Title: Biological Monitoring Method for Urinary Neonicotinoid Insecticides Using LC-MS/MS and Its Application to Japanese Adults

Running title: Biological monitoring of neonicotinoids in Japanese adults

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Abstract: Biological Monitoring Method for Urinary Neonicotinoid Insecticides Using LC-MS/MS and Its Application to Japanese Adults: Jun Ueyama et al., Department of Pathophysiological Laboratory Sciences, Field of Radiological and Medical Laboratory Sciences, Nagoya University Graduate School of Medicine, Nagoya, Japan.

Objectives: Agricultural use of neonicotinoid (NEO) insecticides has been increasing in recent years, but their biological monitoring methods have been scarcely reported. In this study, we developed and validated a rapid and sensitive method for quantifying urinary NEO concentrations using high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). Methods: After phosphate-induced acidification of a urine sample, urinary NEOs were trapped by a solid-phase extraction column and eluted with methanol for acetamiprid, imidacloprid, thiacloprid, thiamethoxam, clothianidin and dinotefuran and with an acetonitrile and methanol solution (1:1, v/v) containing 5% NH₃ for nitenpyram. A separation analysis was performed by LC-MS/MS within 10 min for the sample. This method was applied to first morning urine obtained from 52 Japanese (40.9 ± 10.5 years old, mean ± standard deviation) without occupational NEO exposure. Results: The linear dynamic ranges and their limit of quantification (LOQ, signal to noise ratio = 10) levels were 0.3–20 or 50 µg/l (r = 0.998–0.999) and 0.05–0.36 µg/l, respectively. The absolute recovery was above 64%, and the intra- and inter-day precisions were less than 16.4% (relative standard deviation, %RSD). This method was successfully applied for analysis of NEOs in human urine samples obtained from 52 adults. The frequencies of individuals who showed more than LOD levels was above 90% for imidacloprid, thiamethoxam, clothianidin and dinotefuran, more than 50% for acetamiprid and thiacloprid and 29% for nitenpyram.

Conclusions: These results indicated that our new method could be applied to biological monitoring of NEO exposure even at environmental exposure levels in Japanese adults without occupational spraying histories.
**Key words:** Biological monitoring, LC-MS/MS, Neonicotinoid, Urine
Introduction

Neonicotinoid (NEO) insecticides, the fastest growing class of insecticides in modern crop protection, act selectively on insect nicotinic acetylcholine receptors\(^1,2\). Currently, 7 different active ingredients are available in NEOs: acetamiprid, clothianidin, dinotefuran, imidacloprid, thiacloprid, thiamethoxam and nitenpyram. Launched in the late 1990s, their agricultural use has been increasing in the US\(^3\) and Japan\(^4\), as organophosphorus (OP) and pyrethroid (PYR) insecticides are being phased out primarily due to the emergence of insects resistant to conventional pesticides.

Exposure assessment, which is conducted to evaluate various exposures incurred by workers in the workplace and general populations in their living environments, is a key component of human health risk assessment. Mainly by using high-performance liquid chromatography (HPLC) with a single or tandem mass spectrometry detector (MS/MS), the levels of NEOs in foods and environmental samples such as soil and ground water have been quantitated\(^5-7\). However, methods for biological monitoring of NEO exposure in humans have scarcely been reported. Uroz et al.\(^8\) and the present authors\(^9\) have developed a method of measuring urinary NEO metabolites, 6-chloronicotinic acid (6CN) and 2-chloro-1,3-thiazole-5-carboxylic acid (2CTCA), to monitor the level of NEO exposure. These measurements could play a pivotal role in NEO exposure assessment, but there are two theoretical limitations. One is the difficulty in identifying absorbed compounds from the metabolite information in groups. The other is that dietary intakes of degraded NEOs structurally identical to the metabolites might increase the urinary metabolite levels, which could result in overestimation of NEO exposure.

When NEOs, especially dinotefuran and imidacloprid, are absorbed into the body, they are excreted in urine as an unchanged compound, because they have higher water solubility than organophosphorus and pyrethroids\(^10,11\). Thus, monitoring of urinary NEO, but not
NEO metabolites, may contribute to a more precise estimation of the amount of NEO uptake into the human body. The aim of this study was to develop a new method for simultaneous determination of urinary NEOs using HPLC with MS/MS (LC-MS/MS), which is sensitive enough to be adopted not only in occupational but nonoccupational NEO exposure settings.

