and liquid chromatography/tandem mass spectrometry to quantify pharmaceuticals, pesticides and some metabolites in wastewaters, drinking, and surface waters. J Environ Monit, 10(4), 482–489. 10.1039/b800861b [PubMed: 18385869]

Wang CJ, Liu HJ, Chou TC, Lin CC, Pan WH, Chen PC, & Hsu JF (2022). Simultaneous determination of eight β-adrenergic agonists in human urine by an isotope dilution-online clean-

up system coupled with liquid chromatography-tandem mass spectrometry. Chemosphere, 301, 134778. 10.1016/j.chemosphere.2022.134778 [PubMed: 35504472]
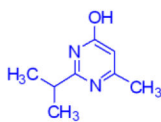
Weaver VM, Kotchmar DJ, Fadrowski JJ, & Silbergeld EK (2016). Challenges for environmental epidemiology research: are biomarker concentrations altered by kidney function or urine concentration adjustment? J Expo Sci Environ Epidemiol, 26(1), 1–8. 10.1038/jes.2015.8 [PubMed: 25736163]

Woollen BH, Marsh JR, Laird WJ, & Lesser JE (1992). The metabolism of cypermethrin in man: differences in urinary metabolite profiles following oral and dermal administration. Xenobiotica, 22(8), 983–991. 10.3109/00498259209049904 [PubMed: 1413886]

Yeh HC, Lin YS, Kuo CC, Weidemann D, Weaver V, Fadrowski J, … Navas-Acien A (2015). Urine osmolality in the US population: implications for environmental biomonitoring. Environ Res, 136, 482–490. 10.1016/j.envres.2014.09.009 [PubMed: 25460670]
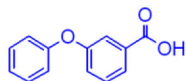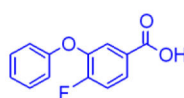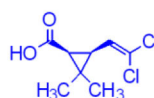
Figure 1.
Urinary metabolite structures and CAS numbers
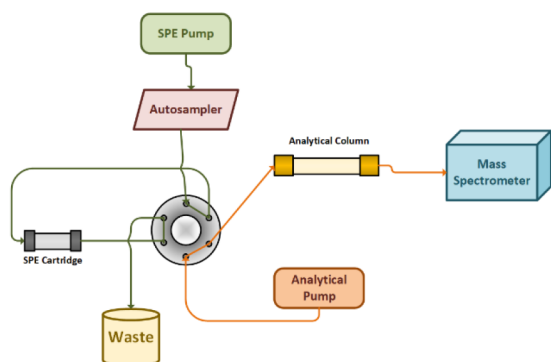
Step 1_Sample load into SPE column and equilibration of analytical column
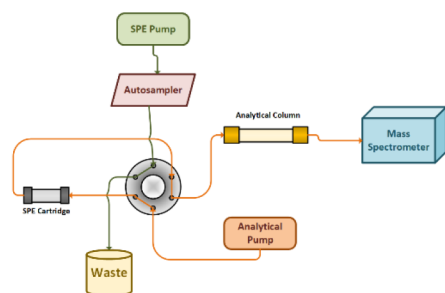
Step 2_Analyte transfer from SPE to analytical column
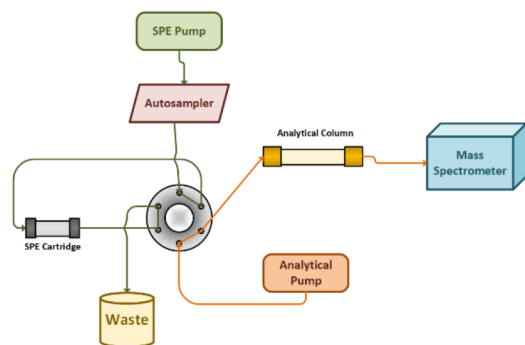
Step 3_Analyte elution from analytical column into the mass spectrometer and clean up and equilibration of SPE column

