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Automatic magnetic solid phase extraction for rapid and high-throughput determination of neonicotinoid insecticides and their metabolites in serum, breast milk and urine samples†

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In this study, an automatic magnetic solid phase extraction method was developed to determine six parent neonicotinoids (NEOs) and three of their metabolites in breast milk, serum and urine samples. As key extraction parameters, the sorbent amount, washing solvent and elution solvent were optimized to 4 mg of HLB packing magnetic sorbent, pure water and acetonitrile, respectively. Recoveries of the analytes ranged between 81% and 121% for bovine milk samples, 64% and 122% for fetal bovine serum samples and 81% and 109% for pooled urine samples, with RSDs of <20%. The intra-day and inter-day variations were 2.7–14.9% and 1.2–13.4%, respectively, for all analytes in the three matrices. The limit of quantitation ranged from 0.002–0.05 ng mL^{-1}, 0.002–0.06 ng mL^{-1} and 0.012–0.348 ng mL^{-1} for the target compounds in bovine milk, fetal bovine serum and pooled urine samples, respectively. The validated method was successfully applied for biomonitoring of NEOs in real samples. Notably, the developed method required only 200 μL of sample and 1.4 mL of organic solvent to prepare a batch of 32 samples in less than 30 min, making it suitable for large-scale epidemiological biomonitoring of human exposure to NEOs or equivalent agrochemicals.

Introduction

Neonicotinoids (NEOs) are a novel class of agrochemicals developed to replace traditional pesticides, such as organophosphate and carbamate insecticides. NEOs have been used to protect a wide range of crops from pest insect attack and for vector control in pets and livestock in more than 120 countries.[1] However, the water solubility, persistence and systemic nature of NEOs have resulted in their detection in the environment and food.[1–3] From a toxicological perspective, the hepatotoxicity, neurotoxicity, reproductive toxicity and genetic toxicity of NEOs have been demonstrated in mammals.[4–7] Therefore, attention has focused on the relationship between NEO exposure and health outcomes in humans. An association between urinary NEOs and symptoms of neurological outcomes (memory loss and finger tremor) has been presented based on a prevalence case–control study.[8] Notably, the levels of NEOs in unborn fetuses and infants should also receive more attention, because unborn fetuses and infants have been suggested to be susceptible stages in human neurodevelopment to chemical exposures. Maternal serum and breast milk, the main sources of nutrition and energy for fetuses and infants, are the main carriers of chemical transfer to the fetuses and infants, respectively. Assessing the risk of NEOs in the human body based on large-scale population studies can provide valuable data support for supervising the use of NEOs.

Measurement of agrochemicals in human matrices is a direct way to assess the occurrence of contaminants in the human body. Urine, serum, and breast milk are three major matrices used in human biomonitoring research, especially for a sensitive population such as pregnant women and infants. Urine is the most commonly used human matrix for NEO biomonitoring.[9] Various pretreatment methods for urine NEO

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† Electronic supplementary information (ESI) available: Parameters of UHPLC-MS/MS analysis for target analytes; schematic representation of an automatic MSPE system; UHPLC-MS/MS typical MRM ion-chromatograms of target analytes in breast milk, serum and urine samples (PDF). See DOI: https://doi.org/10.1039/d4ay01244e

