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### Published

2019

### Journal Title

Science of the Total Environment

### Version

Accepted Manuscript (AM)

### DOI

[10.1016/j.scitotenv.2019.03.040](https://doi.org/10.1016/j.scitotenv.2019.03.040)

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PII: S0048-9697(19)31018-6  
DOI: <https://doi.org/10.1016/j.scitotenv.2019.03.040>  
Reference: STOTEN 31247  
To appear in: *Science of the Total Environment*  
Received date: 24 January 2019  
Revised date: 28 February 2019  
Accepted date: 3 March 2019

Please cite this article as: Y. Tao, D. Phung, F. Dong, et al., Urinary monitoring of neonicotinoid imidacloprid exposure to pesticide applicators, *Science of the Total Environment*, <https://doi.org/10.1016/j.scitotenv.2019.03.040>

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## Urinary monitoring of neonicotinoid imidacloprid exposure to pesticide applicators

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### Abbreviations

**SPE**, Solid-phase Extraction; **LOD**, Limit of Detection; **LOQ**, Limit of Quantification; **ADI**, Acceptable Daily Intake; **ADD**, Absorbed Daily Dose; **MRM**, Multiple Reaction Monitoring; **ME**, matrix effects; **ESI**, electrospray ionization; **IS**, International Standard; **GM**, Geometrical Mean; **ADI**, acceptable daily intake; **ARfD**, acute reference dose

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**Abstract**

Neonicotinoid pesticides have recently drawn attention worldwide owing to their potential adverse effects on non-target organisms and ecosystems. Exposure to imidacloprid, the most widely used neonicotinoid insecticide, is of particular concern among rural populations because of its ubiquitous use in agriculture. Hence, biological monitoring of urinary imidacloprid and its major metabolite 6-chloronicotinic acid (6-CNA) was performed using Polar Enhanced Polymer solid-phase extraction by LC-MS/MS with mean recoveries of 78.3–109.8% and limits of quantitation at 0.029–0.038 ng/mL. Imidacloprid was detected in 100% of urine samples from rural applicators at concentrations of 0.21–8.91 ng/mL (0.06–9.60 µg/g creatinine) and 0.11–24.58 ng/mL (0.66–57.40 µg/g creatinine) before and after pesticide application, respectively. Significant increase in urine concentration (3.52- to 3.77-fold) of imidacloprid and 6-CNA was observed after local imidacloprid field application ( $p \leq 0.001$ ). The estimated absorbed daily dose (ADD) for imidacloprid was 0.52–248.05 µg/kg/d, indicating that attention should be paid to potential health risks for applicators because of increased imidacloprid exposure at level of significance ( $p < 0.05$ ). This study is the first to report ADD estimation for imidacloprid, thereby providing an important reference for further human health risk evaluation.

**Keywords:** *neonicotinoid; imidacloprid; metabolite; 6-chloronicotinic acid; urine; absorbed daily dose*

## 1. Introduction

Neonicotinoids were initially considered harmless to mammals and served as a promising alternative to highly toxic organophosphate and carbamate insecticides. They are currently the most widely used class of insecticides worldwide (Ead et al., 2017). However, the frequent use of neonicotinoids has resulted in their ubiquitous detection in environmental samples (Song et al., 2018; Sousa et al., 2019; Sultana et al., 2018; Zhou et al., 2018) and organisms (Haroune et al., 2015; Taliansky-Chamudis et al., 2017). Alarming evidence has linked neonicotinoid exposure to global pollinator decline (Rundlöf et al., 2015; Stanley et al., 2015; Whitehorn et al., 2012) and may pose a potential threat to aquatic and terrestrial invertebrates and vertebrates (Gibbons et al., 2015; Hallmann et al., 2014; Pisa et al., 2015; Raby et al., 2018; Vijver et al., 2014), including humans (Wang et al., 2015; Zhang et al., 2018).

Imidacloprid [(2E)-1-((6-chloro-3-pyridinyl) methyl)-N-nitro-2-imidazolidinimine], was the first commercially used neonicotinoid pesticide. It has gained worldwide popularity with an increasing share in the insecticide market since its introduction in 1991. Imidacloprid has been extensively produced and used in China with an annual capacity of 25 000 tons (100% a.i.) and a domestic demand of 3000 to 4000 tons/year (China Agrochemicals, 2012; Wang et al., 2015). The tremendous application and environmental accumulation of imidacloprid residue inevitably results in high human exposure (Lonare et al., 2016). Rural farmers are exposed more to imidacloprid owing to their proximity to agricultural land. Potential exposure is even higher for operators

who are directly involved in pesticide application activities. United States Environmental Protection Agency classifies imidacloprid as moderately toxic if ingested (Label Review Manual). A previous study reported disorientation, agitation, incoherence, sweating, and breathlessness as symptoms in a 24-year-old man who accidentally inhaled a pesticide containing 17.8% imidacloprid (Agarwal et al., 2007). In addition, dermatitis was reported in pet owners following the use of veterinary products containing imidacloprid on their pets (WHO). These findings indicated that imidacloprid exposure may cause adverse health effects in humans through even a certain single route in case of high-dose exposure. Pesticide applicators are highly exposed to imidacloprid via ingestion, inhalation, and dermal contact. Thus, the health risks associated with imidacloprid exposure in this population is a high priority.

Animal experiments showed that *in vivo* metabolism of imidacloprid occurred mainly in the liver through two pathways: (i) oxidation cleavage of parent imidacloprid yielding 6-chloronicotinic acid (6-CNA) and (ii) hydroxylation of the imidazoline ring (Solecki R., 2001). The following metabolites may be of toxicological significance: 6-CNA, imidazolidine 4- and 5- hydroxy compounds, olefinic imidacloprid, desnitro-imidacloprid, and the nitrosoimine compound (DPR Medical Toxicology, 2013). While the major urinary metabolites were 6-CNA and its glycine conjugate (Solecki R., 2001). Therefore, parent imidacloprid and the major metabolite 6-CNA were frequently selected for detection in the analysis of biological samples. The commonly used pretreatment methods for biological samples (blood and urine) include liquid-liquid extraction (LLE) (Escrivá et al., 2017; Kavvalakis et al.,

2013; Wang et al., 2015; Yuan et al., 2018), modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) methods (Plassmann et al., 2015; Provas et al., 2015; Taliansky-Chamudis et al., 2017), solid-phase extraction (SPE) (de Oliveira et al., 2013; Hovelmann et al., 2016; Pan et al., 2016; Silveira et al., 2018; Zhang et al., 2018), and dispersive liquid-liquid microextraction (DLLME) (Cunha et al., 2011). Biological monitoring has frequently detected imidacloprid in urine samples (Harada et al., 2016; Kabata et al., 2016; López-García et al., 2017; Ueyama et al., 2015; Ueyama et al., 2014; Yamamuro et al., 2014; Zhang et al., 2018); however, few studies have investigated its metabolites (Nomura et al., 2013; Uroz et al., 2001). Simultaneous determination for imidacloprid and its metabolite in biological sample has been reported only in two studies (Kavvalakis et al., 2013; Wang et al., 2015), and LLE extraction was used for urine matrix in both studies. LLE requires significant volumes of organic solvents to fully extract the target analytes from aqueous-phase layer, and typically entails evaporation for pre-concentration to ensure trace-level detection from biological matrices. Therefore, a greener, high-throughput extraction method is needed for batch analysis of biological samples.

