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Simultaneous Determination of Pyrethroid, Organophosphate and Carbamate Metabolites in Human Urine by Gas Chromatography–Mass Spectrometry (GCMS)

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Abstract: We have developed a rapid, sensitive, and reliable method for simultaneous determination of the urinary metabolites of common insecticides in a single analytical run using gas chromatography–mass spectrometry (GCMS). Thirteen metabolites, one originating from carbamate, six from organophosphates, and seven from pyrethroids, were selected for method validation. Samples at different concentrations (0.5–15 μg/L) were prepared by mixing working solutions containing the analytes with blank urine. After acid hydrolysis for 45 min at 90 °C, samples were processed with liquid–liquid extraction and derivatization by *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) before analysis on GCMS. The limits of detection for all thirteen analytes were below 0.1 μg/L. The recovery rates, evaluated at two concentrations (1, 10 μg/L), were found to be 90.48%, on average. The precision of multiple analyses at three different concentrations (0.5, 5, 15 μg/L) within one day or between 10 days was evaluated, and the resultant relative standard deviations were 8.1% or under. We also applied this method to analyze genuine urine samples collected from 30 human subjects, and successfully detected all the metabolites, with detection frequencies more than 50% for pyrethroid metabolites. In summary, this method is not only as good as others in performance, but is advantageous in terms of cost effectiveness and multiplicity of analytes.

Keywords: carbamate; GCMS; liquid–liquid extraction; metabolites; organophosphate; pyrethroid; urine

1. Introduction

There has been a long history for human beings using a variety of pesticides for agricultural purposes. Other than herbicides and fungicides, insecticides are usually primarily used. Carbamate, organophosphate, and pyrethroids are the most common ingredients of commercial insecticide products used in the residential environments, and have recently become the most popular target insecticides in the domain of risk assessment because of the adverse effects [1]. Carbamate insecticides (CIs) serve as enzyme inhibitors of insects, but could directly bind to the melatonin receptors of mammals to cause an imbalance in circadian rhythm. It has been found that CIs could enhance the risk of diabetes to humans. Organophosphates (OPs) are inhibitors of the neuromuscular enzyme receptor (e.g., acetylcholinesterase) of insects, and also function similarly in humans and many other animals [2]. Recent studies have shown the neurotoxic effects of OPs on children, including behavior change, learning rates, normal physical coordination, and attention deficit hyperactivity disorder, which can be triggered even by routine exposure to low levels of OPs [2–4]. Some OPs, showing a

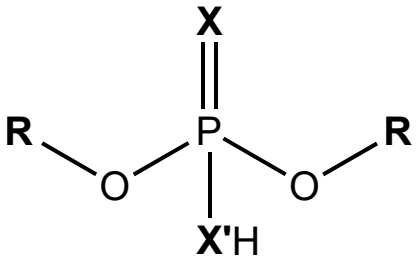
relation with cancer, are regarded as possible carcinogens, as suggested by the International Agency for Research on Cancer (IARC) [5,6].

Pyrethroids (PYRs) are artificial synthetic products of pyrethrins, which have been one of the oldest and most widely used botanical insecticides applied in the control of lice on humans and animals for more than 150 years [1,7]. Artificial pyrethroids, instead of natural pyrethrins, became the major commercial household insecticides worldwide because of their stability to photolysis. The reversible health effects of PYR exposure include allergies of skin and mucosa, headache, and dizziness [8]. In 1999, the US Environmental Protection Agency (EPA) classified PYRs as "likely to be a human carcinogen by the oral route" [9].

Once entering the human body, these insecticides are metabolized and excreted in urine, which can be used for biomonitoring of insecticide exposure [10]. There are six major urinary metabolites of OPs that are the most commonly measured, including dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), dimethyl dithiophosphate (DMDTP), diethyl phosphate (DEP), diethyl thiophosphate (DETP), and diethyl dithiophosphate (DEDTP) (Figure 1a) [11,12]. The major urinary metabolite of CIs is 7-phenol 2,3-dihydro-2,2-dimethyl-7-benzofuranol, also known as 7-phenol [13]. The metabolites of common commercial PYRs are *trans*-chrysanthemumdicarboxylic acid (*trans*-CDCA), *cis*- and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (*cis*/*trans*-DCCA), *cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylic acid (*cis*-DBCA), 3-phenoxybenzoic acid (3-PBA), and 4-fluoro-3-phenoxybenzoic acid (F-BPA) via ester cleavage of original compounds (Figure 1b) [14,15]. A summary of the main metabolites of commerical carbamate, organophosphate, and pyrethroid insecticides is shown in Table 1.

