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ABSTRACT

There is a growing appreciation of the importance of determining chemical exposure levels in early childhood, as well as in embryonic and foetal life, which are now widely believed to be essential for gaining insight into potential health risks associated with these chemicals. To facilitate the assessment of exposure to neonicotinoid insecticides (NEOs) in non-toilet-trained children, a new method using disposable diapers (nappies) was developed for the simultaneous determination of the NEOs acetamiprid and its metabolite N-desmethylacetamiprid, clothianidin, dinotefuran, imidacloprid, thiacloprid, and thiamethoxam (NEO biomarkers). The urine absorbed in disposable diapers was extracted with acetone (diaper urine) and was cleaned using a solid-phase extraction column, before analysis with LC-MS/MS. The absolute recoveries of NEO biomarkers were 19–50%. Good results were observed for the linearity of the matrix-matched calibration curves (r^2 = 0.983–0.996; concentration range LOQ-20 µg L^{-1}) and the precision of intra-day (% relative standard deviation (%RSD): 3.3–12.7%) and inter-day (%RSD: 4.3–19.5%) analyses. The lowest and highest limits of detection of the developed method were 0.07 µg L^{-1} for acetamiprid and 0.75 µg L^{-1} for clothianidin. The developed method was applied for the evaluation of fifty diapered three-year-old children in Japan. Importantly, the study revealed relatively high detection rates for dinotefuran and N-desmethylacetamiprid; 84% and 78% respectively. The highest geometric mean of dinotefuran urinary concentration was 2.01 µg L^{-1}. Thus, a method for determining NEO biomarkers in urine extracted from disposable diapers was established. This is the first report on the simultaneous quantitative analysis of NEO biomarkers of diaper-absorbed urine samples.

Introduction

The widespread use of insecticides to control pests in agriculture has resulted in the contamination of food, water and soil (Stewart et al. 2014; Stone et al. 2014; Watanabe et al. 2018). The main classes of insecticides used in Japan are neonicotinoids (NEOs), organophosphates (OPs), carbamates, and pyrethroids (PYRs) (National Institute for Environmental Studies 2019), and depending on their levels of exposure, these chemicals act on the nervous systems of mammals as well as insects (Costa et al. 2008; Richardson et al. 2019). Among them, the use of NEOs has significantly increased, and they are emerging as the replacements for OPs, PYRs, and carbamates (Cimino et al. 2017). In Japan, the consumption of NEOs has grown

since the late 1990s, and they are being used not only for agriculture but also in other settings, which include public health (National Institute for Environmental Studies 2019). The insecticidal mechanism of action of NEOs involves their binding to the nicotinic acetylcholine receptors (nAChRs) in the insect's central nervous system synapses (Matsuda et al. 2001). Although NEOs were considered to be less neurotoxic towards mammals due to their high selectivity (Tomizawa and Casida 2003), some studies suggest that NEOs act on mammalian nAChRs as well (Tomizawa and Casida 2000; Li et al. 2011; Chen et al. 2014).

The human nervous system develops rapidly in the early stages of childhood, due to which it is anticipated that children would be more vulnerable to neurotoxic

chemicals than adults (Bearer 1998; Tilson 1998; Claudio et al. 2000; Weiss 2000). Therefore, concern for the relation of exposure to insecticides and children's health has increased recently; the potential relations between insecticide exposure and neurotoxicological health effects such as poorer intellectual development (Koureas et al. 2012) and behavioural deficit (Bouchard et al. 2010, 2011; González-Alzaga et al. 2014; Wagner-Schuman et al. 2015) were suggested in previous reports targeting children (7–15 years old). Unfortunately, there is little information about the actual NEO exposure in each individual and whether or not the exposure results in health effects in children.

Urine is frequently used as a biospecimen in human biomonitoring studies to assess chemical exposure because of its advantages such as non-invasive sampling (Yusa et al. 2015). In epidemiological studies among children who cannot control their urination, urine collection bags are commonly used to collect samples. However, this method has some problems. First, the adhesive on the collection bags may irritate the child's skin and elicit rashes. Second, potential leaks from the urine bags due to the child's movement (Davies 2004) may make the parents uncomfortable to volunteer them for such studies. Owing to these drawbacks, it is difficult to collect many urine samples in epidemiological studies to reveal NEO exposure levels of young children using biomonitoring technique, and this may be one of the reasons of slow study progress. As for PYRs and OPs, Saito et al. (2014) and Oya et al. (2017) have successfully established a remarkable method for determining the respective metabolites in urine samples extracted from used diapers (diaper method) which allows the collection of a large number of urine samples from diapered children with minimum effort by the parents. Furthermore, this approach mitigates skin troubles which arise with the use of adhesives present on urine collection bags. Establishing a method for determining NEOs in urine extracted from used diapers will open the door of epidemiological studies in children, especially babies and toddlers who are particularly vulnerable to neurotoxic chemicals due to their rapid development of the nervous system.

The aim of this study was to develop a diaper method for NEOs, i.e., biomonitoring method for simultaneous determination of NEOs in urine extracted from disposable diapers using LC-MS/MS. In this study, biomarkers of six NEOs, including acetamiprid and its metabolite N-desmethylacetamiprid (which showed high detection rates in a previous report (Harada et al. 2016)), clothianidin, dinotefuran, imidacloprid, thiacloprid, and thiamethoxam, were quantified. Nitenpyram, for which the detection needed laborious procedures (Ueyama et al. 2014), was excluded from this study since its detection rates and urinary levels were reportedly very low in the general population in Japan (Osaka et al. 2016).