Biological monitoring of imidacloprid and its major metabolite 6-CNA could reflect *in vivo* exposure levels; on that basis, further dose estimation would be helpful in ascertaining the risk according to urinary concentrations of analytes (Curwin et al., 2007). Mage et al. (2004) and Curwin et al. (2007) adopted an approach to estimate the absorbed daily dose (ADD) of pesticides based on the measurement of parent compound or metabolites in urine. Study on dose estimation of imidacloprid in

biological samples is scarce, and this may decelerate the process of further risk assessment. Therefore, we attempted to preliminarily reveal the potential risks by means of ADD estimation for imidacloprid.

In this study, we established a robust and rapid method for simultaneously measuring imidacloprid and 6-CNA in urine. The commonly used extraction methods were compared to optimize results. The optimized method was then used to analyze 86 urine samples from rural pesticide applicators. The objectives of this study were to 1) detect the concentrations of urinary imidacloprid and its metabolite 6-CNA in the rural population of applicators; 2) compare the exposure level before and after a pesticide application event; and 3) preliminarily conduct dose estimation and risk assessment for imidacloprid exposure. This study is hoping to provide an important reference for further human health risk evaluation.

## **2. Materials and Methods**

### **2.1 Chemicals and Reagents**

Standard solutions of imidacloprid (99.6% purity) and its metabolite 6-CNA (98.7% purity) were obtained from China Standard Material Center (Beijing, China). The internal standard (IS) D<sub>4</sub>-imidacloprid (98.0% purity) was purchased from Dr. Ehrenstorfer (Augsburg, Germany).  $\beta$ -glucuronidase from *Helix pomatia* (125255 units/mL  $\beta$ -glucuronidase and 1095 units/mL sulfatase) was purchased from Sigma-Aldrich (Steinheim, Germany). High performance liquid chromatography (HPLC) grade methanol (MeOH), acetonitrile (ACN), and formic acid (FA) were purchased from Sigma-Aldrich (Steinheim). Analytical grade methanol, ACN,

trichloromethane, glacial acetic acid, ammonium acetate, aqueous ammonia solution, sodium chloride, and anhydrous magnesium sulfate were purchased from Beihua Fine-chemicals Co. (Beijing, China). Ultra-pure water was prepared by a Milli-Q system (Bedford, MA, USA). The clean-up sorbents, primary secondary amine (PSA), octadecylsilane (C<sub>18</sub>), and 0.22- $\mu$ m nylon syringe filters were purchased from Agela Technologies Inc. (Agela, Tianjin, China) along with 60mg/3mL Polar Enhanced Polymer (PEP), Polymer Cation eXchange (PCX), and Polymer Weak Cation eXchange (PWCX) cartridges.

Individual standard stock solutions (100 mg/L) of imidacloprid, 6-CNA, and D<sub>4</sub>-imidacloprid were prepared in HPLC-grade ACN. Further serial dilutions of the stock solutions were prepared with pure ACN to obtain final working solutions at concentrations of 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 ng/mL for imidacloprid and 6-CNA. The working IS solutions were serially diluted to 1 mg/L for D<sub>4</sub>-imidacloprid. All solutions were stored in the dark at -20°C before use.

## 2.2 Field Experiment Design

The study participants comprised rural farmers living in a village adjacent to ten thousand acres of orchard farm in Henan Province, China. The orchard operation uses a smallholder planting model with applicators managing different-sized plots individually. One member who was long occupied in pesticide spraying from each smallholder and would not be absent from the sample collection was nominated as one targeted pesticide applicator. The study participants carried out their tasks as normal. The spray equipment consisted of pressurized knapsack sprayers without

uniform specifications. The spraying time and labor intensity depended on individual management requirements (Table S1), providing an accurate representation of the current application and exposure scenarios for imidacloprid in rural agricultural orchards in Henan Province.

Spot urine samples (n=86) from 43 randomly selected pesticide applicators (age:24–74 years old; 31 males and 12 females) were collected in March 2017 (before imidacloprid spraying) and in May 2017 (after 3–4 imidacloprid spraying events depending on the degree of insect infestation). The participants were required to donate fasting midstream urine samples in the morning. The single spot urine was transferred to a 50-mL high-density polypropylene centrifuge tube. All collected urine samples were immediately stored at -20°C, cold shipped on ice to the laboratory, and stored at -80°C until further analysis. Meanwhile, personal information (Table S3), including age, gender, height, weight, and spraying frequency of neonicotinoid insecticides was obtained by face-to-face farmer interviews. All the study participants provided written informed consent before participation.

### 2.3 Sample Preparation

The flowchart of the procedure for detecting urinary imidacloprid and 6-CNA is presented in Fig. 1. Briefly, a 1-mL aliquot of each urine subsample was pipetted into a 5-mL centrifugal tube and spiked with 100  $\mu$ L of prepared  $\beta$ -glucuronidase (equivalent to 125 units of activity/mL) and 10  $\mu$ L of IS solution (1 mg/L).  $\beta$ -glucuronidase was prepared in advance as follows: 100  $\mu$ L of the initial enzyme (125255 units/mL  $\beta$ -glucuronidase and 1095 units/mL sulfatase) was

dissolved in 2 M acetate buffer. The acetate buffer was prepared by dissolution of 9.7 g of sodium acetate into deionized water, followed by the addition of glacial acetic acid to adjust the pH to 4.5 in a total volume of 100 mL. The samples were incubated at 37 °C and shaken on a rotary disk shaker at 160 r/min for 12 h for deconjugation.

## 2.4 Sample Extraction

In this study, three different pretreatment methods (LLE, QuEChERS, and SPE) were evaluated and compared in terms of extraction efficiency for the target analytes.

**LLE.** The extraction method was performed as follows: a 1-mL aliquot of blank urine was pipetted into a 15-mL centrifuge tube and spiked with 10  $\mu$ L of analyte standard mixture comprising 1 mg/L imidacloprid and 6-CNA and 10  $\mu$ L of IS solution (1mg/L) (five replicates). Then, enzyme-assisted deconjugation was conducted as described above. The resulting urine solution was extracted twice with 5 mL of trichloromethane. For each extraction, the mixtures were vortexed vigorously for 10 min and centrifuged at  $2400 \times g$  for 5 min. Afterward, the combined supernatant was transferred into a 50-mL glass bottle for rotary evaporation to dryness under vacuum. Finally, the residues were reconstituted in 1 mL of ACN.

**QuEChERS.** The modified QuEChERS method was adapted from a previous report (Taliensky-Chamudis et al., 2017) for the extraction of target analytes. Briefly, 0.5 mL of blank urine (five replicates) was pipetted into a 5-mL centrifuge tube, spiked with 10  $\mu$ g/L analyte standard mixture and 10  $\mu$ g/L IS solution, and then vortexed for 30 s before equilibration at 25 °C for 2 h. Extraction was conducted by adding 1.5 mL of glacial acetic acid/ACN (v: v=1:99) into the tube, followed by

vortexing for 1 min. Next, 0.1g sodium chloride was added and the solution was vortexed for another 1 min prior to centrifugation at  $2400 \times g$  for 5 min. The resulting supernatant was transferred to a 2-mL tube containing 50 mg of anhydrous  $MgSO_4$ , 30 mg of PSA, and 20 mg of  $C_{18}$ , vortexed for 1 min, and then centrifuged for 5 min at  $2811 \times g$ . The decanted liquid was passed through a 0.22- $\mu m$  nylon syringe filter prior to instrumental analysis.

**SPE.** Three types of cation exchange columns (PEP, PCX, and PWCX) were evaluated for their extraction efficiency according to the operation manual with minor modifications (the details are shown in Supporting Information).