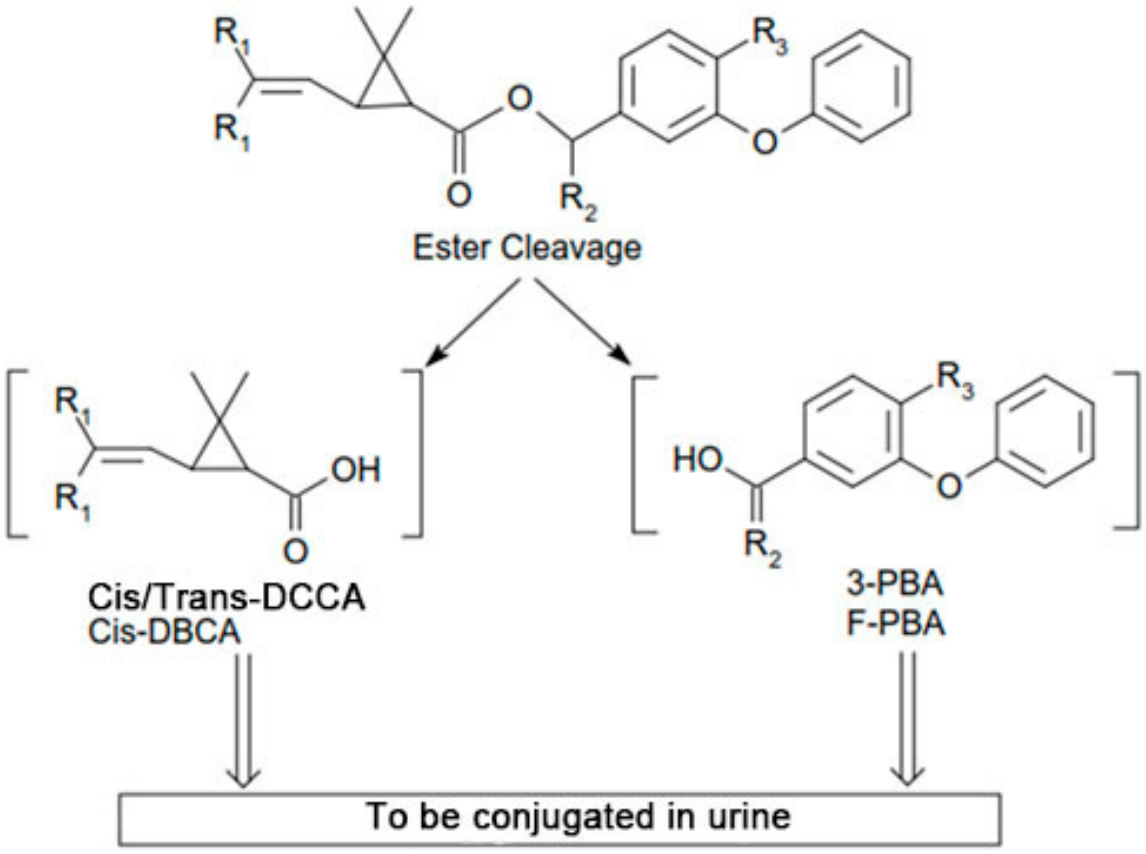
The main assay methods for urinary metabolites of these three insecticides include immunoassay, high performance liquid chromatography–mass spectrometry (LCMS), and gas chromatography–mass spectrometry (GCMS). The immunoassay method is easier to apply and less costly than LCMS or GCMS, but the lower sensitivity and specificity mean it is not favored for precise and accurate analyses [16]. The main LCMS assay method was established in 2004, and became popular recently because of the relatively easy pretreatment of sample extraction [17]; the disadvantage, however, is probably the cost of sample analysis and routine maintenance of LCMS, which is the highest among the assay methods. The early GCMS assay methods with relatively low cost were favored but limited to applications analyzing OP or PYR metabolites individually, and also had relatively high limits of detection and quantification (LODs and LOQs) [18,19]. Simultaneous determination of both insecticide metabolites in human urine was later developed using GC–tandem mass spectrometry (GC/MS/MS), but only analyzed eight metabolites (five from OPs and three from PYRs) [20].

In this study, we developed a rapid, sensitive, and cost-effective extraction assay method using liquid–liquid extraction (LLE) and regular GCMS for simultaneously detecting and quantifying up to 13 urinary metabolites of the common commercial insecticides, including CIs, OPs, and PYRs. This method shows excellent accuracy and precision, good recovery rates of sample extraction, decent LODs as well as LOQs, and method stability and applicability in testing real human urine samples. We are convinced that this assay method could be an efficient tool to facilitate urinary measurements for assessment of exposure to insecticides.