Materials and methods

Chemicals and reagents

The chemical structures of the NEO biomarkers measured in this study are shown in Figure 1. Acetamiprid (purity >99%), clothianidin (purity >99%), dinotefuran (purity >99%), thiacloprid (purity >98%), thiamethoxam (purity >99%), and N-desmethylacetamiprid (purity >98%) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Imidacloprid (purity >98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Isotope-labelled acetamiprid (purity >99.7%, acetamiprid-d_6) was used as the internal standard (IS) for NEO measurement. Acetamiprid-d_6 was purchased from Hayashi Pure Chemical Ind. (Osaka, Japan). Acetonitrile (LC-MS grade), methanol (LC-MS grade), ethyl acetate, phosphoric acid, formic acid, acetic acid, and 1 mol L^{-1} ammonium acetate were purchased from Wako Pure Chemicals (Osaka, Japan). Acetone was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). All reagents were of analytical grade. Bond Elut PCX 30 mg (Agilent Technologies, Inc., Santa Clara, CA, USA), which is a polymeric strong cation exchange solid-phase extraction (SPE) product, and ISOLUTE SLE+ (10 mL) (Biotage AB; Uppsala, Sweden), which is a porous inert diatomaceous earth column, were used for extraction of NEOs from urine.

Urine extraction from diapers

Merries, which are commercial diapers produced by Kao Corporation (Tokyo, Japan), were purchased and adopted in this study
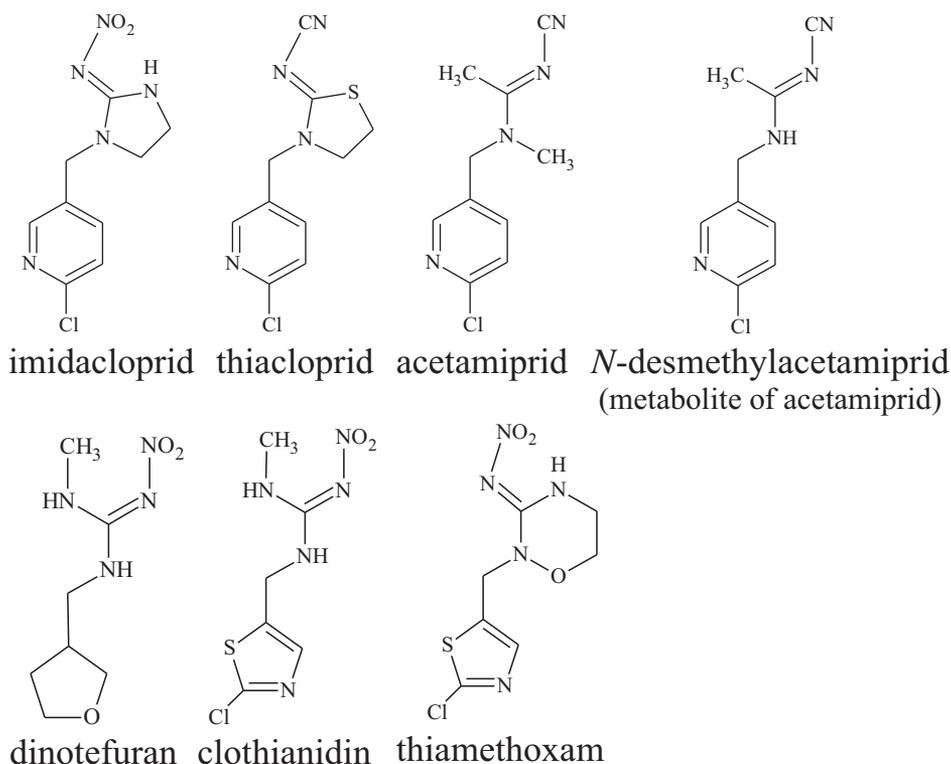
Figure 1. Chemical structures of the six NEOs and the acetamiprid metabolite measured in this study.

because of their higher and more stable pyrethroid metabolite recoveries in comparison with other diapers (Saito et al. 2014). Urine was extracted from the disposable diapers according to our previously reported method (Saito et al. 2014) with slight modifications. The determination of urinary NEOs from diapers is referred to as the 'NEO diaper method' in this paper. This novel method for determining NEO biomarkers by using routine urine samples is based on our previously reported 'NEO direct method' (Ueyama et al. 2014) with minor modifications.