## 2.5 LC-MS/MS

Chromatographic separation and detection were performed on a Waters ACQUITY UPLC (Milford, MA, USA) system interfaced with a triple-quadrupole mass spectrometer (Xevo TQ-S, Waters Corp., USA). The column heater was equipped with an ACQUITY BEH C18 analytical column (2.1 mm  $\times$  50 mm, 1.7  $\mu m$  particle size, Waters, Milford, MA, USA) with an injection volume of 5  $\mu L$ . The column and sample manager were maintained at 40°C and 25°C, respectively. The mobile phases comprised methanol (A) and Milli-Q water (B) acidified with 0.2% formic acid. Gradient elution was at a flow rate of 0.3 mL/min with of the conditions: 0 min, 10% A; 1.6 min, 90% A; 3.1 min, 10% A; 4.0 min, 10% A. The run time was 4 min for each injection. A triple-quadrupole mass spectrometer (Xevo TQ-S, Waters Corp. USA) with an electrospray ionization source was operated in the positive ion mode (ESI+). The optimal MRM transitions and source conditions are listed in Table 1.

Masslynx NT v.4.2 (Waters) software was used for data analysis.

## 2.6 Urinary Creatinine Measurement

Creatinine, a by-product of muscle tissue, is produced in an amount proportional to the muscle mass of an individual. The excretion rate of creatinine in an individual is maintained at a relatively constant level in the absence of renal disease. In this study, the concentrations of imidacloprid and 6-CNA were adjusted using creatinine concentration (represented as  $\mu\text{g/g}$  creatinine) to correct for variable dilutions (i.e. hydration differences) in spot urine samples. Urinary creatinine was measured for each sample using a creatinine (urinary) colorimetric assay kit based on a modified Jaffe reaction (Cayman chemical). The absorbance of creatinine was read at 490 nm in a microplate reader. Each urine sample was subjected to 10-fold dilution with deionized water before measurement.

## 2.7 Absorbed Daily Dose Estimation

Mage et al. (2004) and Curwin et al. (2007) reported an approach for ADD estimation from urinary metabolite concentration. The ADD of pesticide in microgram per kilogram body weight per day ( $\mu\text{g/kg/d}$ ) was calculated according to equation (1):

$$ADD(\mu\text{g/kg/d}) = \frac{C \times Cn \times CF \times R_{mw}}{BW} \quad (1)$$

where  $C$  is the creatinine-adjusted concentration of the pesticide or metabolite (unit:  $\mu\text{g/g}$  creatinine);  $Cn$  is the calculated mass of creatinine excreted per day (unit: g/day);  $CF$  is the correction factor of imidacloprid;  $R_{mw}$  is the ratio of molecular weights between parent compound and pesticide metabolite; and  $BW$  is the body weight (kg).

The mass of creatinine excreted per day  $C_n$  (g/day) was calculated using equation (2) (Ogna et al., 2015):

$$C_n = (266.16 - 47.71 \times sex - 2.33 \times BMI + 0.66 \times age - 0.017 \times age^2) \times BW \times 1.13e^{-4} \quad (2)$$

where  $sex=0$  for male and  $sex=1$  for female;  $BMI$  is the Body Mass Index ( $\text{kg}/\text{m}^2$ );  $age$  is the age of an individual participant;  $BW$  is the body weight (kg); and  $1.13e^{-4}$  represents the mass of creatinine per micro mole ( $\text{g}/\mu\text{mol}$ ).

$CF$  is used for incomplete excretion of pesticide via urine (Curwin et al., 2007). Approximately 75% of imidacloprid is excreted via renal clearance (urine) (DPR Medical Toxicology, 2013), with 6-CNA and its glycine-combined conjugate constituting most of the excreted metabolites (>50%) (Solecki, 2001). In radiolabeled imidacloprid tests, the glycine conjugate of 6-Cl-nicotinic acid and other metabolites have been shown to account for 82% of the total radioactivity in urine (< 82%) (DPR Medical Toxicology, 2013). The exact fraction of the metabolite 6-CNA in urine is unknown, and this might limit further exposure estimation of imidacloprid. Therefore, we chose two extrema (50% and 82%) to evaluate total exposure, resulting in a maximum  $CF = (1/0.75)/0.5=2.667$  and a minimum  $CF = (1/0.75)/0.82=1.626$ .

The ratio of molecular weights was determined as  $R_{mw} = M_{\text{imidacloprid}}/M_{6\text{-CNA}} = 255.7/157.56 = 1.62$ .

## 2.8 Assay Validation

The performance of the method was validated according to the following parameters: accuracy, precision, linearity, limit of quantification (LOQ), matrix effect, and stability (the details are shown in Supporting Information).

### 3. Results and Discussion

#### 3.1 Method Optimization and Performance Validation

The chromatography conditions were optimized (the details are shown in Supporting Information), and methanol/0.2% FA aqueous solution was selected as the mobile phase for imidacloprid and 6-CNA.

The extraction efficiency of three different pretreatment methods was compared for imidacloprid, 6-CNA and D<sub>4</sub>-imidacloprid (Fig. 2) (the details are shown in Supporting Information). PEP column with pure ACN as elution solvent was selected.

In this study, isotope-labeled 6-CNA was not commercially available. As imidacloprid and 6-CNA possess structurally similar groups, D<sub>4</sub>-imidacloprid was used as the joint IS for further calibration and quantification. The recoveries of imidacloprid and 6-CNA were calculated based on two methods: the conventional external standard method and the IS method (in Table 2). The peak area ratios of imidacloprid to D<sub>4</sub>-imidacloprid were not affected by elution solvents (Fig. 3). Therefore, the IS calibration curves prepared in pure ACN were adopted for further quantification.

The developed method was further validated according to the following parameters: accuracy and precision, linearities, LODs and LOQs, matrix effects and stability (the details are shown in Supporting Information).

#### 3.2 Occurrence of Imidacloprid and 6-CNA in Urine Samples

The developed method was used to analyze 86 urine samples from 43 pesticide applicators. Target analytes were detected in 100% of collected urine samples.

Imidacloprid residue is defined as the sum of imidacloprid and its metabolites containing the 6-chloropyridinyl moiety (JMPR report), which was suitable for analyzing imidacloprid residue in products of animal and plant origin. This definition results in a conservative estimate of total imidacloprid concentrations and has been applied for both compliance with maximum residues limits and the estimation of dietary intake. However, no definite provision of this kind is made in biological samples. Moreover, the amount of imidacloprid residue in urine reflects the sum of all exposure pathways and not just exposure through diet. Based on the above considerations, we mainly described the concentrations of imidacloprid and the major metabolite 6-CNA to reflect their level of presence in urine. Urinary imidacloprid and 6-CNA were quantified at 0.57–8.91 ng/mL (0.20–9.60; GM: 2.79  $\mu$ g/g creatinine) and 0.21–2.92 ng/mL (0.06–3.65; geomean (GM): 1.37  $\mu$ g/g creatinine), respectively, before pesticide application. The concentration of imidacloprid and 6-CNA after application was 0.38–24.58 ng/mL (1.21–57.40; GM: 10.52  $\mu$ g/g creatinine) and 0.11–6.88 ng/mL (0.66–16.06; GM: 4.83  $\mu$ g/g creatinine), respectively (Fig. 4). Almost the same trend was observed for urinary concentrations of imidacloprid and 6-CNA before and after application using creatinine-correction (Fig. 4A) and without creatinine-correction (Fig. 4B). The results of paired *t*-test indicated that the concentrations of both analytes significantly increased (3.52- to 3.77-fold) after pesticide spraying compared to samples taken before spraying ( $p < 0.001$  for 6-CNA and  $p = 0.001$  for imidacloprid). Previous studies have shown that imidacloprid could be detected in biological samples from humans (Osaka et al., 2016; Ueyama et al.,