Compound	R	X	X′
DMP	CH_3	O	O
DMTP	CH_3	S	O
DMDTP	CH_3	S	S
DEP	C_2H_5	O	O
DETP	C_2H_5	S	O
DEDTP	C_2H_5	S	S

(**a**)



(**b**)

Figure 1. (**a**) Chemical structures of organophosphate metabolites. (**b**) The main metabolic pathway of pyrethroids in mammals.

Table 1. The main urinary metabolites of common commercial insecticides.

Metabolite	Parent Insecticide	Classification
3-PBA [a]	Cyhalothrin, cypermethrin, deltamethrin, ethonfenprox, esfenvalerate, fenpropathrin, permethrin, phenothrin	PYR
cis/*trans*-DCCA [b]	Cypermethrin, cyfluthrin, permethrin	PYR
cis-DBCA [c]	Deltamethrin	PYR
trans-CDCA [d]	Allethrin, imiprothrin, phenothrin, prallethrin, resmethrin, tetramethrin	PYR
FPBA [e]	Cyfluthrin	PYR
DMP [f]	Azinphos methyl, chlorpyrifos methyl, dichlorvos (DDVP), dicrotophos, dimethoate, fenitrothion, fenthion, methyl parathion, oxydemeton-methyl, phosmet, pirimiphos-methy, temephos, naled, tetrachlorviphos, trichlorfon	OP
DMTP [g]	Azinphos methyl, chlorpyrifos methyl, dimethoate, isazaphos-methyl, fenitrothion, fenthion, methyl parathion, oxydemeton-methyl, phosmet, pirimiphos-methy, temephos,	OP
DMDTP [h]	Azinphos methyl, dimethoate, malathion, phosmet,	OP
DEP [i]	chlorethoxyphos, chlorpyrifos, coumaphos, diazinon, disulfoton, ethion, malathion, parathion, phorate, sulfotepp, terbufos,	OP
DETP [j]	Chlorethoxyphos, chlorpyrifos, coumaphos, diazinon, disulfoton, ethion, parathion, phorate, sulfotepp, terbufos,	OP
DEDTP [k]	Disulfoton, ethion, phorate, terbufos,	OP
7-phenol [l]	Carbofuran	CI

[a] 3-PBA: 3-phenoxybenzoic acid; [b] *cis*/*trans*-DCCA: *cis*- and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid; [c] *cis*-DBCA: *cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylic acid; [d] *trans*-CDCA: *trans*-chrysanthemumdicarboxylic acid; [e] FPBA: 4-fluoro-3-phenoxybenzoic acid; [f] DMP: dimethylphosphate; [g] DMTP: dimethyl thiophosphate; [h] DMDTP: dimethyl dithiophosphate; [i] DEP: diethyl phosphate; [j] DETP: diethyl dithiophosphate; [k] DEDTP: diethyl dithiophosphate; [l] 7-phenol: 7-phenol 2,3-dihydro-2,2-dimethyl-7-benzofuranol.

2. Materials and Methods

2.1. Chemicals and Materials

We analyzed 13 metabolites and used 2-phenoxybenzoic acid (2-PBA) as an internal standard (Table 1). The metabolite compounds and other reagents were purchased from various vendors. *cis*/*trans*-DCCA and DMTP were obtained from Toronto Research Chemicals (TRC, Toronto, Canada); *cis*-DBCA was obtained from Bayer AG (Leverkusen, Germany); 3-PBA, 2-PBA, F-BPA, and DMDTP were obtained from Alfa Aesar (Lancashire, United Kingdom); DMP and DEP were obtained from ACROS (Geel, Belgium); DETP was obtained from Tokyo Chemical Industry Co. (TCI, Tokyo, Japan); *trans*-CDCA, DEDTP, *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA, used for derivatization), 7-phenol, analytical grade *n*-hexane, analytical grade acetonitrile, analytical grade methanol, and hydrogen chloride (97%) were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

2.2. Equipment

2.2.1. Regular Equipment

Volumetric flasks (Witeg: Wertheim, Germany), 5, 10, and 15 mL amber glass tubes with PTFE screw cap (Kimble, Vineland, NJ, USA), amber microvials (Agilent, Santa Clara, CA, USA), pipettes and tips (Rainin, Greifensee, Switzerland) were used for sample distribution and storage. Heater plates (Corning, Corning, NY, USA), a vortex mixer (Fisher Scientific, Hampton, NH, USA), a low temperature centrifuge (ThermoFisher, Waltham, MA, USA), and a miVac Duo centrifugal sampler concentrator (SP Scientific, Ipswich, Suffolk, UK) were used for sample treatment.