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analysis have been reported, such as solid-phase extraction (SPE),[10–12] online extraction method,[13] liquid–liquid micro-extraction and dispersive liquid–liquid microextraction,[14,15] with validation conducted only using urine samples. However, data on NEO residual concentrations in human serum and breast milk are scarce, which may be attributed to the lack of appropriate methodologies for NEO analysis in these human fluids. Compared to urine samples, serum and breast milk samples are challenging matrices to prepare from an analytical perspective owing to the presence of lipids and protein, and a clean-up process is often indispensable to remove interfering compounds within the sample matrix. A protein precipitation method has been used for protein elimination and the extraction of multiple NEOs from human serum and milk samples.[16,17] However, it has been shown to be the least selective preparation method because it retains a wide range of interfering matrices.[18] Therefore, it is necessary to use high resolution instruments, such as Ultra-High Performance Liquid Chromatography-Quadrupole Orbitrap High-Resolution Mass Spectrometers (UHPLC-Q-Orbitrap-HRMS), for quantitative analysis of target NEOs,[16,17] which may increase laboratory costs and reduce accessibility of the method. Compared to the protein precipitation method, SPE offers better selectivity, especially for the detection of low-abundance compounds. Li *et al.* and Yamamuro *et al.* extracted NEOs from serum samples using SPE methods and obtained good sensitivity for compound quantification using an ultra-high performance liquid chromatography system coupled with tandem mass spectrometry (UHPLC-MS/MS).[19,20] To simplify the extraction procedure, a modified QuEChERS method was developed to eliminate lipids and extract NEOs from large volumes of human milk samples.[21] Relatively high accuracy for the extraction of NEOs from hair, milk and serum was achieved using the QuEChERS method,[22–24] but when it comes to urine, relatively low recoveries and significant matrix effects were reported.[14,25] Moreover, two fast and simple liquid–liquid extraction (LLE) methods have been validated for measuring NEOs in small volumes of breast milk.[26,27] However, the above sample preparation methods for NEO analysis require manual operation, and the proficiency of the operator has a significant impact on the results, making it difficult to generalize to large-scale population research. Thus, there is an urgent need for highly automated and high-throughput preparation methods that are suitable for multiple matrices simultaneously.

In recent years, an environmentally friendly method based on magnetic solid phase extraction (MSPE) has received growing attention due to its simplicity, efficiency and sustainability. In MSPE, magnetic materials as sorbents are directly dispersed into the sample solution to ensure a high adsorption efficiency of analytes.[28] After completing extraction, the magnetic sorbents can be easily and rapidly recovered from the solution sample using an external magnet.[28] Compared to conventional SPE, LLE and QuEChERS methods, MSPE is more time efficient and less labor intensive because it eliminates the need for column packing, phase separation, filtration and centrifugation procedures. Currently, MSPE is widely used for the analysis of pollutants in environmental aqueous samples.[29–31] Several studies have also reported the application of MSPE in analyzing compounds in biomatrices.[32–34] However, studies on the application potential of MSPE for the extraction of pesticide residues from human samples are limited. Moreover, instead of the manual MSPE step, an automatic step would make the method more suitable for reliable biomonitoring of large-scale population samples.

The main objective of the present study was to develop an automatic, rapid and high-throughput sample preparation method based on a MSPE technique to measure NEOs, including parent NEOs (p-NEOs) and their metabolites (m-NEOs), which are suitable for the simultaneous analysis of large-scale breast milk, human serum and urine samples. Several key parameters of the automatic MSPE system were optimized. The developed method was validated using blank bovine milk, fetal bovine serum and pooled urine samples, and its feasibility to assess human exposure to NEOs was tested using 10 breast milk, 10 human serum and 10 human urine samples collected from volunteers. This time-saving and labor-saving method is expected to provide technical support for NEO regulation, and contribute to accelerating the development of agriculture and food chemistry towards a human friendly direction.

Materials and methods

Chemicals and reagents

Seven native analytical standards (dinotefuran [DIN], *N*-desmethyl-acetamiprid [*N*-dm-ACE], acetamiprid [ACE], clothianidin [CLO], imidacloprid [IMI], thiamethoxam [THM] and thiacloprid [THD]) and six internal standards (DIN-d_3, ACE-d_3, CLO-d_3, IMI-d_4, THM-d_3 and THD-d_4) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA) with purity > 97%. The remaining two metabolites of IMI, olefin-imidacloprid (Of-IMI) and 5-hydroxy-imidacloprid (5-OH-IMI), were obtained from First Standard (Tianjin, China) with purity > 98%. To compensate for the matrix effects, DIN-d_3 was used as the internal standard for DIN; ACE-d_3 for ACE and *N*-dm-ACE; CLO-d_3 for CLO; IMI-d_4 for IMI, Of-IMI and 5-OH-IMI; THM-d_3 for THM; and THD-d_4 for THD. β-glucuronidase/arylsulfatase was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Formic acid (purity > 98%) was obtained from Anpel Technologies (Shanghai, China); acetonitrile and methanol (LC-MS grade) were purchased from Merck (Darmstadt, Germany) and ultrapure water (18.2 MΩ cm) was prepared using a Milli-Q system (Millipore Corp., Bedford, USA).