The procedure for urine extraction from disposable diapers is shown in Figure 2. Approximately 1.5 g of the urine absorbent (i.e., urine-soaked polymers and flocculent pulp) was removed from the diapers, and placed in a 5 mL syringe, and the combined weight was recorded (wet weight). This syringe and a 10 mL syringe containing acetone were connected head-to-head using a polyethylene tube. The urine sample in the absorbent was then eluted with acetone by manually reciprocating acetone between both syringes. The obtained eluate was evaporated at 40°C on
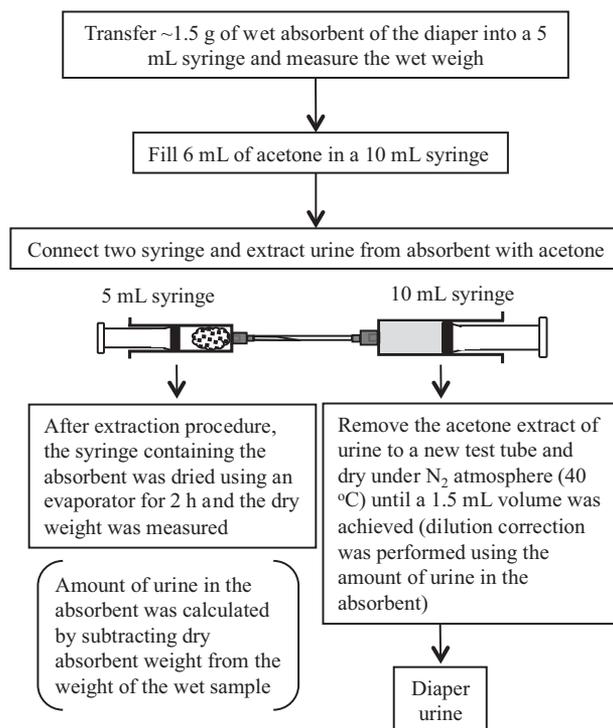
Figure 2. Method for extraction of urine from a disposable diaper.

a heating block under a gentle stream of nitrogen until the volume was reduced to 1.5 mL or less. The volume was adjusted to 1.5 mL with distilled water

(diaper urine). After extraction of urine with acetone, the urine absorbent in the 5 mL syringe was dried under vacuum for more than 1 h, and its weight was obtained (dry weight). The volume of urine in the absorbent was determined from be the difference in wet and dry weights of the urine absorbent.

Sample preparation procedure

The procedure for the NEO diaper method is shown in Figure 3 and Table 1. One millilitre of diaper urine was pipetted into a 10 mL glass test tube, and 1 mL of phosphoric acid solution (2%) and 10 µL of IS solution (5 mg L^{-1} acetamiprid-d$_6$) were added. After gentle shaking, the test tube was incubated at 37°C in a heating bath for 10 min to dissolve any urine crystals. After that, the urine sample was applied to SPE procedure. Preconditioning of the Bond Elute PCX was

achieved by washing with a solution of methanol and acetonitrile (0.5 mL, 1:1, v/v) containing 5% NH$_3$, followed by washing with 0.5 mL of H$_2$O. The SPE cartridge was then loaded with the 2 mL of the acidified urine sample, and the sample was passed through the cartridge and was collected into another new glass tube. The sample thus obtained was then applied onto an ISOLUTE SLE+ column and was eluted with 20 mL of ethyl acetate after leaving to equilibrate for 30 min (this is mainly done for dinotefuran quantitation). The first SPE cartridge (Bond Elute PCX) was washed with 0.5 mL of a formic acid solution (2%) and was eluted with 0.5 mL of methanol. Each fraction was dried using a gentle stream of nitrogen at 40 ºC, and the residues were dissolved in 30 µL of acetonitrile and 60 µL of H$_2$O, respectively. After sonication for 10 min in an ultrasonic bath, the supernatants were mixed together, and the obtained solution was injected into the LC-MS/MS system.
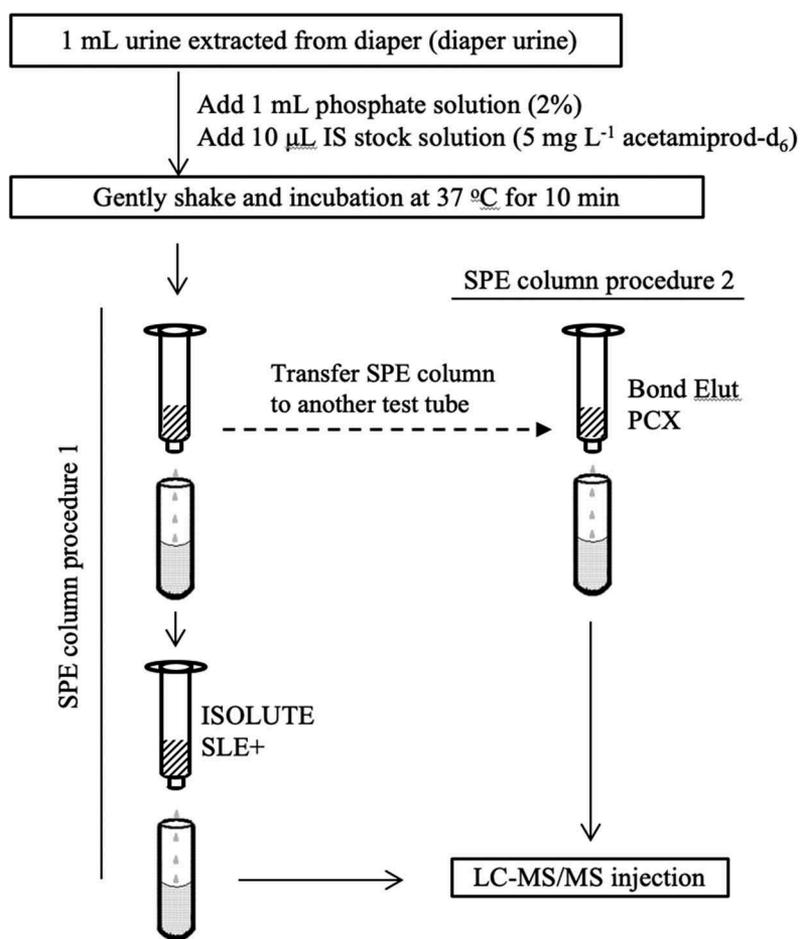
Figure 3. Analytical procedure for analysis of NEOs from diaper urine.