Materials and Methods

Chemicals and reagents

The chemical structures of the measured NEOs are shown in Fig. 1. Acetamiprid (purity >99%), clothianidin (purity >99%), dinotefuran (purity >99%), thiacloprid (purity 98%), thiamethoxam (purity >99%), phosphoric acid, and methanol (LC-MS grade) were obtained from Wako Pure Chemical Industries (Osaka, Japan); imidacloprid (purity >98%) was obtained from Sigma-Aldrich (St. Louis, MO, USA); and nitenpyram (purity >98%) was obtained from AccuStandard (New Haven, CT, USA). Isotope-labeled cotinine (1.0 mg/ml methanol, cotinine-d3) and acetamiprid (purity 99.7%, acetamiprid-d6) were used as internal standards (IS) for nitenpyram and other NEO measurement, respectively. Cotinine-d3 and acetamiprid-d6 were purchased from Cerilliant (Round Rock, TX, USA) and Hayashi Pure Chemical Ind. (Osaka, Japan), respectively. LC-MS grade acetonitrile, formic acid, acetic acid and 1 mol/l ammonium acetate were purchased from Wako Pure Chemicals (Osaka, Japan). All other reagents were of analytical grade. A polymeric strong cation exchange solid-phase extraction (SPE) product, Bond Elut PCX (30 mg) (Agilent Technologies, Inc., Santa Clara, CA, USA), was used for NEO extraction from urine.

Preparation of stock solutions

NEO standard reagents were diluted to a concentration of 1000 mg/l in ethanol, and further diluted with the same respective solvents to prepare working standard solutions at
concentrations ranging from 0.1 to 10 mg/l. These standard solutions were stored in the dark at -30°C and were used within 3 months. Pooled urine, collected from three healthy volunteers who were neither medicated nor occupationally exposed to NEO beforehand, was prepared by twofold dilution with H₂O to minimize background concentrations of dinotefuran in the original urine. The pooled urine was used for the basic methodological experiment to evaluate NEO stability in the reproducibility experiment.

Sample preparation procedure

A flow chart of the procedure for determining urinary NEOs is shown in Fig. 2. One milliliter of urine was pipetted into a 10-ml screw-top glass test tube, and 1 ml phosphoric acid solution (2%) and IS solution (10 mg/l acetamiprid-d₆ and 5 mg/l cotinine-d₃) were added. After gentle shaking, the test tube was incubated at 37°C in a heat bath for 10 min for degradation of some urine crystals. After that, the urine sample was applied to an SPE procedure.

In this method, the SPE procedure was composed of 2 steps. Preconditioning was achieved by washing with 0.5 ml methanol and acetonitrile (1:1, v/v) solution containing 5% NH₃ followed by washing with 0.5 ml H₂O. Then, the SPE was loaded with the 2 ml prepared urine sample and this was followed by a wash with 0.5 ml formic acid solution (2%). The SPE cartridge was dried in a vacuum for 3 min and eluted with 0.5 ml methanol. The eluate (fraction A) contained acetamiprid, imidacloprid, thiacloprid, thiamethoxam, clothianidin, dinotefuran and acetamiprid-d₆ (SPE procedure step 1). Furthermore, the SPE column was washed with 0.5 ml acetonitrile, and the nitenpyram and cotinine-d₃ were eluted by 0.5 ml methanol and acetonitrile (1:1, v/v) containing 5% NH₃ (fraction B, SPE procedure step 2).

Each fraction was dried with a gentle nitrogen stream at 37°C, and the residues were
dissolved in 170 µl acetonitrile. After sonication for 10 min in an ultrasonic bath and centrifugation (12,000 × g for 5 min), the supernatants were injected into an LC-MS/MS system.