2015; Wang et al., 2015); however, the urinary exposure levels reported were almost always lower than that in the current study. This demonstrates that pesticide applicators at the Henan site were subjected to higher risk of imidacloprid exposure. Higher usage of imidacloprid for pest control (Agrochemical Market Trends) and paucity of proper protective measures in developing countries may inevitably lead to higher exposure of pesticide applicators after pesticide spraying activity. In addition, some other factors such as lifestyle, including smoking and food ingestion, might potentially contribute to the differences in urinary pesticide concentrations of applicators before and after pesticide application. Tan et al. conducted research on food intake pathway by monitoring neonicotinoid residues in 49 kinds of vegetables and 24 kinds of fruits in China and found that the detection rate of imidacloprid was 100% (Tan et al., 2016). Lu et al. reported that imidacloprid was the most commonly detected neonicotinoid in vegetables and fruits, with 66% detection in Chinese dietary exposure assessment, thus, imidacloprid has become part of the dietary staple, with possible health implications for individuals (Lu et al., 2018). Ingestion of imidacloprid from food sources may play a crucial role in contributing to the high concentration in urine. Further research is warranted to make certain the extent of contribution of these factors to urinary imidacloprid concentration.

The median level of imidacloprid in urine samples was calculated as 4.66 ng/mL (10.44  $\mu\text{g/g}$  creatinine), and the GM concentration was 4.47 ng/mL (10.52  $\mu\text{g/g}$  creatinine) (calculated from IBM SPSS Statistics 23.0) after pesticide application. For 6-CNA, the median and GM values were 2.24 ng/mL (4.43  $\mu\text{g/g}$  creatinine) and 2.05

ng/mL (4.83  $\mu\text{g/g}$  creatinine), respectively. The values were much higher than those reported in the study by Wang et al. (2015), in which the GM concentration of imidacloprid in rural adults engaged in pesticide spraying increased from 0.18 ng/mL (before spraying) to 0.54 ng/mL (after spraying) in a village in the Shandong Province in China. Notably, no evident change in concentration level was observed for urinary 6-CNA after pesticide spraying (GM value at 0.08 ng/mL) in that study. Several factors contribute to the concentration differences observed in these studies. In the current study, the estimated application rate was 105–525 g/day (Table S1), which in many cases was higher than 200–300 g/day reported by Wang et al. (2015). In addition, lack of proper protective measures is a common problem contributing to agro-chemical exposure in developing countries (China included). Differences in professional practices with regards to imidacloprid application also likely contributed to the differences in urine concentrations across provinces.

### 3.3 Imidacloprid ADD Estimation

As summarized in Table 3, prior to pesticide spraying, the minimum imidacloprid ADD values for applicators ranged from 0.52–37.59  $\mu\text{g/kg/d}$ , with a mean of 15.93  $\mu\text{g/kg/d}$  (median:15.82  $\mu\text{g/kg/d}$ ; GM:13.80  $\mu\text{g/kg/d}$ ), and the maximum ADD values were 0.85-61.66  $\mu\text{g/kg/d}$  with a mean of 26.13  $\mu\text{g/kg/d}$  (median:25.94  $\mu\text{g/kg/d}$ ; GM:22.64  $\mu\text{g/kg/d}$ ). The acceptable daily intake (ADI) value was set as 0.06 mg/kg per day (expressed as 60  $\mu\text{g/kg/d}$ ) and the acute reference dose (ARfD) was 400  $\mu\text{g/kg/d}$  for imidacloprid (WHO/FAO). The ADD values before pesticide spraying were lower than the chronic guidelines suggested by the above international agency

except for one case (61.66  $\mu\text{g}/\text{kg}/\text{d}$ ) exceeding 60  $\mu\text{g}/\text{kg}/\text{d}$ . This indicated that limited health risk of imidacloprid exposure was observed for the tested population before spraying with imidacloprid with Hazard Quotients (HQs) of  $<1$  (calculated as shown in Table 4).

After pesticide spraying, the minimum imidacloprid ADD values ranged from 7.65–151.23  $\mu\text{g}/\text{kg}/\text{d}$  (mean:57.86  $\mu\text{g}/\text{kg}/\text{d}$ ; median:47.73  $\mu\text{g}/\text{kg}/\text{d}$ ; GM:48.60  $\mu\text{g}/\text{kg}/\text{d}$ ), and the maximum ADD values were 12.56–248.05  $\mu\text{g}/\text{kg}/\text{d}$  (mean:94.91  $\mu\text{g}/\text{kg}/\text{d}$ ; median:78.23  $\mu\text{g}/\text{kg}/\text{d}$ ; GM:79.71  $\mu\text{g}/\text{kg}/\text{d}$ ). The results showed that the minimum imidacloprid ADD derived from stoichiometric estimates based on the measurement of 6-CNA exceeded the ADI value in 14 of 43 urine samples (32.6%), and none of the 43 urine samples exceeded the ARfD value. The maximum imidacloprid ADD estimated based on the measurement of 6-CNA in 32 of 43 urine samples (74.4%) was higher than the ADI value, and none of the 43 urine samples exceeded the ARfD value. The HQs of imidacloprid after pesticide application are presented in Table 4. By referring to the ARfD value, the HQs ranged within 0.02–0.62 ( $<1$ ), indicating almost no acute health effects caused by imidacloprid exposure. While the 50<sup>th</sup> and 75<sup>th</sup> percentiles HQs were 0.80–2.09, indicating that some exposure values exceeded the corresponding ADI guideline. The maximum HQs were 2.52–4.13, indicating higher exposure risk based on the chronic guidelines recommended by the international agency. These results showed that exposure to imidacloprid had the potential to cause increased health risks, especially following pesticide application.

Usually, the ADI value is established based on long-term (lifetime period) exposure, and the ARfD value is set based on short-term (certain day) exposure; however, broad toxicological thresholds existed in these two guidelines. In the present study, the estimated ADD values at the sampling day after pesticide application seem to not accurately represent the acute (application-day) or chronic (lifetime) status for imidacloprid exposure. Nevertheless, comparison with guideline values better reflected the degree of imidacloprid exposure and potential risks and significantly increased dose in vivo would be an adverse indicator. Agricultural workers may be exposed to imidacloprid once or several times per season via different exposure pathways including dermal contact, air inhalation and diet intake, and risk assessments based on aggregate exposure are greatly needed. It is likely that this population experienced chronic high exposure to the chemical as evidenced from the concentrations detected in urine samples collected prior to spraying events. Further risk evaluation is urgently needed by monitoring exposure levels over different points of time. In this study, we used the extreme value range to conduct ADD estimation protocol for risk assessment, and the results indicate that there are exposure risks for pesticide applicators. To our knowledge, this is the first ADD estimation for imidacloprid. Increased understanding of the metabolism of imidacloprid could help improve the accuracy of ADD estimates and eliminate the need for the range of values used here.

#### **4. Conclusion**

This study established a robust and rapid method for simultaneously measuring

imidacloprid and its metabolite 6-CNA in urine using UPLC-MS/MS. The isotope-labeled IS calibration proved effective in compensating for the matrix effects of imidacloprid and 6-CNA in the complex urine matrix. High sensitivity with low LOQs was achieved, ensuring the detection frequencies of trace-level analytes in urine samples. Exposure data from pesticide applicators indicated that this population experiences significant exposure to imidacloprid, especially after a spraying event. The results of the ADD range estimation revealed that the potential health risks associated with the estimated ADD values exceeded the guidelines for imidacloprid. Further exposure assessment studies are warranted to ensure the safe use of imidacloprid in agriculture to protect human health.