Table 1. Supplementary explanation of Figure 1 (SPE column procedure at-a-glance).

SPE column procedure 1 (for dinoteturan)	SPE column procedure 2 (for the other neonicotinoids)
1. Condition Bond Elut PCX −0.5 mL methanol and acetonitrile (1:1, v/v) containing 5% NH₃ −0.5 mL H₂O	
2. Apply sample	
3. Collect pass through liquid into a new glass tube	3. Wash Bond Elute PCX cartridge with 0.5 ml formic acid solution (2%)
4. Transfer liquid from 3. into ISOLUTE SLE+	4. Elute into a new glass tube with 0.5 ml methanol
5. Wait 30 min	5. Dry with N₂ gas at 40°C
6. Elute with 20 ml ethyl acetate	6. Add 30 µl acetonitrile and 60 µl H₂O
7. Dry with N₂ gas at 40°C	7. Mix well and sonicate 10 min
8. Add 30 µl acetonitrile and 60 µl H2O	
9. Mix well and sonicate 10 min	
Combine extracts from both procedures Analyse by LC-MS/MS	

LC-MS/MS analysis

LC-MS/MS analysis was conducted on an Agilent 1200 Infinity LC coupled with an Agilent 6430 Triple Quadrupole LC-MS System (Agilent Technologies). The LC operating conditions were as follows: LC columns, CAPCELLPAK C18 AQ (150 mm × 2.0 mm i.d., 3 µm silica; Shiseido Company, Tokyo, Japan) and CAPCELLPAK C18 AQ (10 mm × 2.0 mm i.d., 3 µm silica; Shiseido Company); mobile phase, H_2O containing 0.1% acetic acid and 5 mmol L^{-1} ammonium acetate (A), and acetonitrile (B); total flow rate of mobile phase, 0.2 mL min^{-1}; total run time including equilibration, 14 min. The initial mobile phase composition was 98% A and 2% B. The percentage of mobile phase B was increased to 10% after 1 min and was linearly increased to 95% over the next 9 min. The mobile phase composition was then allowed to return to the initial conditions, which was followed by equilibration for 4 min. The injection volume was 10 µL.

The MS/MS analysis was carried out in the positive ion mode using electrospray ionisation (ESI) source with multiple reaction monitoring (MRM). The nebuliser gas pressure, source temperature, and the gas flow were 35 psi, 325°C, and 10 L min^{-1}, respectively. The capillary voltage was 4000 V (positive mode), and high-purity nitrogen gas was used in the collision cell. Table 2 shows the optimised MRM parameters and retention times for 7 NEO biomarkers and IS. The raw chromatograph and mass spectral data were processed with the MassHunter Workstation software (Agilent). The peak area ratios of each NEO biomarker to the IS was used for quantitation.

Method validation

About 150 mg of a urine absorber removed from an unused disposable diaper was used for each analytical run, and 1.5 mL of pooled urine spiked with known concentrations of each analyte of interest was applied to the absorber. The urine absorbers were then incubated for 1 hour at 37°C to simulate actual analyses by the NEO diaper method.

In the validation study, the concentration of NEO in the standard spiked-urine sample was selected to be at around 1 µg L^{-1} according to our past study, in which the 95th percentile concentrations of NEOs in urine samples obtained from 223 three-year-old children were around 1 µg L^{-1} (Osaka et al. 2016). The absolute recoveries were determined at concentrations from 0.6 to 3 µg L^{-1}. For each concentration, the standard solution mixture of NEO biomarkers was spiked at three different points in the measurement procedure, a spike into urine before application onto the absorbents (i), a spike into the extracted diaper urine (ii), and a spike into the sample prepared just before the injection into the LC-MS/MS (iii). The extraction efficiencies and recoveries of the measurement procedure were calculated by comparing analyte/IS peak area ratios. The partial absolute recovery of NEO biomarkers from diapers by acetone extraction was calculated as the mean ratio of the results for (ii) and those for (i). The total absolute recovery percentage through all the measurement procedure was calculated as the ratio of (iii) to (i).

Calibration curves were constructed by plotting the analyte/IS peak area ratios (Y axis) versus the concentrations (X axis) of 7 calibration samples ranging from 0.1 to 20 µg L^{-1}. Linear regression analysis of the calibration plot provided a slope and intercept from which unknown sample concentrations were determined. The calibration samples were prepared by spiking standard solutions into the pooled urine, which was then poured onto the urine absorbent. The

Table 2. Compound-specific mass spectrometer settings.