Sample collection

Breast milk ($n = 10$) samples were randomly collected from women who delivered naturally without pregnancy complications and had a single birth, and serum ($n = 10$) and urine ($n = 10$) samples were randomly collected from early pregnant women with a single fetus at the Guangdong Women and Children Hospital, South China. Each sample was collected from a different woman, aged between 26 and 32 years old, with

a BMI of 19.5 to 26.6 kg m^{-2}. The participating women were informed about the goals, and all participants agreed to complete interviewer-administered questionnaires and voluntarily provide breast milk, serum and urine samples. Breast milk samples were collected 2–5 days after delivery. Venous blood samples were drawn from volunteers into 5 mL vacutainer anticoagulant-free serum tubes. Serum samples were isolated by centrifugation at 3000 rpm for 10 min within 3 h of collection, before being transferred to 2 mL Eppendorf tubes. First morning urine samples were directly collected into 10 mL polypropylene (PP) tubes. All samples were cryogenically transported and stored at −80 °C until analysis. These biological fluid samples were primarily collected for clinical testing purposes, with the remaining specimens subsequently utilized for our study. The present study was approved by the Ethics Committee of Guangdong University of Technology (approval number GDUTXS2023007).

Automatic MSPE system

A schematic of the automatic MSPE system (Agilebio, Suzhou, China) is shown in Fig. S1.† The system utilized 96-well plates without fixed carriers, magnetic SPE materials and magnetic bars. The system automatically performed activation, sample loading, washing and elution by absorbing and desorbing the magnetic SPE materials with magnetic bars. Organic solvents were added to the wells in rows 1 and 7 to activate the magnetic SPE materials. After mixing with annular tubes, magnetic bars were used to draw the magnetic SPE materials from the wells in rows 1 and 7 to the wells containing organic solvents in rows 2 and 8, respectively, to achieve material equilibrium. Subsequently, the magnetic bars were used to transfer the activated magnetic SPE materials from the wells in rows 2 and 8 to the wells containing sample solutions in rows 3 and 9, respectively. Afterwards, the magnetic bars were used to draw the magnetic SPE materials from the wells in rows 3 and 9 to the wells containing organic solvents in rows 4 and 10, respectively, for washing. Finally, the magnetic bars were used to move the magnetic SPE materials from the wells in rows 4 and 10 to the wells containing organic solvents in rows 5 and 11, respectively, for target compound elution. The solvents or samples in the wells were brought in full contact with the magnetic sorbent beads by performing vigorous vertical vibrations of the strip tip comb to remove impurities from the magnetic SPE materials during the activation and washing processes. This also allowed the target compounds to adsorb to and elute from the magnetic SPE materials during the sample loading and elution processes, respectively. Thus, in this system, a single 96-well plate could be used to process up to 16 samples simultaneously. The capacity of each well was less than 2 mL. All the above processes could be controlled through the instrument panel to enable automatic operation.

Sample preparation

Prior to preparation, 200 μL of each breast milk, serum or urine sample was diluted with 600 μL of ultrapure water containing 2% formic acid and added to the wells in rows 3 and 9. Next, each sample was spiked with 100 pg of each labelled internal standard solution in methanol. The magnetic HLB sorbent (30–50 μm, Agile Bio, Suzhou, China) was selected as the extraction and purification material due to the broad-spectrum application of HLB packing. The sorbent beads were activated and equilibrated with 800 μL of methanol and 600 μL of ultrapure water for 2 min, respectively. Subsequently, the magnetic HLB sorbent was transferred into the sample wells and allowed to extract the target compounds for 2 min, before being magnetically separated and washed with 600 μL of ultrapure water for 2 min. The target NEOs were eluted with 600 μL of acetonitrile for 2 min. The above steps were repeated once automatically. The entire system took less than 30 min to process a batch of 32 samples with two 96-well plates. The eluate was magnetically separated from the sorbent and then evaporated using a vacuum centrifugal concentrator (25 °C, 1400 rpm, Ji Aim, Beijing, China). Residues were reconstructed with 100 μL of ultrapure water containing 25% acetonitrile for UHPLC-MS/MS analysis.