2.2.2. GCMS Operating Condition

Processed samples were analyzed in selection ion model (SIM) using an Agilent 6890 gas chromatograph (Agilent, Santa Clara, CA, USA) coupled with an Agilent 5973-II mass spectrometer (Agilent, Santa Clara, CA, USA) and equipped with an autosampler (Agilent, Santa Clara, CA, USA). The inlet condition was set at 270 °C with a split model (20:1), and 99.995% helium was used as carrier gas at a constant flow of 0.8 mL/min through a Restek RTX-35 column (30 m × 0.25 mm × 0.25 µm, Restek, Bellefonte, PA USA). The injection volume was 1 µl with a pre- and post-washing program to avoid cross-contamination. Oven condition was set to start and hold for 1 min at 70 °C, to increase temperature to 160 °C at a rate of 8 °C/min and to 300 °C at 10 °C/min, and to hold for 2 min at 300 °C; the post-run was set for 3 min at 330 °C. For the mass spectrometry conditions, electron energy was set at 70 eV and the ion source was at 250 °C with 260 °C interface. We applied one deionized water sample as blank to ensure that there was no interference with SIM detection, and the mass spectrometer was calibrated every 7 days or after 100 sample runs.

2.3. Internal Standard Solution

To prepare the stock solution of the internal standard (IS), 10 mg of 2-PBA was dissolved in 10 mL of methanol (1000 mg/L). One milliliter of the stock solution was diluted in a 100 mL glass volumetric flask with fresh deionized water to be used as the internal standard solution (10 mg/L). The 2-PBA IS solution was used to spike urine samples (see the following Section 2.4, Section 2.5, and Section 2.7).

2.4. Sample Extraction

2.4.1. Acid Hydrolysis

Each 2 mL urine sample was spiked with 20 µL IS solution in a 10 mL amber screw cap tube. Acidic hydrolysis was performed by adding 5 mL hydrogen chloride (50%) and heating in a 90 °C water bath for 45 min for deconjugation.

2.4.2. Liquid–Liquid Extraction (LLE)

After cooling acidified urine sample to room temperature, 5 mL of *n*-hexane was added to each, followed by shaking well for 10 min using a vortex system, followed by centrifugation at 3000× *g* for 5 min. The upper organic layer was collected and transferred into a 15 mL amber screw cap tube, and 5 mL *n*-hexane was added to the remainder for re-extraction, to minimize sample loss. The total 10 mL organic layer was concentrated and dried in a miVac Duo concentrator using a programmed temperature control system set at 40 °C under 1 kPa for 30 min.

2.4.3. Derivatization

The residue was dissolved with 100 µL acetonitrile in an amber microvial, and 20 µL MTBSTFA (>97%) was added. After gentle mixing at room temperature (25 °C) for 3 min using a vortex mixer, the solution was heated for approximately 45 min at 80 °C for derivatization. The final solution was centrifuged at 3000*g* for 5 min, and the organic layer was transferred to an amber microvial. All processed samples were stored at −20 °C prior to analysis.

2.5. Calibration and Preparation Procedures

Thirteen separate starting solutions were prepared by dissolving 10 mg of each metabolite compounds, which were 3-PBA, *cis*/*trans*-DCCA, *cis*-DBCA, *trans*-CDCAPBA, DMP, DMTP, DMDTP, DEP, DETP, DEDTP, and 7-phenol, with acetonitrile in thirteen separate 10 mL glass volumetric flasks (1 g/L). The multicomponent stock solution was prepared by adding 100 µL of each starting solution into a 10 mL glass volumetric flask and filling with acetonitrile to the mark (10 mg/L). Two working solutions

of different concentrations were prepared by this multicomponent stock solution with acetonitrile, and other solutions used in this study were prepared following the methods shown as follows:

Working solution I

One milliliter of the multicomponent stock solution was diluted in a 20 mL glass volumetric flask (500 μg/L).

Working solution II

One hundred microliter (100 μL) of the multicomponent stock solution was diluted in a 20 mL glass volumetric flask (50 μg/L).

Blank I

We collected urine from 10 volunteers who were not exposed to any insecticides in Hualien city (eastern Taiwan, ROC), pooled them together followed by dilution with distilled deionized water (1:1 (v/v)), and filtered the diluted pooled urine through a 0.2 μm syringe filter. The filtered sample was extracted and screened for the target metabolites using the previously described method to confirm the blankness. The Blank I sample was dispensed into several 100 mL glass volumetric flasks and stored at −20 °C.

Blank II

We also collected urine from 15 other non-exposed volunteers residing in Hsinchu city, Taichung city, and Changhua city (western Taiwan, ROC), pooled them together followed by dilution with distilled deionized water (1:1 (v/v)), and filtered the diluted pooled urine through a 0.2 μm syringe filter. The filtered sample was confirmed to be blank, dispensed into different 100 mL glass volumetric flasks, and stored at −20 °C.