Compounds	Fragmentor (V)	Collision energy (eV)	Precursor ion (m/z)	Product ion (m/z)	Retention time (min)
Acetamiprid	120	18	223	126 (Q)	8.3
				56 (C)	
Clothianidin	80	16	250	169 (Q)	8.0
				131 (C)	
Dinotefuran	90	3	203	129 (Q)	6.3
				157 (C)	
Imidacloprid	140	10	256	209 (Q)	8.1
				175 (C)	
Thiacloprid	160	22	253	126 (Q)	8.8
				99 (C)	
Thiamethoxam	70	8	292	211 (Q)	7.4
				132 (C)	
N-desmethylacetamiprid	110	20	209	126 (Q)	7.9
				90 (C)	
Acetamiprid-d_6	100	24	229	126 (Q)	8.3
				62 (C)	

Abbreviations: C, confirmation ion. Q, quantification ion. IS, internal standard.

concentrations of the NEO biomarkers before absorption were back-calculated using the measured concentrations from the urine extracted from the diapers, and the errors relative to their spiked concentrations (relative error, %) were evaluated. The absolute matrix effects were represented as matrix factor, which was calculated by dividing the peak response (area) of NEO biomarkers with urine matrix by the peak response in the absence of urine matrix.

The intra-day precision for our proposed method (NEO diaper method) was examined by analysing the assays of the pooled urine samples which were spiked with the standards at various concentrations (0.8, 1.5, and 3 μg L^{-1}) and was then put in urine absorbents (n = 5 for each). The inter-day precision was examined at concentrations of 0.8, 1.5, 3, and 5.7 μg L^{-1} for 5 consecutive days (single measurement in each day).

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as the values at which the signal-to-noise ratios were 3 and 10, respectively. The ratios of the analyte signal to the noise in chromatogram were measured by the MassHunter Workstation software (Agilent Technologies).

Stability of NEO biomarker concentrations in urine at body temperature

The stabilities of NEO biomarkers in the urine absorbents were examined at 37°C, assumed to be the body temperature or room temperature conditions in mid-summer. The urine absorbents containing NEO biomarker-spiked pooled urine (0.8 and 3 μg L^{-1}) were incubated at 37°C for 6, 12, and 24 h, respectively, and the NEO biomarker stabilities were assessed by comparing the analyte/IS peak area ratios of the incubated samples with those of non-incubated ones (n = 3 for each).

Freeze-thaw and storage stabilities of NEO biomarker concentrations in diaper urine

Freeze-thaw stabilities and 1-month storage stabilities of NEO biomarkers in the extracted pooled urine were evaluated at concentrations of 0.8 and 3 μg L^{-1}. These assays were performed according to the approach provided by the U.S. Food and Drug Administration for Bioanalytical Method Validation Guidance for Industry (FDA 2018). The NEO biomarker-spiked pooled urine in the urine absorbent was extracted and stored at −80°C. The sample container for the diaper urine was completely thawed unassisted in running water, and the supernatants were then frozen again and were cooled to −80°C. After three freeze-thaw cycles, the NEO biomarkers were measured and were compared with the concentrations of those in urine which did not undergo the freeze-thaw cycles (n = 3 for each). Moreover, the extracted urine was analysed after storage for 2 weeks and 1 month at −80°C and the results were compared with those obtained from fresh diaper urine without subjecting to any storage procedure (n = 3 for each).

Application of the established method to field study samples

This method was applied for the analysis of samples from 50 three-year-old diapered children (31 males and 19 females) born in Aichi, Japan. Children participating in the Aichi regional sub-cohort of the Japan Environment and Children's Study (JECS-A) (Ebara et al. in revision) were recruited into our study at the time of their three-year-old check-up, which was provided by the local governments. The informed consent of the guardians was obtained for their children's participation in the study. Each child was requested to wear a designated disposable diaper ('Merries') overnight, and the diaper was sent to the laboratory at Nagoya City University the next morning by refrigerated carrier. The urine was extracted from collected diapers within the day and was stored at −80°C (Oya et al. 2017). The urine samples thus obtained, were delivered to our laboratory at Nagoya University and were stored at −80°C until the NEO biomarker analyses. The samples were collected over the season from May to November. When the dinotefuran concentrations were calculated to be over 20 μg L^{-1}, the diaper urine was re-analysed after a 2-fold dilution with H_2O.

The Ethics Committees of the Nagoya University Graduate School of Medicine (2013-0007) and the Nagoya City University Graduate School of Medical Sciences approved the study protocol (1116).

Results and discussion

Method development

The hydrolysis procedure using enzymes or heating under acidic conditions was not performed, because NEO biomarkers we measured were excreted in urine mostly as a free form (Ford and Casida 2006a, 2006b). Prior to the present study, we found that acetone remaining in the diaper urine decreases the recoveries of NEO biomarkers, particularly for dinotefuran, when SPE column procedures 1 and 2 alone (Figure 3, data not shown) were employed. Addition of an SPE column procedure 3 increased the recovery of the dinotefuran without interfering with the chromatographic peaks resolution. The ISOLUTE SLE+ column comprising diatomaceous earth provided the cleanest samples in comparison with the other diatomaceous earth columns (data not shown). However, 20 ml of ethyl acetate was necessary to elute dinotefuran with high recoveries. Further improvement of the method is desirable to reduce the cost of the solvent and make the procedure more environmentally friendly. Pooled urine samples, spiked with or without NEO stock solutions, were analysed to evaluate chromatographic interference. No interference was detected between analytes or IS peaks (Figure 4).