**LC-MS/MS analysis**

LC-MS/MS analysis was run on an Agilent 1200 infinity LC coupled with an Agilent 6430 Triple Quadrupole LC/MS System (Agilent Technologies, Inc., Santa Clara, CA, USA). The LC operating conditions were as follows: LC column, Scherzo SM-C18 (Intakt, Kyoto, Japan), 100 mm × 2 mm i.d., 3 µm silica; mobile phase, H₂O containing 17 mmol/l acetic acid and 5 mmol/l ammonium acetate (A), and acetonitrile containing 17 mmol/l acetic acid and 5 mmol/l ammonium acetate (B); total flow rate of mobile phase, 0.2 ml/min; total run time including equilibration, 10 min. The initial mobile phase composition was 98 % mobile phase A and 2% mobile phase B. The percentage of mobile phase B was changed linearly over the next 2 min until 5%. Over the next 2 min, the percentage was increased to 70% linearly. After that the percentage was maintained for 3 min, the mobile phase composition was allowed to return to the initial conditions and allowed to equilibrate for 3 min. The injection volume was 3 µl.

The MS/MS was operated with an electrospray ionization (ESI) source in the positive ion mode with multiple reaction monitoring (MRM). The nebulizer gas pressure was set at 35 psi with the source temperature of 325°C and the gas flow at 10 l/min. The capillary voltage was 4000 V (positive mode). High-purity nitrogen gas was used as collision cell gas.

Table 1 shows the optimized MRM parameters and retention times for NEOs and their ISs. The raw chromatograph and mass spectrogram data were processed with the MassHunter Workstation software (Agilent).
**Assay validation**

The bioanalytical method was validated in terms of selectivity, linearity, accuracy, precision, extraction recovery, matrix effect and stability.

To determine and calculate absolute recoveries, we spiked NEOs at two different stages in the NEO measurement procedure, i.e., in the beginning of the extraction procedure (urine sample) and prior to the LC-MS/MS analysis. We compared the IS ratios obtained at these two stages.

The matrix effect for NEO ionizations was determined at the same concentrations of NEO-added prepared sample derived from pooled urine and NEO-added acetonitrile solution (1 and 5 µg/L of acetonitrile, n=6 for each concentration).

Calibration curves using pooled urine are presented as the NEO/IS peak area ratio versus the concentrations of calibration samples ranging from 0.3 to 50 µg/l (8 concentrations) for dinotefuran and 0.3 to 20 µg/l (7 concentrations) for the others. The linearity of the calibration curve was determined by linear regression analysis. A calibration curve with a correlation coefficient \( r \geq 0.990 \) was considered to be linear.

The within-run precision of our method was examined through an assay of pooled urine spiked with NEOs at concentrations of 0.3, 0.6, 1.3, 5 and 20 µg/l (n = 5 for each).

Moreover, the between-run precision was examined through a duplicate assay of the pooled urine spiked with NEO metabolites at concentrations of 1.3 and 5 µg/l for 5 consecutive days (duplicate assay). The limit of detection (LOD) levels were calculated as values resulting in a signal-to-noise ratio of 3. The limit of quantitation (LOQ) levels were defined as the lowest concentrations for which the NEO signal-to-noise ratio was \( \geq 10 \) and the precision, the percent of relative standard deviation (%RSD), was <20.

**Sample stability**
Freeze and thaw stability and short-term storage stability of NEO in pooled urine were evaluated at concentrations of 1.25 and 5 µg/l. NEO-spiked pooled urine samples were incubated at 4°C and 37°C for 24 h and then stored at -80°C until NEO analysis. NEO stabilities were assessed by comparing the NEO peak areas of 4°C or 37°C incubated samples with those of non-incubated samples. Other NEO-spiked pooled urine samples were stored at -80°C for 24 h and thawed unassisted at room temperature (approximately 25°C). When completely thawed, the urine samples were frozen under the same conditions again. After three freeze-thaw cycles were performed, each NEO was measured and compared with those for urine samples that did not undergo freezing (n = 3 for each). NEO stabilities in prepared samples were evaluated by comparing the NEO peak areas obtained from samples stored at 4°C for 24 h with those stored for 0 h (n = 3 for each).

Application of methods to field study samples

Our method was applied to first morning urine obtained from 52 Japanese (41 males and 11 females) who worked in 2 companies located in Aichi Prefecture in central Japan (40.9 ± 10.5 years old, mean ± standard deviation). According to the results of a questionnaire, they comprised a population exposed to NEOs at environmental background levels in their daily lives from unidentified sources in general but not occupational settings. Collected urine samples were transported at 4°C to our laboratory and stored at -80°C until NEO analyses. The Ethics Committees of the Nagoya University Graduate School of Medicine approved the study protocol. Prior to enrollment in the study, an informed consent form was signed by each subject giving the right to use personal information for research purposes.