Figure 2.
Dual pump switching scheme for SPE clean up and chromatography steps
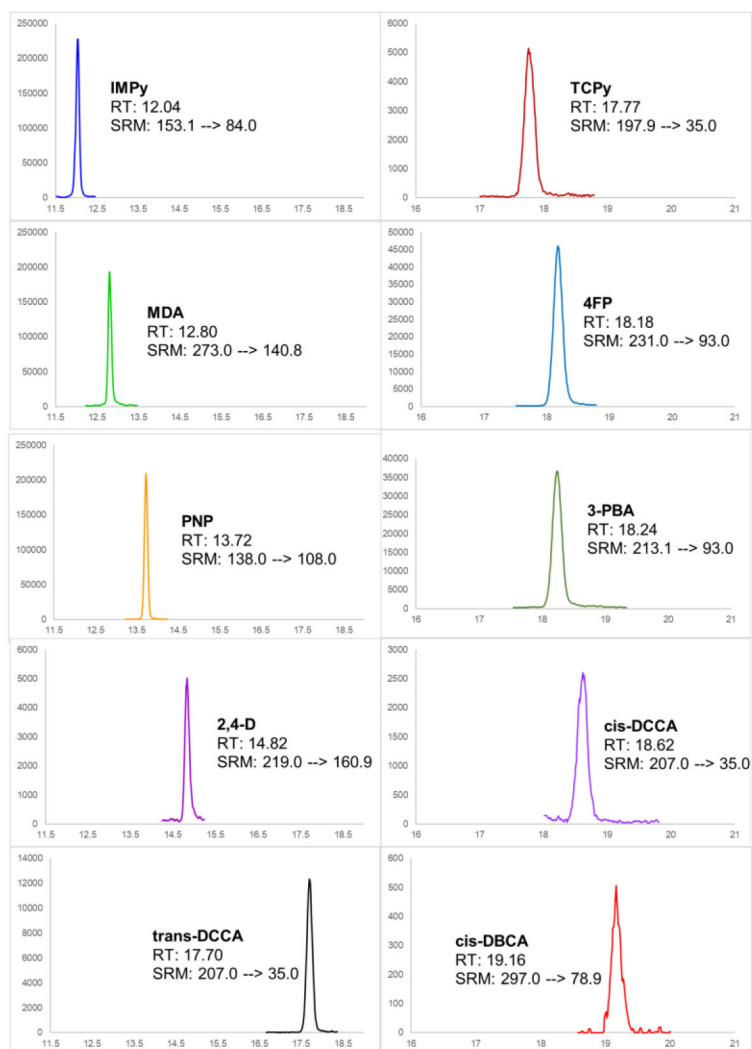
Figure 3.
Extracted ion chromatograms of a fortified low QC in urine. Concentrations for the analytes range from 0.5 to 2.1 ng/mL as shown in Table 3. RT: retention time in minutes. SRM: selected reaction monitoring ions.

Table 1.

Analytical methods based on liquid chromatography-mass spectrometry for analysis of pesticide metabolites.

Author	Sample preparation	Instrumentation	Sample volume (mL)
Current work	Online SPE	LC-MS/MS	0.20
Gao et al., 2022	Offline SPE	LC-MS/MS	1.00
Jagani et al., 2022	Offline SPE	LC-MS/MS	0.20
Fišerová et al., 2021	Offline SPE	LC-MS/MS	0.50
López-García et al., 2019	Offline SPE, TurboFlow, Online SPE	LC–HRMS	10, 0.5, 0.5
Li & Kannan, 2018	Offline SPE	LC-MS/MS	0.50
Garí et al., 2018	Offline SPE	LC-MS/MS	1.00
Roca et al., 2014	QuEChERS	LC–HRMS	5.00
Davis et al., 2013	Offline SPE	LC-MS/MS	1.00
Baker et al., 2004	Offline SPE	LC-MS/MS	5.00
Olsson et al., 2004	Offline SPE	LC-MS/MS	2.00
Olsson et al., 2003	Offline SPE	LC-MS/MS	2.00
Baker et al., 2000	Liquid-Liquid Extraction	LC-MS/MS	10.00

Table 2.