Calibration standards (concentration range: 0.5 to 150 μg/L)

The standards were prepared from two working solutions. Nine calibration concentrations were prepared in acetonitrile, between 0.5 and 150 μg/L (i.e., 0.5, 1, 2, 5, 10, 20, 50, 100, and 150 μg/L). Aliquots of 2 mL Blank I were processed with acid hydrolysis and LLE, and the residues after concentration were added separately to standards at different concentrations. The prepared standard solutions were processed with derivatization prior to GCMS analysis.

Samples for various tests were prepared in low (L), medium (M), and high (H) concentrations. The detailed preparation methods are described as follows.

Group A

Multicomponent testing samples of two concentrations were prepared by spiking working solution II with Blank I (Group-AL: 0.5 μg/L, n = 10; Group-AH: 5 μg/L, n = 10).

Group B

Group B is designed for testing the extraction effectiveness as compared to Group A, serving as control. The concentrations were prepared to be as same as in Group A (0.5 and 5 μg/L), but with a different preparation method that was the same as the preparation of standards. The urinary residues of Blank I after acid hydrolysis, LLE, and concentration were supplemented with 0.5 and 5 μg/L in acetonitrile to become Group BL (0.5 μg/L, n = 10) and Group BH (5 μg/L, n = 10), respectively. The Group B solutions were then processed with derivatization, followed by GCMS analysis.

Group C

Group C was used for testing the within-day precision. The multicomponent test samples at three concentrations were prepared by spiking working solution II with Blank I (Group DL: 0.5 μg/L, n = 5; Group DM: 5 μg/L, n = 5; Group DH: 15 μg/L, n = 5).

Group D

Group D was used to test whether there was a difference between Blank I and II. The multicomponent testing samples with the same concentrations as in Group CL were prepared by spiking working solution II with Blank II (0.5 μg/L, n = 5).

Group E

Group E was prepared for testing the between-day precision. The multicomponent testing samples were considered duplicates of those in Group C, with the same preparation method, but used to check the precision between days (Group-EL: 0.5 μg/L, n = 5; Group-EM: 5 μg/L, n = 5; Group-EH: 15 μg/L, n = 5).

2.6. Storage of Sample Solutions

The stock solutions and working solutions could be stored at −20 °C for more than 6 months. Urine samples could be stored at −80 °C for more than 1 year. The final samples after derivatization could also be stored at 4 °C for more than 2 months.

2.7. Method Validation

2.7.1. Efficiency of Extraction (Method Accuracy)

The recovery rate was calculated by dividing the measured concentration of a sample by GCMS analysis by its prepared concentration. The Group AL/H and Group BL/H samples were prepared to be the same in concentration, but the metabolite compounds in the former went through LLE and those in the latter did not. Comparing the recovery rates derived from the two groups could determine whether there was a significant loss during LLE, or the efficiency of extraction.

2.7.2. Method Precision

The method precision was assessed by calculating the relative standard deviations (RSDs) of analyses of multiple samples of the same concentrations. The within-day precision was determined using a total of 20 samples containing Group C and Group D at three concentrations (0.5, 5, and 15 μg/L), which had to be analyzed within 24 hours. The between-day precision was determined using 15 samples of Group E at three concentrations (0.5, 5, and 15 μg/L), and the analyses were scattered throughout a 10-day span.

3. Results and Discussion

3.1. Separation on GCMS Chromatogram, Limit of Detection (LOD), and Limit of Quantification (LOQ)

The 13 insecticide metabolites plus one IS were distinctly eluted between 9 and 22 min (Figure 2), starting with 7-phenol at 9.34 min and ending with 3-PBA at 21.92 min. The selection of RTX-35 column enhanced the resolution for separating *cis*/*trans*-DCCA, and generally reduced noise compared to other similar columns we tried (e.g., HP5-MS, DB5-MS, DB35-MS, RTX-65). LOD and LOQ for each compound in spiked urine samples were calculated by multiplying the signal-to-noise ratio by 3 and 10, respectively. All LOD and LOQ values, with information of retention time and mass detection, were listed in Table 2.