Precision, recovery rate, linearity, and sensitivity

The values for all validation data are summarised in Table 3. First, the NEO recovery rates (residual NEO biomarker levels) in the urine absorbent of a diaper, after urine extraction with acetone were examined. We observed ~50% recovery loss for NEOs in the NEO biomarker extraction procedure from the diaper. Saito et al. (2014) reported acetone to be a suitable solvent for releasing metabolites from polymers with ~100% absolute recoveries for the pyrethroid metabolite 3-phenoxybenzoic acid (3-PBA). However, a lower recovery rate was observed for another pyrethroid metabolite, *trans*-chrysanthemumdicarboxylic acid, which is more hydrophilic in comparison with 3-PBA. In the present study, the most highly hydrophilic NEO dinotefuran (LogKow −0.549) showed the lowest recovery rate, suggesting that hydrophilic compounds might not be recovered with high efficiency from diaper absorber when using acetone. The absolute recovery rates with the present method ranged from 19 to 50%. However, the matrix-matched calibration curves, composed of pooled urine spiked with NEO biomarker standards and urine absorbent, resolves the quantitative accuracy, as shown in the result of minimal relative errors (Table 3). Saito et al. (2014) had shown that the calibration slopes or absolute recoveries of urinary PYR metabolites in their diaper method were different depending on the diaper brands. Therefore, the diaper brands used for preparing calibration curves for the determination of NEO biomarkers need to be matched with the actual diaper samples obtained from children. The usage of IS acetamiprid-d$_6$ successfully corrected the variations of all NEO biomarkers
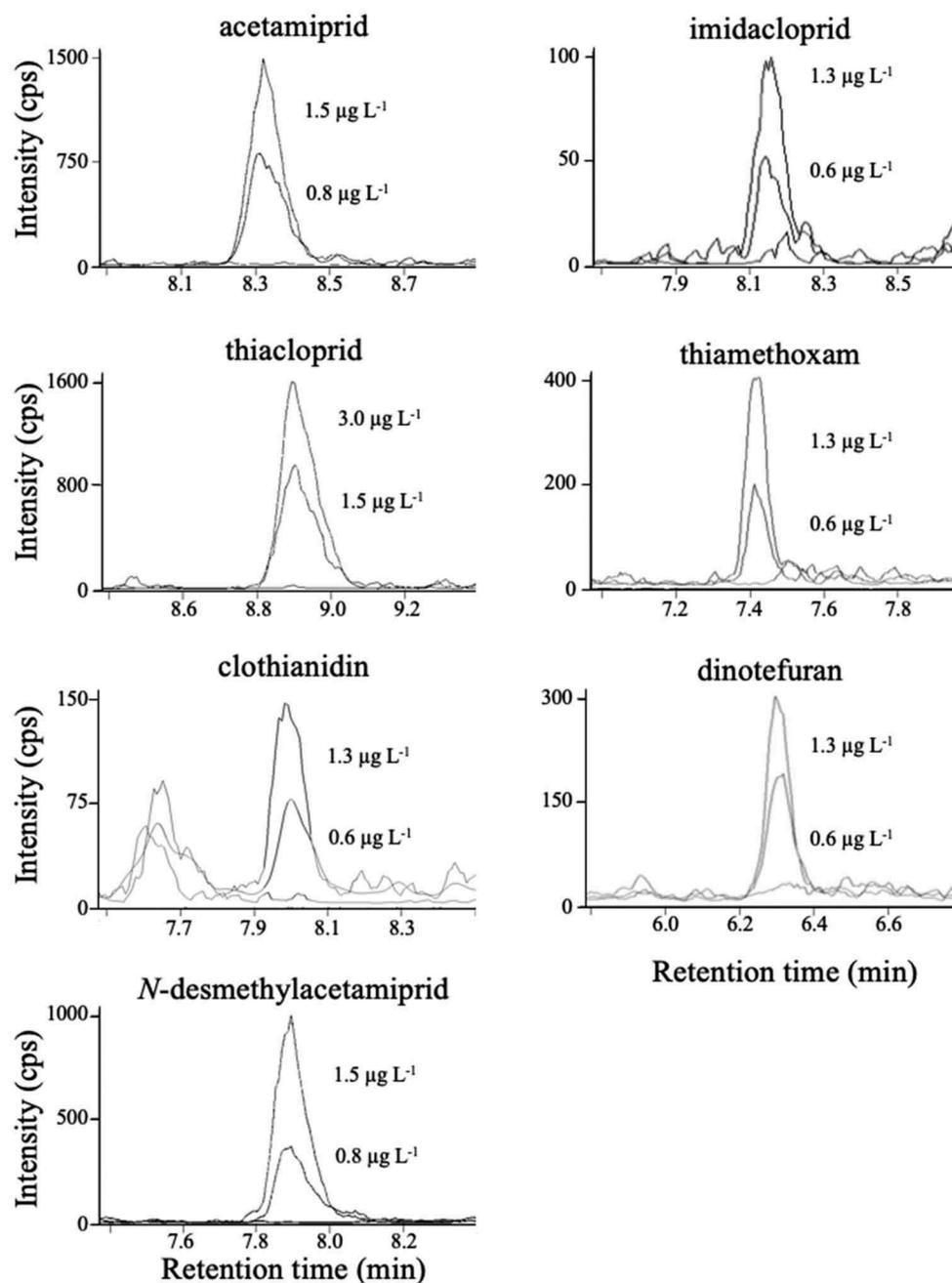
Figure 4. MRM chromatograms of NEOs extracted from urine absorbent, which contained pooled human urine samples spiked with spiked with NEO standards: blank, 0.8 and 1.5 μg L^{-1} for acetamiprid and N-desmethylacetamiprid; blank, 1.5 and 3 μg L^{-1} for thiacloprid; and blank, 0.6 and 1.3 μg L^{-1} for dinotefuran, clothianidin, imidacloprid, and thiamethoxam. Y- and X-axis represent counts per second (cps) of ions and retention time (min), respectively.