Results and discussion

Method development
The supplier-recommended SPE protocol was slightly modified for urinary NEO extraction. Use of methanol as an extraction solution for NEO, concentration with N₂ gas and a centrifugation procedure ensured high sensitivity for determining NEOs without interfering with chromatograph peaks. However, simultaneous extraction of all the measured NEOs could not be achieved because of the relatively strong ionic binding of nitenpyram to the SPE column.

HPLC and ESI-MS/MS settings were optimized to allow maximal sensitivity. Although formic acid has been used for determination of NEOs in field products in some reported methods, the mobile phase using acetic acid improved the detection sensitivity substantially for all chemical structures except for that of dinotefuran. Moreover, higher NEO peaks were obtained in the ESI-positive mode than in the ESI-negative mode. The other MS/MS parameters, such as collision energy and product ions for NEOs and ISs, are summarized in Table 1.

Pooled urine samples with or without spiked NEOs were analyzed to evaluate chromatographic interference. No interference with analytes or internal standard peaks was detected (Fig. 3). We confirmed that the concentration of one NEO component was not affected by the concentration of the other NEOs (data not shown).

**Method validation**

The values for absolute recovery, precision, LOD and LOQ are summarized in Table 2. For the within-run precision, the %RSD was 16.4% or less at the concentration of 0.3 µg/l. For the between-run precision, the %RSD was between 2.4 and 9.3% at the concentration of 1.3 µg/l. The lowest %RSD values, both for the within- and between-run precisions, were found for acetamiprid. This high precision may be due to the use of acetamiprid-d₆ as an IS, suggesting that an isotope-labeled IS could similarly improve the precision in determining
The absolute recoveries of NEOs from pooled urine in our method ranged from 64 to 95%. Using a large volume of extraction solution could not increase the recovery rate. Although some recovery rates of NEOs were relatively low, the extents of recoveries of analytes were reproducible; urine-based calibration curves should contribute to the ability to obtain reliable quantitative data from human urine samples. A matrix effect on NEO ionization was not observed; the recoveries of NEOs ranged from 89 to 103%.

Calibration curves were constructed by plotting the peak area ratio of the each NEO to the IS (y-axis) versus the concentration of the corresponding NEO (x-axis). The regression equations were $y=0.0275x+0.0021 \ (r=0.9998)$ for acetamiprid, $y=0.0012x+0.0004 \ (r=0.9989)$ for imidacloprid, $y=0.0049x+0.0009 \ (r=0.9999)$ for thiacloprid, $y=0.0106x+0.0026 \ (r=0.9995)$ for thiamethoxam, $y=0.0029x+0.0008 \ (r=0.9996)$ for clothianidin, $y=0.0115x+0.0002 \ (r=0.9983)$ for dinotefuran and $y=0.0012x+0.0001 \ (r=0.9992)$ for nitenpyram.

It is difficult to compare LOD and LOQ values in our study with those in other studies, because this is the first report to develop a method for determining NEOs from urine samples. However, the LOD levels for our method are approximately the same as the LOD values in pesticide residue analyses$^{6,13}$.

**Sample stability**

The stability of NEOs in human urine was assessed for typical storage/handling conditions. After three freeze-thaw cycles, changes in the ratios of peak areas were negligible. With regard to long-term stability at 4°C (24 h) and 37°C (24 h), the peak ratios were comparable to those of the fresh sample (90.2-104.6% for 4°C and 90.2-104.6% for 37°C). These observations suggest that NEOs likely are stable following storage under typical processing.
conditions, but careful handling may be required at room temperature to prevent NEO degradation by proliferated bacteria.