Parameters for selected reaction monitoring

Analyte	Retention Time (min)	Polarity	Precursor ion (m/z)	Product ion (m/z)	CE (V)
2,4-D_Q	14.8	–	219	160.9	15.31
2,4-D_C	14.8	–	219	124.9	27.4
2,4-D_L	14.8	–	225	166.9	14.59
3-PBA_Q	18.44	–	213.1	93	22.28
3-PBA_C	18.44	–	213.1	169.1	12.62
3-PBA_L	18.44	–	219.1	98.9	26.95
4FP_Q	18.4	–	231	93	25.62
4FP_C	18.4	–	231	187	14.02
4FP_L	18.4	–	237.1	193.1	13.6
cis-DBCA_Q	19.46	–	297	78.9	10.23
cis-DBCA_C	19.46	–	297	80.9	10.23
cis-DBCA_L	19.46	–	300	78.9	15
cis-DCCA_Q/*trans*-DCCA_Q	18.4	–	207	35	11.71
cis-DCCA_C/*trans*-DCCA_C	18.4	–	208.9	37	11.71
cis-DCCA_L/*trans*-DCCA_L	18.4	–	212	37	10.23
IMPy_Q	12.1	+	153.1	84	18.11
IMPy_C	12.1	+	153.1	70.1	20.46
IMPy_L	12.1	+	157.1	88.1	18.45
MDA_Q	13	–	273	140.8	10.23
MDA_C	13	–	273	157	18.91
MDA_L	13	–	277	140.8	10.23
PNP_Q	13.8	–	138	108	17.32
PNP_C	13.8	–	138	91.9	23.69
PNP_L	13.8	–	144	114	18.07
TCPy_Q	17.9	–	197.9	35	15.42
TCPy_C	17.9	–	197.9	37	16.67
TCPy_L	17.9	–	198.9	35	15.95

CE: collision energy in volts, _Q: quantitation ion, _C: confirmation ion, _L: labeled analog quantitation ion.

Table 3.

Method validation data.

Analyte	LOD (ng/mL)	Accuracy[a]				Total Precision [b]	
		5 ng/mL	10 ng/mL	20 ng/mL	30 ng/mL	QCL ng/mL (%RSD)	QCH (ng/mL) (%RSD)
2,4-D	0.15	93.8	96.6	98.9	102	0.6 (8.6)	4.4 (8.0)
3-PBA	0.1	90.8	95.8	98.7	101	0.4 (8.7)	3.9 (7.3)
4FP	0.1	92.7	96.2	99.1	101	0.5 (8.3)	3.9 (7.3)
cis-DBCA	0.5	92.9	95.4	95.5	99.8	1.7 (11.5)	10.9 (9.8)
cis-DCCA	0.5	93.7	97.2	96.1	99.4	2.1 (9.4)	9.2 (9.2)
IMPy	0.1	94.1	97.0	100	102	0.8 (6.7)	5.4 (5.9)
MDA	0.5	90.5	93.7	93.9	95.3	1.3 (11.5)	7.1 (7.4)
PNP	0.1	92.5	95.1	95.7	98.2	0.5 (7.8)	6.5 (6.4)
TCPy	0.1	95.1	97.9	97.4	99.1	0.6 (11.3)	10.5 (9.3)
trans-DCCA	0.6	97.2	99.7	99.1	102	2.1 (7.6)	9.7 (7.9)

LOD: Limit of detection; RSD=Relative standard deviation

[a] N=6 three replicate samples prepared in two individual runs

[b] N = 20, two identical instrument set-ups used by two analysts over one month.

Table 4.

Urinary pesticide metabolites concentrations (ng/mL)

Analyte	Self-Reported Organic Diet (N=50)					Self-Reported Use of Pesticides (N=40)				
	%>LOD	Min	Max	Median	IQR	%>LOD	Min	Max	Median	IQR
2,4-D	69%	<LOD	1.9	0.23	0.36	85%	<LOD	1.2	0.24	0.27
3-PBA	100%	0.16	19	0.78	2.1	97%	<LOD	26	0.76	1.5
4FP	18%	<LOD	1.5	0.07	0	13%	<LOD	0.65	0.07	0
cis-DBCA	5%	0.35	3.7	0.35	0	5%	0.35	0.85	0.35	0
cis-DCCA	43%	0.35	5.7	0.35	0.37	44%	0.35	17	0.35	0.75
IMPy	41%	<LOD	1.3	0.070	0.16	28%	<LOD	2.0	0.07	0.039
MDA	24%	0.35	2.8	0.35	0	6%	0.35	58	0.35	0
PNP	100%	0.16	4.4	0.68	1.09	100%	0.14	5.9	0.94	1.2
TCPy	100%	0.38	4.9	1.4	1.8	100%	0.18	18	1.5	2.5
trans-DCCA	51%	0.42	12	0.64	0.88	54%	0.42	53	0.67	1.8

LOD=Limit of detection

%>LOD: percentage of samples above LOD

IQR=interquartile range

LOD= 0.1 ng/mL for 4FP, TCPy, 3PBA, IMPY, and PNP; 0.5 ng/mL for cis-DBCA, cis-CCCA, and MDA; 0.15 ng/mL for 2,4-D; and 0.6 ng/mL for trans-DCCA.