Instrument analysis

The UHPLC-MS/MS conditions were based on the published literature with some modifications.[35] Separation of the nine target chemicals was achieved using an ExionLC™ system (UHPLC system) with a Poroshell 120 EC-C18 column (100 mm × 4.6 mm, 2.7 μm; Agilent Technologies, Santa Clara, CA, USA).[36] The injection volume was 10 μL and the flow rate of the mobile phase was set at 0.35 mL min^{-1}. Solvent A (0.01% formic acid in water) and solvent B (acetonitrile) were used as the mobile phase. The gradient elution program was initialized with 5% solvent B and held for 2 min, then increased to 99% solvent B within 6 min and held for 2 min, and finally it returned to the initial mobile phase conditions and equilibrated for 4.5 min before the next run. An API 6500 triple quadrupole tandem mass spectrometry system (QTRAP MS/MS System, Sciex, Washington DC, WA, USA) was operated in positive ion mode to identify and quantify the target chemicals. Ionization parameters were obtained through the automatic flow injection analysis optimization process. The source temperature was set at 500 °C, and the ion spray voltage was set at 5500 V. The curtain gas (CUR), collision gas (CAD), ion source gas 1 (GAS1), and ion source gas (GAS2) for target compounds were set to 25 psi, 9 psi, 50 psi and 50 psi, respectively. Further details of the mass transitions and MRM parameters are provided in Table S1.†

Method validation

Method validation was performed using blank bovine milk, fetal bovine serum and pooled urine as quality control (QC) samples, which were evaluated for the absence of target analytes using the above method. It should be noted that the pooled urine sample consisted of samples from 10 anonymous healthy non-smoking donors from universities in the Guangzhou Higher Education Mega Center, China, and was diluted three times with pure water after pooling. The method specificity, selectivity, method detection limit (MDL), method quantitation

limit (MQL), linearity, recovery, precision, accuracy and matrix effects were evaluated. The specificity and selectivity of the method were assessed by comparing chromatograms of QC samples with those of QC samples spiked with known concentrations of NEOs after preparation. The MDL and MQL of each analyte were defined as the concentrations corresponding to signal-to-noise (S/N) ratios of 3 and 10, respectively, in the QC samples throughout the entire pretreatment process. The concentrations used to generate the calibration curves were 0.01, 0.02, 0.05, 0.10, 0.50, 1.00, 5.00, 10.0, 25.0 and 50.0 ng mL^{-1} of NEOs in the prepared QC samples, along with DIN-d_3, ACE-d_3, CLO-d_3, IMI-d_4, THM-d_3 and THD-d_4 as internal standards (1 ng mL^{-1}). The linearity of the signal for each analyte was evaluated using the corresponding calibration curve. The method precision was determined by analyzing five replicate QC samples at three spiked concentrations [low level QC (LQC): 0.1 ng mL^{-1}, medium level QC (MQC): 1.0 ng mL^{-1}, and high level QC (HQC): 10 ng mL^{-1}] during a single day (repeatability) and over a period of 3 days (reproducibility). Extraction recoveries were determined at concentrations of 0.1 ng mL^{-1}, 1.0 ng mL^{-1} and 10 ng mL^{-1} for the target compounds. For each concentration, three replicate QC samples were spiked with appropriate concentrations of analytes before preparation (pre-spiked samples). Three additional replicate QC samples were analyzed with analytes only added after the preparation was completed (post-spiked samples). Recoveries were calculated by comparing the measured concentrations of pre-spiked samples with those of post-spiked samples, expressed as a percentage (eqn (1)).[20] The matrix effect of each analyte was calculated by comparing the peak area of the analyte in post-spiked samples against that measured in the solvent used for reconstitution (eqn (2)).[20]

$$\% \text{ recovery} = \frac{\text{measured concentration of pre-spiked sample}}{\text{measured concentration of post-spiked sample}} \times 100 \quad (1)$$

$$\% \text{ matrix effect} = \frac{\text{peak area of analyte in post-spike sample} - \text{peak area of analyte in standard solution}}{\text{peak area of analyte in standard solution}} \times 100 \quad (2)$$

Quality assurance and quality control

Instrumental QC involved regular injection of solvent blanks and standard solutions for every batch of 10 field samples. The QC values were $100 \pm 20\%$ of the nominal values, indicating low batch-analysis variability. For method QC, a set of QC samples were evaluated using procedural blanks and spiked matrices for every batch of 10 field samples. The recoveries of NEOs in spiked matrix samples (blank bovine milk, fetal bovine serum and pooled urine samples) were 74.6–117%. The concentrations of the analytes in the field samples were not corrected for recovery. None of the analytes were detected in the procedural blanks. When the concentration of the analytes exceeds the range of the calibration curve, the sample will be diluted and re-quantified.