#### **Conflict of interest**

All authors declare that there is no conflict of interest.

#### **Acknowledgments**

This work was financially supported by The National Key Research and Development Program of China (2016YFD0200204).

#### **Ethical approval**

The study was approved by the Institutional Review Board of Chinese Academy of Agricultural Sciences.

#### **References**

Agarwal, R., Srinivas, R., 2007. Severe neuropsychiatric manifestations and rhabdomyolysis in a patient with imidacloprid poisoning. *American Journal of*

Emergency Medicine 25, 844-845.

Agrochemical Market Trends. Imidacloprid: Global Product Intelligence (2016-2021).

China Agrochemicals, 2012. China Pesticides Products Reports.

<http://www.agrochemex.org/wp-content/uploads/2012/02/2012.12.pdf>.

Cunha, S. C., Fernandes, J. O., 2011. Quantification of free and total bisphenol A and bisphenol B in human urine by dispersive liquid-liquid microextraction (DLLME) and heart-cutting multidimensional gas chromatography-mass spectrometry (MD-GC/MS). *Talanta* 83, 117-125.

Curwin, B. D., Hein, M. J., Sanderson, W. T., Striley, C., Heederik, D., Kromhout, H., et al., 2007. Pesticide dose estimates for children of Iowa farmers and non-farmers. *Environ. Res.* 105, 307-315.

de Oliveira, D. M., Pinto, C. B., Sampaio, G. R., Yonekura, L., Catharino, R. R., Bastos, D. H., 2013. Development and validation of methods for the extraction of phenolic acids from plasma, urine, and liver and analysis by UPLC-MS. *J. Agric. Food. Chem.* 61, 6113-6121.

DPR Medical Toxicology, 2013. California Environmental Protection Agency Department of Pesticide Regulation Medical Toxicology Branch. Summary of Toxicology Data Imidacloprid, t20131118.

Ead, M., Mulhauser, B., Mullet, M., Mutabazi, A., Glauser, G., Aebi, A., 2017. A worldwide survey of neonicotinoids in honey. *Science* 358, 38-39.

Escrivá, L., Manyes, L., Font, G., Berrada, H., 2017. Mycotoxin Analysis of Human Urine by LC-MS/MS: A Comparative Extraction Study. *Toxins* 9, 330.

- Gibbons, D., Morrissey, C., Mineau, P., 2015. A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. *Environ. Sci. Pollut. R.* 22, 103-118.
- Hallmann, C. A., Foppen, R. P. B., Turnhout, C. A. M. V., Kroon, H. D., Jongejans, E., 2014. Declines in insectivorous birds are associated with high neonicotinoid concentrations. *Nature* 511, 341-343.
- Harada, K. H., Tanaka, K., Sakamoto, H., Imanaka, M., Niisoe, T., Hitomi, T., et al., 2016. Biological Monitoring of Human Exposure to Neonicotinoids Using Urine Samples, and Neonicotinoid Excretion Kinetics. *PLoS One* 11, e0146335.
- Haroune, L., Cassoulet, R., Lafontaine, M. P., Belisle, M., Garant, D., Pelletier, F., et al., 2015. Liquid chromatography-tandem mass spectrometry determination for multiclass pesticides from insect samples by microwave-assisted solvent extraction followed by a salt-out effect and micro-dispersion purification. *Anal. Chim. Acta* 891, 160-170.
- Hovelmann, Y., Hickert, S., Cramer, B., Humpf, H. U., 2016. Determination of Exposure to the Alternaria Mycotoxin Tenuazonic Acid and Its Isomer allo-Tenuazonic Acid in a German Population by Stable Isotope Dilution HPLC-MS(3). *J. Agric. Food. Chem.* 64, 6641-6647.
- Kabata, R., Nanayakkara, S., Senevirathna, S., Harada, K. H., Chandrajith, R., Hitomi, T., et al., 2016. Neonicotinoid concentrations in urine from chronic kidney disease patients in the North Central Region of Sri Lanka. *J. Occup. Health* 58,

128-133.

- Kavvalakis, M. P., Tzatzarakis, M. N., Theodoropoulou, E. P., Barbounis, E. G., Tsakalof, A. K., Tsatsakis, A. M., 2013. Development and application of LC–APCI–MS method for biomonitoring of animal and human exposure to imidacloprid. *Chemosphere* 93, 2612-2620.
- Label Review Manual. U.S Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances, Office of Pesticide Programs. <http://www.epa.gov/oppfead1/labeling/lrm/chap-07.pdf>, updated Aug 2007.
- Lonare, M., Kumar, M., Raut, S., More, A., Doltade, S., Badgujar, P., et al., 2016. Evaluation of ameliorative effect of curcumin on imidacloprid-induced male reproductive toxicity in wistar rats. *Environ. Toxicol.* 31, 1250-1263.
- López-García, M., Romero-González, R., Lacasaña, M., Garrido, F. A., 2017. Semiautomated determination of neonicotinoids and characteristic metabolite in urine samples using TurboFlow™ coupled to ultra high performance liquid chromatography coupled to Orbitrap analyzer. *J. Pharm. Biomed. Anal.* 146, 378-386.
- Lu, CS, Chang, CH, Palmer, C, Zhao, MR, Zhang, Q, 2018. Neonicotinoid Residues in Fruits and Vegetables: An Integrated Dietary Exposure Assessment Approach. *Environ. Sci. Technol.* 52, 3175-3184.
- Mage, D. T., Allen, R. H., Gondy, G., Smith, W., Barr, D. B., Needham, L. L., 2004. Estimating pesticide dose from urinary pesticide concentration data by creatinine correction in the Third National Health and Nutrition Examination

- Survey (NHANES-III). *J Expo Sci Environ Epidemiol.* 14, 457-465.
- Nomura, H., Ueyama, J., Kondo, T., Saito, I., Murata, K., Iwata, T., et al., 2013. Quantitation of neonicotinoid metabolites in human urine using GC-MS. *J. Chromatogr. B* 941, 109-115.
- Ogna, V. F., Ogna, A., Vuistiner, P., Pruijm, M., Ponte, B., Ackermann, D., et al., 2015. New anthropometry-based age- and sex-specific reference values for urinary 24-hour creatinine excretion based on the adult Swiss population. *BMC Medicine* 13, 1-10.
- Osaka, A., Ueyama, J., Kondo, T., Nomura, H., Sugiura, Y., Saito, I., et al., 2016. Exposure characterization of three major insecticide lines in urine of young children in Japan-neonicotinoids, organophosphates, and pyrethroids. *Environ. Res.* 147, 89-96.
- Pan, Y., Jing, J., Yeung, L. W., Sheng, N., Zhang, H., Yao, B., et al., 2016. Associations of urinary 5-methyl-2'-deoxycytidine and 5-hydroxymethyl-2'-deoxycytidine with phthalate exposure and semen quality in 562 Chinese adult men. *Environ. Int.* 94, 583-590.
- Pisa, L. W., Amaral-Rogers, V., Belzunces, L. P., Bonmatin, J. M., Downs, C. A., Goulson, D., et al., 2015. Effects of neonicotinoids and fipronil on non-target invertebrates. *Environ. Sci. Pollut. R.* 22, 68-102.
- Plassmann, M. M., Schmidt, M., Brack, W., Krauss, M., 2015. Detecting a wide range of environmental contaminants in human blood samples--combining QuEChERS with LC-MS and GC-MS methods. *Anal. Bioanal.Chem.* 407,

7047-7054.