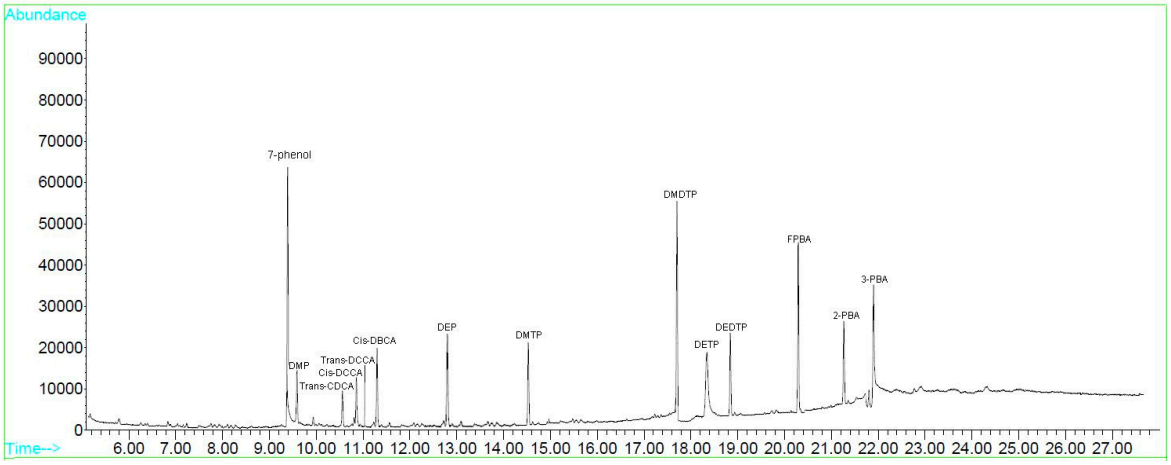


Figure 2. GCMS chromatogram of 13 insecticide metabolites plus one IS in a spiked urine sample (0.1 μg/L).

3.2. Calibration Procedure

All standards at nine different concentrations that were used to set up calibration curves were prepared with blank urine (0.5, 1, 2, 5, 10, 20, 50, 100, and 150 μg/L), and the calibration curves were linear for all 13 pesticide metabolites and one IS. The coefficients of determination (i.e., R^2 values) for calibration curves were higher than 0.995, indicating excellent linear relationships within the detection range.

Table 2. Analytical data of GCMS for tested insecticide metabolites.

Metabolite	RT (min)	DI (*m*/*z*)	LOD (μg/L)	LOQ (μg/L)
7-phenol	9.34	245.0	0.050	0.2
DMP	9.63	306.1	0.050	0.2
DEP	12.81	334.7	0.100	0.3
DMTP	14.54	322.2	0.050	0.2
DMDTP	16.73	338.2	0.100	0.3
DETP	17.38	350.4	0.100	0.3
DEDTP	19.21	366.0	0.050	0.2
trans-CDCA	10.63	331.0	0.050	0.2
cis-DCCA	10.86	323.0	0.025	0.1
trans-DCCA	11.08	323.0	0.025	0.1
cis-DBCA	11.15	368.9	0.025	0.1
FPBA	20.37	364.0	0.025	0.1
2-PBA	21.28	364.0	0.025	0.1
3-PBA	21.92	382.0	0.025	0.1

RT: retention time; DI: detected ion; LOD: limit of detection; LOQ: limit of quantification.

3.3. Accuracy of the Method and Sample Extraction Efficiency

We applied LLE to avoid any possible loss caused by other frequently used extraction methods (e.g., solid phase extraction (SPE)), and used SIM for detection to reduce interference from impurities as well as to enhance the sensitivity. As a result, the recoveries for 0.5 μg/L solutions containing 13 target metabolites were 89.3%~94.5% (average recovery rate: 92.3%), and that for 5 μg/L (93.4% on average) were even better (Table 3). The high recovery rates associated with low RSDs (<7%) for 13 target metabolites plus one IS indicate the accuracy and consistency of the method; the low relative errors (<5%) for comparison between Groups A and B also confirm the excellent LLE efficiency. The merit may have been, in part, attributable to the autoprogrammed vacuum concentration system (miVac Duo concentrator), which is able to reduce the cross-contamination usually caused by the traditional

drying method (i.e., manual use of rotary evaporator and nitrogen drying). Additionally, no significant difference in concentration between Groups A and B (identical to standard solutions) also eliminates any possibility of matrix interference in the analysis.

Table 3. Comparison of recovery rates between Groups A and B (n = 10).

Metabolite	Group A L		Group B L		-	Group A H		Group B H		-
	RR (%)	RSD (%)	RR (%)	RSD (%)	RE (%)	RR (%)	RSD (%)	RR (%)	RSD (%)	RE (%)
3-PBA	94.5	5.3	96.2	2.6	1.77	93.6	5.2	95.4	2.7	1.89
cis-DCCA	93.3	6.1	96.0	3.1	2.81	94.4	2.1	97.4	1.7	3.08
trans-DCCA	94.5	3.2	97.2	2.4	2.78	95.3	2.5	97.7	2.6	2.46
cis-DBCA	90.7	4.2	94.2	4.3	3.72	91.5	2.6	96.2	3.6	4.89
trans-CDCA	92.5	3.4	95.5	2.7	3.14	92.1	4.6	95.9	1.8	3.96
FPBA	89.3	6.5	93.9	3.7	4.90	92.3	4.3	93.2	5.3	0.97
DMP	92.5	3.4	101.5	4.3	8.87	94.5	3.9	97.4	3.3	2.98
DMTP	90.6	4.3	96.5	4.1	6.11	91.3	5.4	95.2	4.2	4.10
DMDTP	94.4	4.3	103.3	2.5	8.62	93.9	2.6	97.7	2.4	3.89
DEP	92.8	3.8	97.6	3.9	4.92	95.5	5.4	97.3	1.1	1.85
DETP	93.4	2.5	94.2	1.1	0.85	93.6	4.3	94.1	2.1	0.53
DEDTP	90.2	3.3	94.4	2.4	4.45	94.4	3.5.	98.5	2.3	4.16
7-phenol	92.6	3.1	96.2	3.5	3.74	92.7	3.9	97.5	3.1	4.92
2-PBA	91.5	4.6	92.5	3.3	1.08	92.4	4.1	92.1	3.2	0.33
Average	92.34	4.14	96.37	3.14	4.13	93.39	3.92	96.11	2.81	2.86