Application of the method to field study samples

The newly developed method was applied to urine samples of 52 adults, and the resulting data are shown in Table 3. We confirmed that concentrations of urinary NEO were log-normally distributed (data not shown) and that the respective interquartile ranges (IQR) were <LOD-0.06 µg/l for acetamiprid, 1.0-3.1 µg/l for imidacloprid, <LOD-0.23 µg/l for thiacloprid, 0.3-1.4 µg/l for thiamethoxam, 0.4-1.0 µg/l for clothianidin, 1.2-3.5 µg/l for dinotefuran and <LOD-0.17 µg/l for nitenpyram. The detection frequencies of NEOs were more than 50%, except for nitenpyram. Thus, this method could be applied to biological monitoring of NEO exposure even at environmental exposure levels in individuals without occupational spraying histories.

The measured results strongly suggest that exposure to several NEOs had occurred in the Japanese adults in their daily lives. The median and maximum NEO concentrations in urine were relatively higher for dinotefuran (2.3 and 27.4 µg/l, respectively) and imidacloprid (1.9 and 8.2 µg/l). On the other hand, the detection frequency of nitenpyram was lower (29%).

According to recent data in Japan, dinotefuran and imidacloprid ranked first and second, respectively, among the seven NEO components, and nitenpyram ranked the lowest in terms of the amount of shipped⁴. Although the sample size was small in this study, the detection frequencies and concentration levels of urinary NEOs seem to reflect the amount of NEOs shipped in Japan.

Limitations of this study and future directions

The following limitations should be mentioned. The main limitation was that only one
isotope-labeled NEO, acetamiprid-d₆, was used as an IS, because the remaining 6
isotope-labeled NEOs were commercially unavailable. The use of these substances as ISs
might help to improve the measurement reliability. Although the level of confirmation ions
and quantification ions and retention time were monitored carefully to determine whether the
peak in the MRM chromatogram was NEO specific or not, it was difficult to identify NEO
peaks at concentrations near the LOD levels. This problem might be resolved using the 7
isotope-labeled NEOs. Finally, comprehensive evaluation of occupational and
environmental NEO exposures is warranted using various urine samples including historical
samples.

Conclusions

It was suggested that our method is sensitive enough to measure urinary NEOs at
environmental exposure levels in individuals without occupational NEO exposure histories.
Since simultaneous measurement of NEOs themselves in the urine makes it possible to
identify each of the NEOs and to estimate their exposure amounts, such monitoring studies
need to be conducted to precisely assess the risk to human health posed by exposure.
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Conflict of interest: The authors declare that they have no conflict of interest.
References


Figure legends

Fig. 1. Chemical structures of the seven NEOs measured in this study.

Fig. 2. Optimized analytical procedure for urinary NEOs. Nitenpyram and other NEOs were extracted by two eluents separately. One included methanol for acetamiprid, imidacloprid, thiacloprid, thiamethoxam, clothianidin and dinotefuran (SPE procedure step 1, left), and the other included methanol and acetonitrile (1:1, v/v) containing 5% NH₃ for nitenpyram (SPE procedure step 2, right).

Fig. 3. MRM chromatograms of NEOs in pooled human urine samples at three concentrations: <LOQ, 1.3 and 2.5 µg/l for acetamiprid, thiacloprid, thiamethoxam, clothianidin and nitenpyram; <LOQ, 2.5 and 5.0 µg/l for imidacloprid; and <LOQ, 3.0 and 4.6 µg/l for dinotefuran.
<table>
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<th>Compounds</th>
<th>Fragmentor (V)</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Collision energy (eV)</th>
<th>Retention time (min)</th>
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<td>223</td>
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C, confirmation ion. Q, quantification ion.
Table 2. Recovery, precision, LOD and LOQ data for the analytical procedure.

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<tr>
<th>Concentration (µg/l urine)</th>
<th>n</th>
<th>Acetamiprid</th>
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<th>Thiacloprid</th>
<th>Thiamethoxam</th>
<th>Clothianidin</th>
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Within-run