- Provatas, A. A., Yevdokimov, A. V., King, C. A., Gatley, E. L., Stuart, J. D., Evers, D. C., et al., 2015. Rapid QuEChERS extraction with novel phospholipid cleanup: A streamlined ultra high performance liquid chromatography with ultraviolet detection approach for screening polycyclic aromatic hydrocarbons in avian blood cells and plasma. *J. Sep. Sci.* 38, 2677-2683.
- Raby, M., Zhao, X., Hao, C., Poirier, D. G., Sibley, P. K., 2018. Chronic effects of an environmentally-relevant, short-term neonicotinoid insecticide pulse on four aquatic invertebrates. *Sci. Total Environ.* 639, 1543.
- Rundlöf, M., Andersson, G. K., Bommarco, R., Fries, I., Hederström, V., Herbertsson, L., et al., 2015. Seed coating with a neonicotinoid insecticide negatively affects wild bees. *Nature* 521, 77-80.
- Silveira, A. L., MI, G. D. O., Rocha, D. G., Dracz, S., Borgati, T. F., Mag, L., et al., 2018. Multiresidue determination of anabolic agent residues: steroids, stilbenes and resorcylic acid lactones, in bovine urine by GC-MS/MS employing microwave assisted derivatization. *J. Agric. Food. Chem.* 32, 8630-8638.
- Solecki R., 2001. Toxicological evaluations. Imidacloprid. Joint Meeting on Pesticide Residues.
- Song, S., Zhang, C., Chen, Z., He, F., Wei, J., Tan, H., et al., 2018. Simultaneous determination of neonicotinoid insecticides and insect growth regulators residues in honey using LC-MS/MS with anion exchanger-disposable pipette

extraction. *J. Chromatogr. A* 51-61.

- Sousa, J. C. G., Ribeiro, A. R., Barbosa, M. O., Ribeiro, C., Tiritan, M. E., Pereira, M. F. R., et al., 2019. Monitoring of the 17 EU Watch List contaminants of emerging concern in the Ave and the Sousa Rivers. *Sci. Total Environ.* 649, 1083-1095.
- Stanley, D. A., Garratt, M. P. D., Wickens, J. B., Wickens, V. J., Potts, S. G., Raine, N. E., 2015. Neonicotinoid pesticide exposure impairs crop pollination services provided by bumblebees. *Nature* 528, 548-550.
- Sultana, T., Murray, C., Kleywegt, S., Metcalfe, C. D., 2018. Neonicotinoid pesticides in drinking water in agricultural regions of southern Ontario, Canada. *Chemosphere* 202, 506-513.
- Taliansky-Chamudis, A., Gómez-Ramírez, P., León-Ortega, M., García-Fernández, A. J., 2017. Validation of a QuEChERS method for analysis of neonicotinoids in small volumes of blood and assessment of exposure in Eurasian eagle owl (*Bubo bubo*) nestlings. *Sci. Total Environ.* 595, 93-100.
- Tan Y., Zhang Q., Zhao C., Wang X., Li J., Wang D., et al., 2016. Residues of Neonicotinoid Pesticides in Vegetables and Fruit and Health Risk Assessment of Human Exposure via Food Intake. *Asian J TOXICOL.* 11, 67-81.
- Ueyama, J., Harada, K. H., Koizumi, A., Sugiura, Y., Kondo, T., Saito, I., et al., 2015. Temporal Levels of Urinary Neonicotinoid and Diaklyphosphate Concentrations in Japanese Women Between 1994-2011. *Environ. Sci. Technol.* 49, 14522-14528.

- Ueyama, J., Nomura, H., Kondo, T., Saito, I., Ito, Y., Osaka, A., et al., 2014. Biological monitoring method for urinary neonicotinoid insecticides using LC-MS/MS and its application to Japanese adults. *Journal of Occupational Health* 56, 461-468.
- Uroz, F. J., Arrebola, F. J., Egea-González, F. J., Martínez-Vidal, J. L., 2001. Monitoring of 6-chloronicotinic acid in human urine by gas chromatography-tandem mass spectrometry as indicator of exposure to the pesticide imidacloprid. *Analyst* 126, 1355-1358.
- Vijver, M. G., Pj, V. D. B., 2014. Macro-invertebrate decline in surface water polluted with imidacloprid: a rebuttal and some new analyses. *PLoS One* 9, e89837.
- Wang, L., Liu, T., Liu, F., Zhang, J., Wu, Y., Sun, H., 2015. Occurrence and profile characteristics of the pesticide imidacloprid, the preservative parabens, and their metabolites in human urine from rural and urban China. *Environ. Sci. Technol.* 49, 14633-14640.
- Whitehorn, P. R., O'Connor, S., Wackers, F. L., Goulson, D., 2012. Neonicotinoid pesticide reduces bumble bee colony growth and queen production. *Science* 336, 351-352.
- WHO. Toxicological Evaluations: Imidacloprid; International Programme on Chemical Safety, World Health Organization. <http://www.inchem.org/jmpr/jmprmono/2001pr07.htm>, updated Feb 2004.
- Yamamuro, T., Ohta, H., Aoyama, M., Watanabe, D., 2014. Simultaneous determination of neonicotinoid insecticides in human serum and urine using

diatomaceous earth-assisted extraction and liquid chromatography–tandem mass spectrometry. *J. Chromatogr. B* 969, 85-94.

Yuan, Y., Wang, Q., Yang, M., Xu, Y., Chen, W., Zou, X., et al., 2018. Application of response surface methodology to vortex-assisted dispersive liquid-liquid extraction for the determination of nicotine and cotinine in urine by GC-MS/MS. *J. Sep. Sci.* 10, 2261-2268.

Zhang, Q., Wang, X., Li, Z., Jin, H., Lu, Z., Yu, C., et al., 2018. Simultaneous determination of nine neonicotinoids in human urine using isotope-dilution ultra-performance liquid chromatography-tandem mass spectrometry. *Environ. Pollut.* 240, 647–652.

Zhou, Y., Lu, X., Fu, X., Yu, B., Wang, D., Zhao, C., et al., 2018. Development of a fast and sensitive method for measuring multiple neonicotinoid insecticide residues in soil and the application in parks and residential areas. *Anal. Chim. Acta* 1016, 19-28.

### Figure captions

**Fig. 1.** Optimized analytical procedure for urinary imidacloprid and 6-CNA.

**Fig. 2.** Effects of different pretreatment methods on the recoveries of target analytes.

**Fig. 3.** Comparison of different elution solvents on the signal response of target analytes.

**Fig. 4.** Concentration levels of urinary imidacloprid and 6-CNA without creatinine-correction (A) and with creatinine-correction (B).

Note: The concentration sum of imidacloprid ( $\Sigma$  IMI) was calculated as:  $\Sigma$

$$\text{IMI} = C_{\text{Imidacloprid}} + C_{6\text{-CNA}} \times M_{\text{imidacloprid}} / M_{6\text{-CNA}} \text{ (M-molecular weight)}$$

\* represents  $p \leq 0.05$ ; \*\* represents  $p \leq 0.01$ ; \*\*\* represents  $p \leq 0.001$ .