L: 0.5 μg/L; H: 5 μg/L; RR: recovery rate; RSD: relative standard deviation; RE: relative error.

3.4. Precision of the Method

We analyzed Group C samples prepared with pooled blank urine (Blank I) at three concentrations (0.5, 5, and 15 μg/L) within 24 h to determine the RSD representing the within-day precision. The RSDs for the low concentration (0.5 μg/L) ranged between 1.7% and 5.2%, and that for the other two concentrations (5 and 15 μg/L) also yielded good results showing no more than 5.2% (Table 4). To confirm the method precision, we used a different source of blank urine (Blank II) to prepare Group D samples for testing. Five samples at 0.5 μg/L were analyzed and resulted in RSDs ranging between 1.6% and 4.8% (Table 4), which were similar to those derived from Group CL; The results of Groups C and D and the mutual comparison indicate that the method is precise in analyzing insecticide metabolites in urine which, as a medium, had no effect on or interference with the analysis.

Table 4. The within-day and between-day precisions of urine samples at different concentrations (n = 5).

Metabolite	Within-Day RSD (%)				Between-Day RSD (%)		
	Group CL	Group CM	Group CH	Group D	Group EL	Group EM	Group EH
3-PBA	2.6	3.9	4.6	4.5	2.9	3.2	3.1
cis-DCCA	4.7	3.4	2.3	3.7	3.5	3.6	2.6
trans-DCCA	4.6	4.5	3.5	4.8	4.2	3.2	3.1
cis-DBCA	4.1	2.8	2.6	3.6	2.8	2.4	2.5
trans-CDCA	3.8	3.3	3.7	2.8	3.4	3.7	3.7
FPBA	4.8	2.6	3.3	3.6	4.5	5.3	3.8
DMP	3.9	4.1	5.1	3.2	3.8	4.1	2.6
DMTP	5.2	3.4	3.5	3.3	2.3	3.4	3.3
DMDTP	4.7	4.1	1.6	2.2	4.6	4.2	3.1
DEP	4.5	3.3	3.5	2.3	3.3	3.1	2.4
DETP	4.4	2.7	1.3	1.6	1.8	2.9	3.1
DEDTP	3.6	1.2	2.4	2.8	2.9	2.3	1.7
7-phenol	1.7	2.3	1.8	3.9	1.0	1.7	1.9
Average	4.05	3.20	3.02	3.25	3.15	3.32	2.84

The between-day precision was determined by Group E samples at three concentrations (0.5, 5, and 15 μg/L). The precision (RSDs) ranged between 1.0% and 4.6% for 0.5 μg/L (n = 10), 1.7% and 5.3% for 5 μg/L (n = 10), and 1.6% and 4.8% for 15 μg/L (n = 10), and indicates that this method is also precise for analysis up to 10 days, during which the processed samples are stable (Table 4).

3.5. Testing Result of Genuine Urine Samples

We used 30 human urine samples to evaluate the applicability of this method. The samples, collected in a human exposure study that was approved by an institutional review board, were originally intended for analysis of insecticide metabolites; thus, this study was in compliance with the relevant laws and institutional guidelines. Data of the 30 human samples are summarized in Table 5, with the average concentrations of the target metabolites being within a range between 2.14 and 17.33 μg/L. Among the metabolites, *cis*/*trans*-DCCA, *cis*-DBCA, *trans*-CDCA, and FPBA were detected with frequencies more than 50%, indicating common exposure to pyrethroids. This finding may result from the use of household insecticides, ingredients of which are usually pyrethroids. A previous study on insecticide residues in the indoor dust of homes in a rural county of Taiwan was in support of our findings in urine, showing frequent detection of a variety of pyrethroids [21]. The mean concentration of spiked IS (i.e., 2-PBA) was found to be 97.14 μg/L, which was not significantly different to the theoretical value (100 μg/L) (P = 0.971), suggesting the applicability of this method to analysis of urinary insecticide metabolites.