Results and discussion

Optimization of extraction parameters and sample preparation

In this study, we devised a fast and automatic MSPE method using small sample and solvent volumes as an alternative to lengthy QuEChERS, SPE and LLE approaches. Due to the viscosity of breast milk, serum and urine samples, dilution with ultrapure water was used to promote the dispersion of the magnetic HLB sorbent in the sample solution. Each 200 μL sample was diluted with 600 μL of water to maintain the extraction efficiency and ensure that the magnetic bar was sufficiently covered. Additionally, 2% formic acid was added to the water to facilitate the adsorption of the analytes on the HLB sorbent.[37] The sorbent amount, washing solvent and elution solvent are key parameters of the automatic MSPE system that must be optimized to obtain satisfactory extraction results. During the optimization of the sorbent amount, 600 μL of water and 600 μL of acetonitrile were used as the washing solvent and elution solvent; during the optimization of the washing solvent, 3 mg of sorbent and 600 μL of acetonitrile were used as the sorbent amount and elution solvent; during the optimization of the elution solvent, 3 mg of sorbent and 600 μL of water were used as the sorbent amount and washing solvent, respectively.

Optimization of sorbent amount

As presented in Fig. 1a, the recoveries of NEOs and their metabolites in spiked bovine milk samples increased from below 60% to above 80% with increasing sorbent loadings from 1 mg to 4 mg, except for THD (from 75% to 98%) and DIN (from 5% to 41%). The largest increase was observed for THM (from 33% to 91%) and Of-IMI (from 45% to 104%). The recoveries of target analytes in spiked bovine milk samples with 5 mg sorbent loading were comparable to or lower than those with 4 mg sorbent loading. This may be attributed to the excessive amount of adsorbent in the limited reaction space (2 mL of each well), leading to low efficiency of solvent or sample contact with the adsorbent. However, in contrast to the bovine milk samples, the recoveries of target analytes in spiked fetal bovine serum samples fluctuated between 80% and 113% (less than 20% variation for each analyte) with increasing sorbent loadings from 1 mg to 5 mg, except for DIN and 5-OH-IMI (Fig. 1b). The recoveries of DIN and 5-OH-IMI increased from 23% to 63% and 34% to 63%, respectively, with increasing sorbent loading from 1 mg to 4 mg, reaching the highest recoveries of 75% and 69%, respectively, when the sorbent loading was 5 mg. Similarly, the recoveries of the target analytes in spiked pooled urine
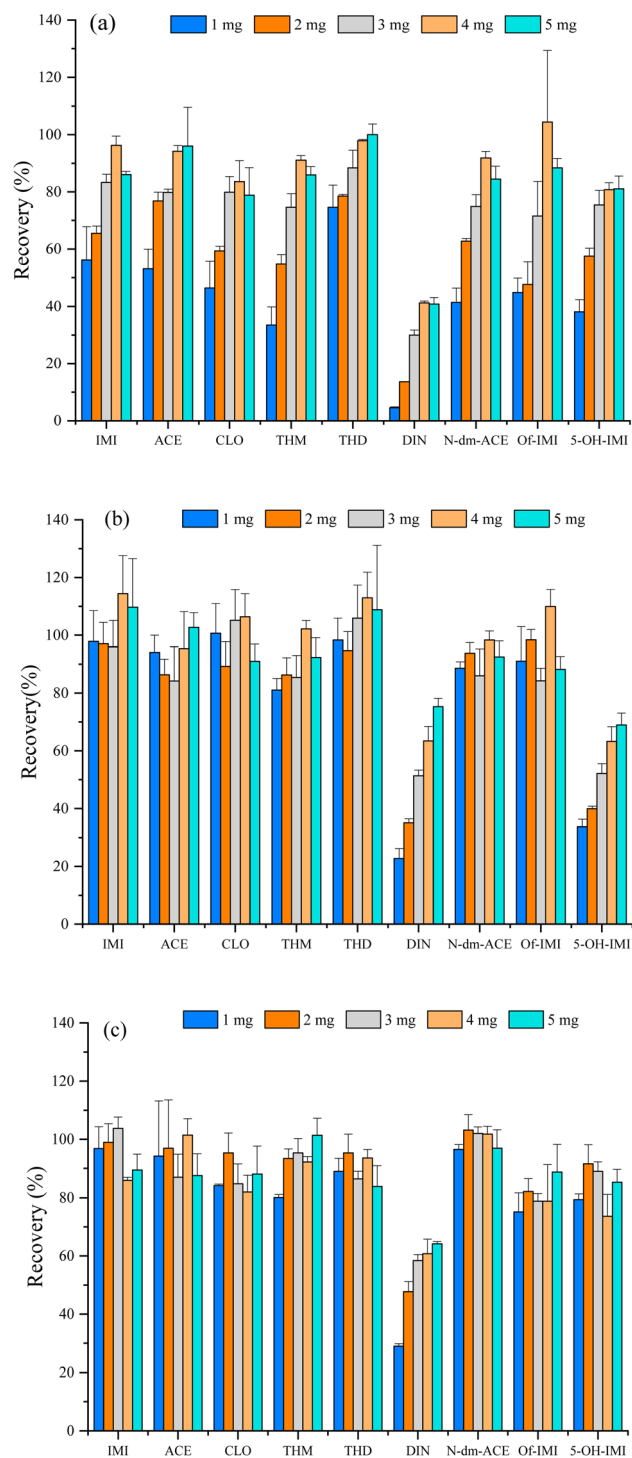
Fig. 1 Effect of sorbent amount on the recoveries of p-NEOs and m-NEOs in spiked (10 ng mL^{-1}) (a) bovine milk, (b) fetal bovine serum, and (c) pooled urine samples.