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**Table 1** Experimental parameters for Multiple Reaction Monitoring (MRM).

compound	Molecular formula	MW	t <sub>R</sub> (min)	Ion source	CV (V)	Quantification ion transition	CE 1 (eV)	Confirmatory ion transition	CE 2 (eV)	Ion ratio <sup>a</sup>
imidacloprid	C <sub>9</sub> H <sub>10</sub> ClN <sub>5</sub> O <sub>2</sub>	255.66	2.20	ESI+	22	256.1→175.01	17	256.1→209.05	20	1.47
D4-imidacloprid (IS)	C <sub>9</sub> H <sub>6</sub> D <sub>4</sub> ClN <sub>5</sub> O <sub>2</sub>	259.69	2.20	ESI+	21	260.0→213.01	14	260.0→179.02	17	1.03
6 CNA	C <sub>6</sub> H <sub>4</sub> Cl <sub>2</sub> NO <sub>2</sub>	157.55	2.24	ESI+	21	157.8→121.9	20	157.8→78	15	2.45

MW-molecular weight; t<sub>R</sub>-retention time; CV-cone voltage; CE-collision energy; <sup>a</sup>- area of quantification ion/area of qualitative ion.

**Table 2** Accuracy (recovery, %) and precision (RSD, %) for the targeted compounds in urine at three spiked levels.

Compound	Spiked level(ng/mL)								
	1			10			100		
	Recovery (%)	RSD <sup>a</sup> (%)	RSD <sup>b</sup> (%)	Recovery (%)	RSD <sup>a</sup> (%)	RSD <sup>b</sup> (%)	Recovery (%)	RSD <sup>a</sup> (%)	RSD <sup>b</sup> (%)
imidacloprid <sup>1</sup>	94.9	5.9	8.0	89.6	7.3	7.2	78.3	4.9	5.7
6-CNA <sup>1</sup>	109.8	3.8	3.4	98.1	3.0	3.5	90.3	2.9	2.7
imidacloprid/D4 -imidacloprid <sup>2</sup>	105.6	2.6	7.5	90.9	2.7	5.0	99.8	4.1	5.3
6-CNA/D4-imid acloprid <sup>2</sup>	110.6	2.3	2.6	93.9	2.7	3.4	105.2	2.7	5.7

RSD, the relative standard deviations.

<sup>a</sup> Intra-day (n=5).

<sup>b</sup> Inter-day (n=15).

<sup>1</sup>(external standard)Recovery= $\frac{\text{native analyte response from extract}}{\text{native analyte response from standard}} \times 100\%$

<sup>2</sup>(internal standard)Recovery= $\frac{(\text{native analyte response from extract})/(\text{IS response from extract})}{(\text{native analyte response from standard})/(\text{IS response from standard})} \times 100\%$

**Table 3** Imidacloprid Absorbed Daily Dose (ADD) estimated from urinary 6-CNA.

Number	Before pesticide spraying			After pesticide spraying		
	Urinary 6-CNA ( $\mu\text{g/g}$ creatinine)	ADD <sub>min</sub> ( $\mu\text{g}$ $\text{kg}^{-1}\text{d}^{-1}$ )	ADD <sub>max</sub> ( $\mu\text{g kg}^{-1}\text{d}^{-1}$ )	Urinary 6-CNA ( $\mu\text{g/g}$ creatinine)	ADD <sub>min</sub> ( $\mu\text{g kg}^{-1}\text{d}^{-1}$ )	ADD <sub>max</sub> ( $\mu\text{g kg}^{-1}\text{d}^{-1}$ )
A1	1.98	19.60	32.14	8.18	80.89	132.68
A2	2.33	26.17	42.92	4.13	46.44	76.18
A3	1.67	18.30	30.02	5.32	58.35	95.71
A4	0.69	7.30	11.98	3.29	34.81	57.09
A5	1.81	19.97	32.76	6.97	76.96	126.23
A6	0.96	11.40	18.70	2.88	34.11	55.95
A7	1.46	17.45	28.61	12.64	151.23	248.05
A8	3.65	37.59	61.66	10.10	104.03	170.64
A9	1.41	16.87	27.68	6.38	76.32	125.18
A10	2.47	24.19	39.68	8.99	88.21	144.69
A11	2.21	17.04	27.95	16.06	123.70	202.90
A12	2.07	22.21	36.43	6.79	72.73	119.30
A13	2.79	30.02	49.23	7.74	83.28	136.60
A14	0.06	0.52	0.85	8.98	72.99	119.73
A15	1.48	12.09	19.83	9.04	73.93	121.26
A16	1.36	11.62	19.06	6.60	56.41	92.52
A17	2.05	15.82	25.94	2.98	22.94	37.62
A18	3.08	25.58	41.95	6.38	52.96	86.87
A19	2.11	16.30	26.74	15.92	122.76	201.35
A20	1.19	11.49	18.85	5.94	57.46	94.24
A21	0.74	8.25	13.52	4.23	47.43	77.79
A22	0.73	7.96	13.05	4.74	51.35	84.22
A23	1.59	17.28	28.35	2.30	25.03	41.05
A24	0.98	9.50	15.59	1.78	17.31	28.39
A25	1.50	17.07	28.01	10.56	120.51	197.66
A26	2.65	28.96	47.51	4.34	47.47	77.86
A27	1.98	14.63	24.00	7.37	54.57	89.50
A28	1.87	20.86	34.21	3.92	43.72	71.71
A29	1.29	14.75	24.19	3.68	42.16	69.15
A30	2.00	21.53	35.31	4.43	47.73	78.28
A31	1.71	18.95	31.09	4.12	45.64	74.86
A32	0.96	9.21	15.11	4.33	41.67	68.35
A33	2.45	26.29	43.12	4.20	45.03	73.86
A34	1.49	15.83	25.97	4.59	48.70	79.88
A35	1.59	13.38	21.95	2.14	18.01	29.53
A36	0.93	9.87	16.18	3.28	34.88	57.21
A37	1.24	14.48	23.74	0.66	7.65	12.56
A38	0.82	8.47	13.90	1.19	12.35	20.25

A39	0.75	8.70	14.28	9.73	112.88	185.15
A40	1.15	10.94	17.95	2.05	19.53	32.03
A41	0.72	8.08	13.25	3.74	41.92	68.76
A42	0.97	10.12	16.60	4.31	44.93	73.70
A43	1.03	8.34	13.67	3.34	27.08	44.42

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$ADD_{max}$  was estimated maximal value for ADD;

$ADD_{min}$  was estimated minimal value for ADD. ADD was calculated in Eq. (1) and (2) as described in “Absorbed Daily Dose Estimation”.

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**Table 4** Hazard Quotient of imidacloprid exposure after pesticide application.

Guideline	Hazard Quotient <sup>a</sup>					Hazard Quotient <sup>b</sup>				
	Minimum	25 <sup>th</sup>	50 <sup>th</sup>	75 <sup>th</sup>	Maximum	Minimum	25 <sup>th</sup>	50 <sup>th</sup>	75 <sup>th</sup>	Maximum
60 $\mu\text{g}/\text{kg}/\text{d}$ (ADI, WHO/FAO)	0.13	0.58	0.80	1.27	2.52	0.21	0.95	1.30	2.09	4.13
400 $\mu\text{g}/\text{kg}/\text{d}$ (ARfD, WHO/FAO)	0.02	0.087	0.12	0.19	0.38	0.03	0.14	0.20	0.31	0.62

Hazard Quotient (HQ)=ADD/Guideline dose.

<sup>a</sup> HQ was calculated according to the values of ADD<sub>minimum</sub> in Table 4;

<sup>b</sup> HQ was calculated according to the values of ADD<sub>maximum</sub> in Table 4.