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Between-run

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<th>Precision (%RSD&lt;sup&gt;b&lt;/sup&gt;)</th>
<th>1.3</th>
<th>5</th>
<th>0.7</th>
<th>4.1</th>
<th>0.7</th>
<th>4.1</th>
<th>0.01</th>
<th>0.05</th>
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<td>2.4</td>
<td>1.6</td>
<td>0.7</td>
<td>5.9</td>
<td>0.7</td>
<td>5.9</td>
<td>0.12</td>
<td>0.36</td>
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<td>8.4</td>
<td>2.8</td>
<td>4.1</td>
<td>1.6</td>
<td>4.1</td>
<td>1.6</td>
<td>0.06</td>
<td>0.20</td>
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<td>2.8</td>
<td>5.9</td>
<td>0.4</td>
<td>5.9</td>
<td>0.4</td>
<td>0.04</td>
<td>0.13</td>
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<td>2.8</td>
<td>1.6</td>
<td>0.04</td>
<td>1.6</td>
<td>0.04</td>
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<td>0.10</td>
<td>2.8</td>
<td>0.10</td>
<td>0.10</td>
<td>0.34</td>
</tr>
</tbody>
</table>

<sup>a</sup>Recovery was obtained by adding the standards just before the separation analysis step. <sup>b</sup>Relative standard deviation. <i>n</i>, number of observations. LOD, limit of detection. LOQ, limit of quantitation.
Table 3. Concentrations of urinary NEOs (μg/l) among Japanese adults (n=52)

<table>
<thead>
<tr>
<th></th>
<th>&gt;LOD (%)^a</th>
<th>GM(GSD)^b</th>
<th>5th</th>
<th>25th</th>
<th>50th</th>
<th>75th</th>
<th>95th</th>
<th>Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetamiprid</td>
<td>56</td>
<td>N.C^d</td>
<td>&lt;LOD^c</td>
<td>&lt;LOD</td>
<td>0.02</td>
<td>0.06</td>
<td>0.18</td>
<td>0.36</td>
</tr>
<tr>
<td>Imidacloprid</td>
<td>96</td>
<td>1.54 (2.7)</td>
<td>0.3</td>
<td>1.0</td>
<td>1.9</td>
<td>3.1</td>
<td>6.9</td>
<td>8.2</td>
</tr>
<tr>
<td>Thiacloprid</td>
<td>67</td>
<td>N.C</td>
<td>&lt;LOD^c</td>
<td>&lt;LOD</td>
<td>0.14</td>
<td>0.23</td>
<td>0.35</td>
<td>0.50</td>
</tr>
<tr>
<td>Thiamethoxam</td>
<td>100</td>
<td>0.56 (2.8)</td>
<td>0.1</td>
<td>0.3</td>
<td>0.5</td>
<td>1.4</td>
<td>3.0</td>
<td>6.2</td>
</tr>
<tr>
<td>Clothianidin</td>
<td>96</td>
<td>0.57 (3.4)</td>
<td>0.1</td>
<td>0.4</td>
<td>0.7</td>
<td>1.0</td>
<td>3.5</td>
<td>6.6</td>
</tr>
<tr>
<td>Dinotefuran</td>
<td>100</td>
<td>2.27 (2.6)</td>
<td>0.5</td>
<td>1.2</td>
<td>2.3</td>
<td>3.5</td>
<td>18.3</td>
<td>27.4</td>
</tr>
<tr>
<td>Nitenpyram</td>
<td>29</td>
<td>N.C</td>
<td>&lt;LOD^c</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>0.17</td>
<td>0.85</td>
<td>1.03</td>
</tr>
</tbody>
</table>

^aPercent of detection frequency. ^bGeometric mean (geometric standard deviation). ^cLower than the level of limit of detection. ^dData not calculated due to its low detection frequency.
Fig. 1.

Chloropyridinyl neonicotinoids

Acetamiprid

Imidacloprid

Chlorothiazolylmethyl neonicotinoids

Clothianidin

Thiamethoxam

Tetrahydrofuranylmethyl neonicotinoid

Thiacloprid

Nitenpyram

Dinotefuran
Fig. 2.

1 ml urine

Add 1 ml phosphate solution (2%)
10 µl IS stock solution

Incubation at 37°C for 10 min

SPE procedure step 1

Transfer SPE column to another test tube

Fraction A
acetamiprid, imidaclorpid, thiacloprid,
thiamethoxam, clothianidin, dinotefuran

Dry with N₂ gas at 37°C

Add 170 µl
CH₃CN (100%)

Mix well and sonication for 10 min

Centrifuge
(10 min, 12,000 × g)

SPE procedure step 2

Fraction B
nitenpyram

Supernatant is injected into LC-MS/MS (nitenpyram and other NEOs are analyzed separately)
Fig. 3.