fluctuated between 75% and 105% (less than 20% variation for each analyte) with increasing sorbent loadings from 1 mg to 5 mg, except for DIN (Fig. 1c). The recovery of DIN increased from 29% to 61% with increasing sorbent loadings from 1 mg to 4 mg, reaching the highest recovery of 64% when the sorbent loading was 5 mg. These results suggest that the sorbent amount had a large influence on the extraction of p-NEOs and

m-NEOs in bovine milk and the extraction of DIN in all the three matrices. Accordingly, 4 mg of sorbent loading was selected as the optimal sorbent amount when preparing samples using an automatic MSPE system, with average recoveries ranging from 41–104%, 63–114% and 61–109% for bovine milk, fetal bovine serum and pooled urine samples, respectively.

Optimization of washing solvent

The effect of using different washing solvents on the performance of an automatic MSPE system for extracting the target analytes from bovine milk, fetal bovine serum and pooled urine
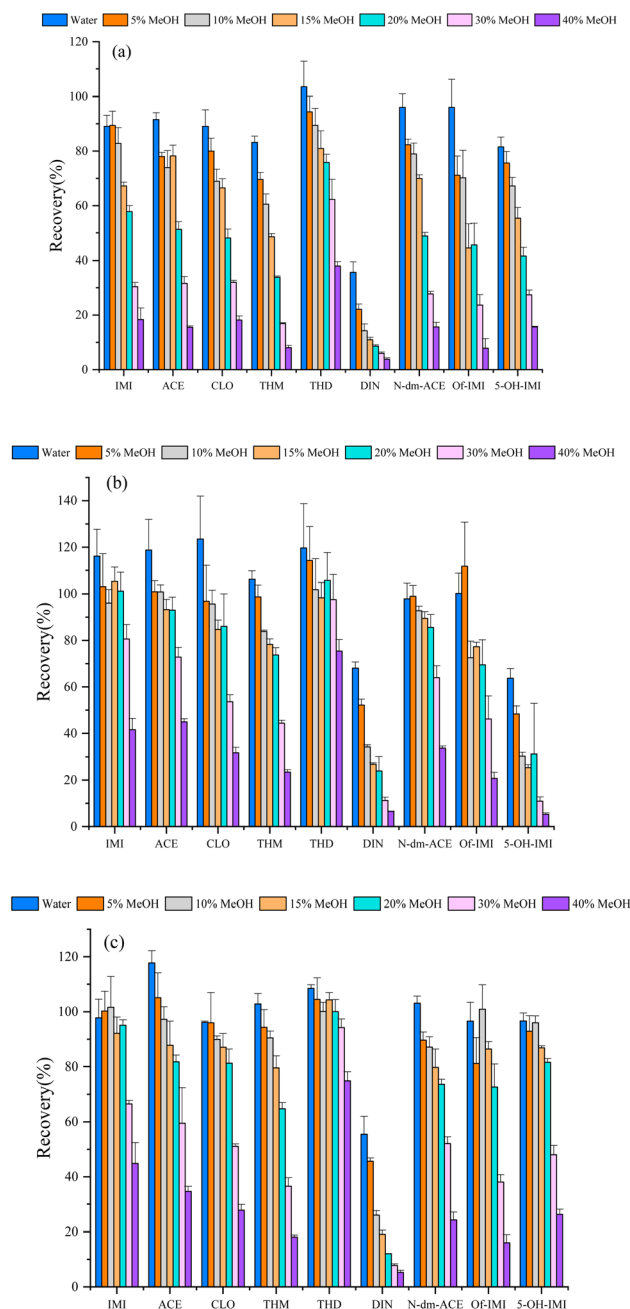