among the sample preparation procedure due in part to roughly same absolute recovery rate of IS (63%). The recoveries corrected by IS were more than 72% at 1.3 μg L^{-1} of each NEO biomarker. On the other hand, the recoveries were lower for 5.0 μg L^{-1} of clothianidin and dinotefuran even after IS correction. This problem might be resolved by using isotope-labelled NEO biomarkers.

Next, the linearity and correlation coefficients of the various samples were obtained from the calibration curves at concentrations ranging from the blank levels to 20 μg L^{-1}, and were found to be sufficient. The concentration

Table 3. Precision, recovery rate, linearity, LOD, and LOQ data of the analytical procedure.

	Concentration (µg L^{-1} urine)	n	Acetamiprid	Clothianidin	Dinotefuran	Imidacloprid	Thiacloprid	Thiamethoxam	N-desmethyl acetamiprid
Absolute recovery rate (%)									
All of the protocol	0.8	3	40	50	19	40	35	33	43
	3.0	3	42	48	25	41	36	35	37
Extraction rate of	0.6	2	60	65	36	51	51	51	71
neonicotinoids from diaper by	1.3	2	51	58	45	49	47	49	73
acetone	2.5	2	64	62	47	53	51	53	60
IS-corrected recovery rate (%)									
All of the protocol	1.3	2	88	96	72	76	83	86	113
	5.0	2	94	66	66	83	80	92	101
Within-run									
Precision (%RSD)	0.8	5	5.5	11.2	8.2	10.1	7.6	8.7	6.9
	1.5	5	6.4	10.9	12.4	7.2	6.7	12.7	3.3
	3.0	5	3.9	12.1	10.7	5.3	5.8	8.4	5.3
Between-run									
Precision (%RSD)	0.8	5	5.1	11.4	17.8	11.6	11.3	13.8	7.1
	1.5	5	5.3	15.4	14.8	14.1	11	14.8	6.8
	3.0	5	5.5	13.5	17.1	13.9	11.9	19.5	4.7
	5.7	5	5.6	9.8	18.1	16.7	4.3	14.9	6.2
Relative error (%)									
	0.6	2	0.1	13.6	-^{a}	-^{a}	7.9	0.9	15.8
	1.3	2	13.4	0.7	4.4	0.7	0.9	10.0	6.9
	3.0	2	4.0	13.8	9.2	7.6	2.9	12.1	4.5
	10.0	2	12.1	15.0	16.4	3.3	7.8	14.5	2.4
Calibration curve (for environmental PYR exposure)									
Slope			51.1	717.3	203.4	1015.8	76.7	168.0	83.6
Intercept			0.01	0.05	0.43	0.39	0.24	0.07	0.13
r^2			0.994	0.988	0.983	0.985	0.996	0.990	0.993
LOD (µg L^{-1}) (S/N = 3)			0.07	0.75	0.34	0.40	0.08	0.21	0.08
LOQ (µg L^{-1}) (S/N = 10)			0.22	2.25	1.02	1.21	0.23	0.62	0.23

Abbreviations: *n*, number of observations. RSD, relative standard deviation. LOD, limit of detection. LOQ, limit of quantitation. Note: ^{a}more than 20%.

range of calibration curves was considered applicable to more than 95% of children (Osaka et al. 2016). The urine samples, for which the calculated dinotefuran concentrations were over 20 µg L^{-1}, were adequately diluted with purified water and were reanalysed. The lowest matrix factors (means higher matrix effect) were 52% for imidacloprid, 44% for N-desmethylacetamiprid, 63% for dinotefuran, and 62% for thiacloprid (the others were more than 74%), which suggested that matrix-matched calibration curves might ameliorate these effects.

LOD values ranged from 0.07 (acetamiprid) to 0.75 µg L^{-1} (clothianidin). These values were higher than the LOD levels reported by Saito et al. (2014), during their urinary PYR metabolite analyses using GC-MS detection. The sensitivity of the NEO diaper method was lower than that of the direct method, which might be due in part to possible absorption of some NEO biomarkers into urine absorbents even after the extraction procedure with acetone.

Stability

The stabilities of NEO biomarkers in urine absorbents were assessed at 37°C for 6, 12, and 24 h at the 0.8 and 3 µg L^{-1} concentrations. The analyte/IS peak area ratios were comparable to those of the non-incubated samples (81–107% for 0.8 µg L^{-1} and 93–120% for 3 µg L^{-1}), and no significant differences were observed (Table 4). Furthermore, the NEO biomarkers in the diaper absorbents appeared to be stable at temperatures below 37°C and within 24 h. However, given that it is difficult to control the storage conditions of all diapers in an epidemiological study, the urine samples absorbed on diapers should be extracted as soon as possible.