Table 5. Data of genuine urine samples (n = 30).

Metabolites	DF (%)	Mean ± SD (μg/L)	Median (μg/L)	Maximum (μg/L)
3-PBA	36.5	11.34 ± 1.15	8.52	23.61
cis-DCCA	61.3	5.66 ± 2.41	2.71	71.30
trans-DCCA	68.5	17.33 ± 5.12	10.19	34.60
cis-DBCA	72.6	16.91 ± 4.12	4.41	24.52
trans-CDCA	57.4	14.74 ± 3.33	7.24	21.69
FPBA	81.3	5.12 ± 2.54	1.25	27.87
DMP	14.5	7.41 ± 5.51	4.23	10.91
DMTP	13.2	4.39 ± 1.16	2.11	5.24
DMDTP	14.1	5.33 ± 1.07	1.36	6.94
DEP	6.6	2.14 ± 3.45	2.45	7.17
DETP	8.8	6.26 ± 2.21	4.47	8.23
DEDTP	3.3	3.33 ± 1.77	1.21	7.11
7-phenol	24.5	7.65 ± 2.36	3.39	34.49
2-PBA	100	97.14 ± 1.26	95.26	104.32

DF: detection frequency; SD: standard deviation.

3.6. Comparison with Other Assay Methods

Urinary metabolites are the most frequently used biomarkers for assessment of exposure to common insecticides. The aim of this study was to develop a rapid, sensitive, and cost-effective method for routine urinary analysis. We used LLE, instead of the commonly used SPE method, to retain as many metabolites in urine as possible; thus, the extraction efficiency could be preserved [22,23]. In the meantime, conducting LLE was relatively time saving, compared to SPE cartridges dripping. Additionally, our method did not need large quantities of organic solvents, SPE cartridges, or expensive equipment, such as tandem mass spectrometry, indicating the cost effectiveness. This assay method is comparable to others, with even more metabolites being analyzed simultaneously than that of others (Table 6).

There are two common methods applied to determine the urinary metabolites of pyrethroids. One is GCMS, the average LOD of which was found to be 0.3~0.5 μg/L before the year 2000, and after, improved to 0.02~0.1 μg/L [18,24,25]. The other is LC-tandem mass spectrometry (LC/MS/MS), the average LOD of which was about 0.015 μg/L [26]. Due to the lower LOD and time consumption

compared with using GCMS, LC/MS/MS has become a more popular analytical method, along with using SPE as a pre-analysis treatment. However, in view of the relative high costs for using LC/MS/MS, this method, using LLE and GCMS, is much more cost effective, albeit with slightly elevated LODs.

We also conducted a comparison with previous studies regarding method precision. Leng showed the within-day RSDs to be between 4.7% and 10% among six metabolites at 0.2 μg/L, and the between-day RSDs to be between 6.7% and 13.3% among five metabolites at 0.2 μg/L [25]. Schettgen demonstrated the between-day RSDs ranging from 13.3% to 17.9% among five metabolites at 0.4 μg/L [24]. Our method resulted in relatively stable results for repetitive routine analyses within 24 hours and over days.

Table 6. Comparison of assay methods for urinary insecticide metabolites among recent studies.

Type	No. of Metabolites	Detected Assay	LOD (μg/L)	Year & Source
OP	6	SPE & GCMS	0.1~0.15	2008 [23]
OP	6	LLE & LC/MS/MS	0.004~0.1	2014 [27]
PYR	5	SPE & GCMS	0.006~0.01	2009 [28]
PYR	5	LLE & LC/MS/MS	0.015	2012 [26]
CI	4	SPE & GC/MS/MS	0.04	2006 [13]
OP & PYR	OP = 5, PYR = 5	SPE & LC/MS/MS	<0.05	2013 [29]
OP & PYR	OP = 5, PYR = 3	SPE & GC/MS/MS	0.008~0.83	2017 [20]
CI, OP & PYRs	CI = 1, OP = 6, PYR = 7	LLE & GCMS	0.025~0.1	This study

SPE: solid phase extraction; LLE: liquid–liquid extraction; MS/MS: tandem mass spectrometry.

4. Conclusions

This assay method has shown high reliability and reproducibility for analyzing urinary insecticide metabolites. Regarding performance, it is as functional as the others, and even better in terms of cost effectiveness and multiplicity of analytes. The results of genuine urine sample analysis showing frequent detection of metabolites of pyrethroids is in agreement with the finding of an environmental study in Taiwan, suggesting the applicability of the method. As the exposure to insecticides in the current climate-changing world is inevitable, we believe that this method could facilitate the assessment of exposure to insecticides considering multiple aspects.

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