**Highlights**

Optimization was conducted for imidacloprid and 6-CNA in urine using IS calibration

Imidacloprid and 6-CNA was detected in 100% of urine samples from rural applicators

Urinary concentration of imidacloprid increased significantly after spraying event

The ADD range estimation was firstly reported for imidacloprid

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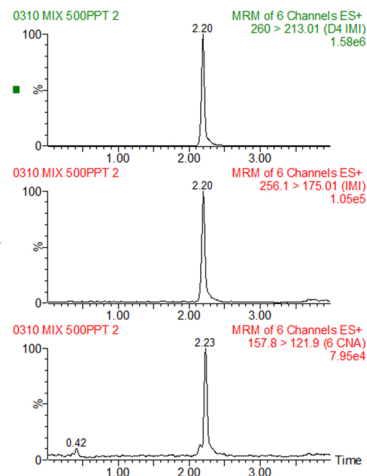
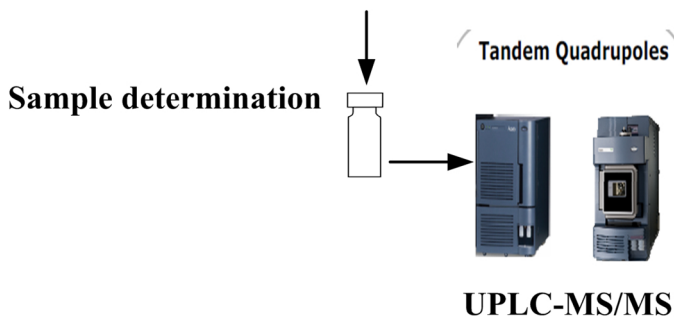
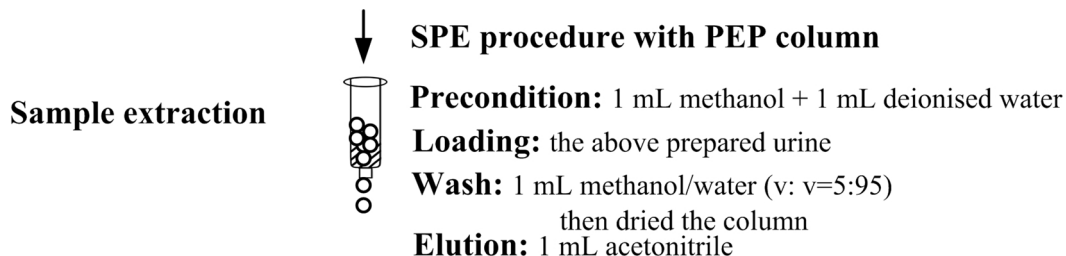
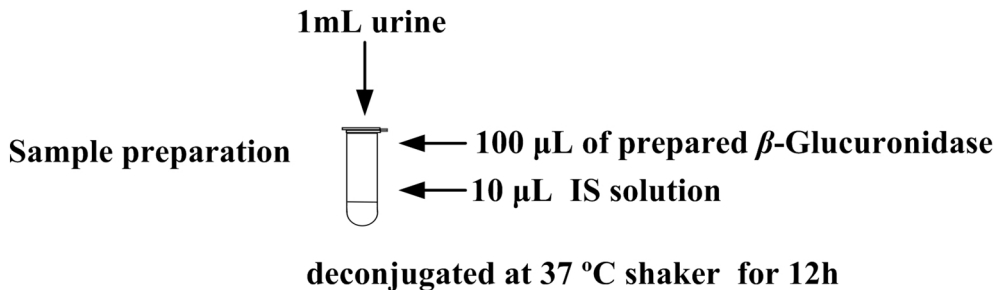


Figure 1

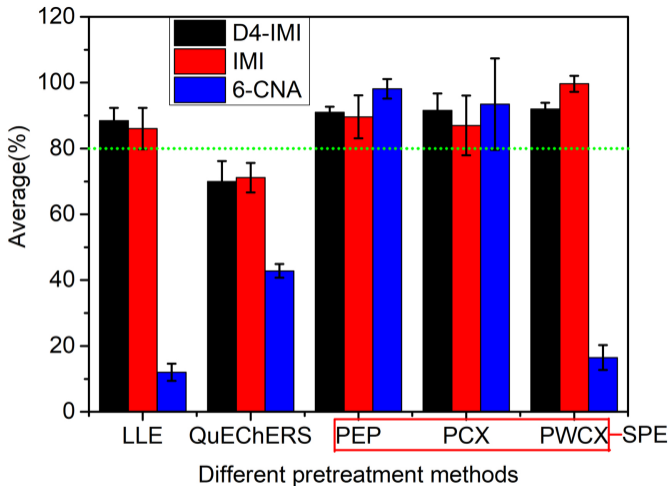


Figure 2

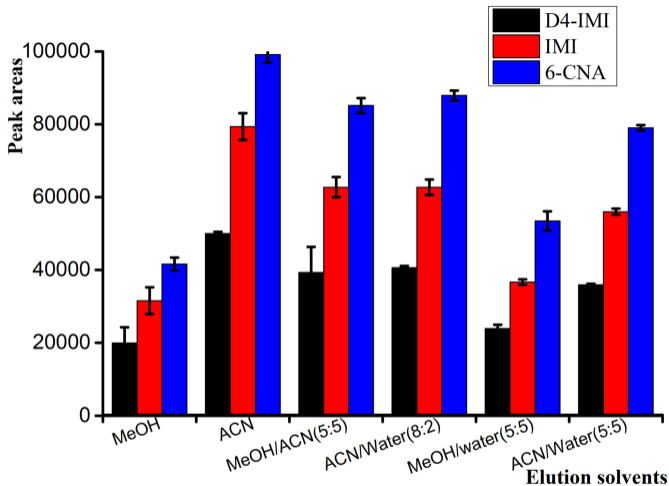
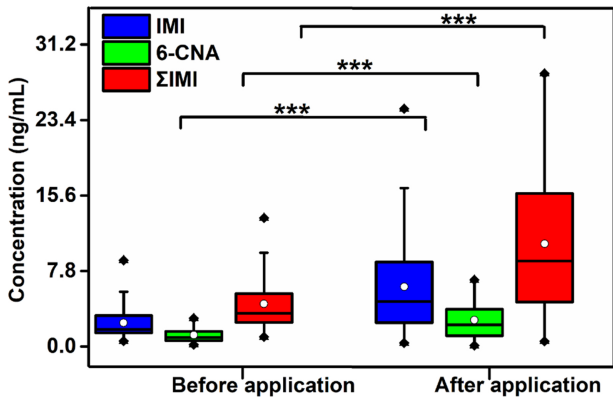


Figure 3

A



B

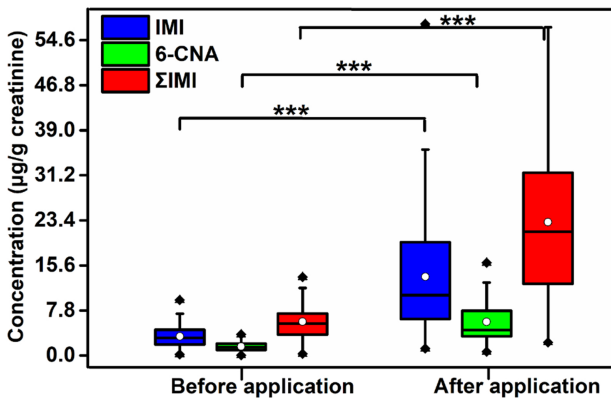


Figure 4