The stabilities of the NEOs in the urine extracted from the absorbents were studied by following three freeze-thaw cycles and 1-month storage at −80°C at 0.8 and 3 µg L^{-1} concentrations. The peak area ratios of NEO biomarkers were approximately the same as the control under each condition (Table 4). Further, the NEO biomarkers in extracted urine were stable

Table 4. NEO stabilities in diaper and diaper urine.

	Concentration (µg L^{-1} urine)	n	Storage time	Acetamiprid	Clothianidin	Dinotefuran	Imidacloprid	Thiacloprid	Thiamethoxam	N-desmethyl acetamiprid
Stability in diapers at 37°C (%)										
	0.8	3	6h	101	103	103	89	100	101	102
	0.8	3	12h	99	96	101	105	97	99	107
	0.8	3	24h	102	96	99	81	98	95	97
	3	3	6h	103	106	100	101	101	93	103
	3	3	12h	106	103	109	120	102	97	104
	3	3	24h	105	99	103	101	98	94	99
Stability in extracted urine from diaper										
Three freeze-thaw cycles (%)	0.8	3		109	102	92	109	106	88	103
	3	3		100	104	94	105	102	93	107
Stability at − 80°C (%)	0.8	3	2weeks	101	106	97	102	94	96	97
	0.8	3	1month	101	94	102	93	101	103	92
	3	3	2weeks	103	98	95	94	98	103	99
	3	3	1month	101	97	104	97	93	100	91

Abbreviation: n, number of observations.

through a few freeze-thaw cycles, and also for at least 1 month.

Application of the method to field study samples

The established method was applied to study the urine samples from diapers of 50 three-year-old toddlers (Table 5). The detection rates (>LOD%) of dinotefuran and N-desmethylacetamiprid were relatively high (84% for dinotefuran and 78% for N-desmethylacetamiprid) compared to the other NEO biomarkers. Previously, a detection rate of 100% in N-desmethylacetamiprid was reported in Japanese adults (Harada et al. 2016). In children, when we studied the urinary NEO biomarkers levels in three-year-old toddlers living in the same prefecture as in the present study, but not the same city (Osaka et al. 2016), the highest detection rate (57.8%) was found for dinotefuran. However, there was no data for urinary N-desmethylacetamiprid in the study. Dinotefuran is a relatively new NEO insecticide, which was registered in Japan in 2002, and its domestic shipments in Japan have dramatically increased in the past decade, which attest to its burgeoning use (National Institute for Environmental Studies 2019). Therefore, there is a growing concern about the individual exposure levels of NEOs, including that of dinotefuran, and about the magnitude of the risk of health effects such exposures bring about, despite the fact that the average current intake of NEOs in Japanese adults is estimated to be below 1% of the acceptable daily intake (Harada et al. 2016). The median concentration of urinary dinotefuran detected in the present study (1.99 µg L^{-1}) was higher than that from our previous study (0.44 µg L^{-1}) (Osaka et al. 2016). This might be caused by the differences in the sample collection year between the studies (2012 for the previous study and 2017 for the present study), or the difference in the urine collection methods. Furthermore, the diaper method, established in the present study, was effective in providing us with the exact first-morning-void from children. Although the primary routes of exposure to NEOs are not well understood, the study revealed that the intakes of dinotefuran into three-year-old children in Japan

Table 5. Detection rates, geometric means, and percentiles of urinary NEOs concentrations (µg L^{-1}).

	>LOD (%)[a]	GM	Selected percentiles					Max.
			5th	25th	50th	75th	95th	
Acetamiprid	10	NC[b]	<LOD	<LOD	<LOD	<LOD	0.13	0.70
Clothianidin	18	NC[b]	<LOD	<LOD	<LOD	<LOD	1.21	1.99
Dinotefuran	84	2.01	<LOD	0.82	1.99	4.44	24.30	77.79
Imidacloprid	0	NC[b]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Thiacloprid	2	NC[b]	<LOD	<LOD	<LOD	<LOD	<LOD	0.08
Thiamethoxam	24	NC[b]	<LOD	<LOD	<LOD	<LOD	0.62	1.10
N-desmethylacetamiprid	78	0.34	<LOD	0.08	0.26	1.53	3.09	14.56

Abbreviations: LOD, limit of detection. GM, geometric mean. NC, not calculated. <LOD, Lower than level of limit of detection.
Notes: [a]Percent of detection frequency. [b]GM was not calculated due to low detection rate (less than 60% of the samples).

is considerably higher than the other related neonicotinoids. Overall, this novel method will contribute to effective and efficient biomonitoring of urinary NEO biomarkers in diapered children.

Conclusions

The present method allows the simultaneous determination of the presence and quantification of seven NEO biomarkers in urine with high reliability among diapered children. On the basis of the stability of the biomarkers observed both in the urine absorbed on the diapers and in the extracted urine from the diapers, this method would be expected to help us assess NEO exposure in diapered children in epidemiological settings.

Disclosure statement

There are no conflicts of interest to declare.

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