Fig. 2 Effect of washing solvent on the recoveries of p-NEOs and m-NEOs in spiked (10 ng mL^{-1}) (a) bovine milk, (b) fetal bovine serum, and (c) pooled urine samples.

samples is shown in Fig. 2. The recoveries of the target analytes in spiked bovine milk samples were more than 80% when using pure water as the washing solvent, except for DIN (36%) (Fig. 2a). When the percentage of methanol in the washing solvent was increased to 40%, the recoveries of all target compounds decreased to less than 40%. Similarly, in spiked fetal bovine serum samples, the highest recoveries of each target analyte (68–123%) were obtained when pure water was used as the washing solvent, except for N-dm-ACE and Of-IMI, which showed the highest recoveries when 5% methanol in pure water was used as the washing solvent (Fig. 2b). The recoveries of N-dm-ACE and Of-IMI in spiked fetal bovine serum samples were 98% and 100%, respectively, when using pure water as the washing solvent. When the percentage of methanol in the washing solvent exceeded 15%, the recoveries of the target analytes in spiked fetal bovine serum samples decreased sharply with the increasing percentage of methanol. For the spiked pooled urine sample, the recoveries of the target analytes were more than 80% when the washing solvent contained less than 15% methanol, except for DIN (Fig. 2c). The recoveries of the target analytes in spiked urine samples sharply decreased with the increasing percentage of methanol from 20% to 40%, consistent with the results of the spiked fetal bovine serum samples. To summarize, the recoveries of the target compounds in the three matrices showed a decreasing trend with increasing methanol content in the washing solvent. This may have been due to the polarity of the analytes, as methanol could have reduced retention of the analytes on the sorbent. Moreover, as shown in Tables S2–S4,† the washing solutions had little effect on the matrix effect of most of target compounds. Thus, pure water was selected as the optimal washing solvent during sample preparation with an automatic MSPE system, showing average recoveries of 36–103%, 64–123% and 55–117% for bovine milk, fetal bovine serum and pooled urine samples, respectively.

Optimization of elution solvent

Different solvents (600 μL each), i.e., methanol, acetonitrile, ethyl acetate, dichloromethane and methyl tert-butyl ether, were evaluated to optimize the elution efficiencies of the target analytes. The sorbent was found to agglomerate or adhere to the interior wall surface of plates when ethyl acetate, dichloromethane and methyl tert-butyl ether were used as solvents to elute the target analytes, which could lead to poor dispersal of the sorbent and adverse effects on the performance of the system. As shown in Fig. 3, the recoveries of the target compounds in the three spiked matrices using acetonitrile as the elution solvent were slightly higher than or comparable to those using methanol as the elution solvent. Moreover, in practice, the drying time of acetonitrile was faster than that of methanol during the subsequent vacuum-drying process, although methanol is more volatile than acetonitrile. It may be because a small amount of water was carried over when the magnetic SPE materials were transferred from the rinsing (pure water) to the elution process. Hydrogen bonds formed between water and methanol molecules need more energy to break and
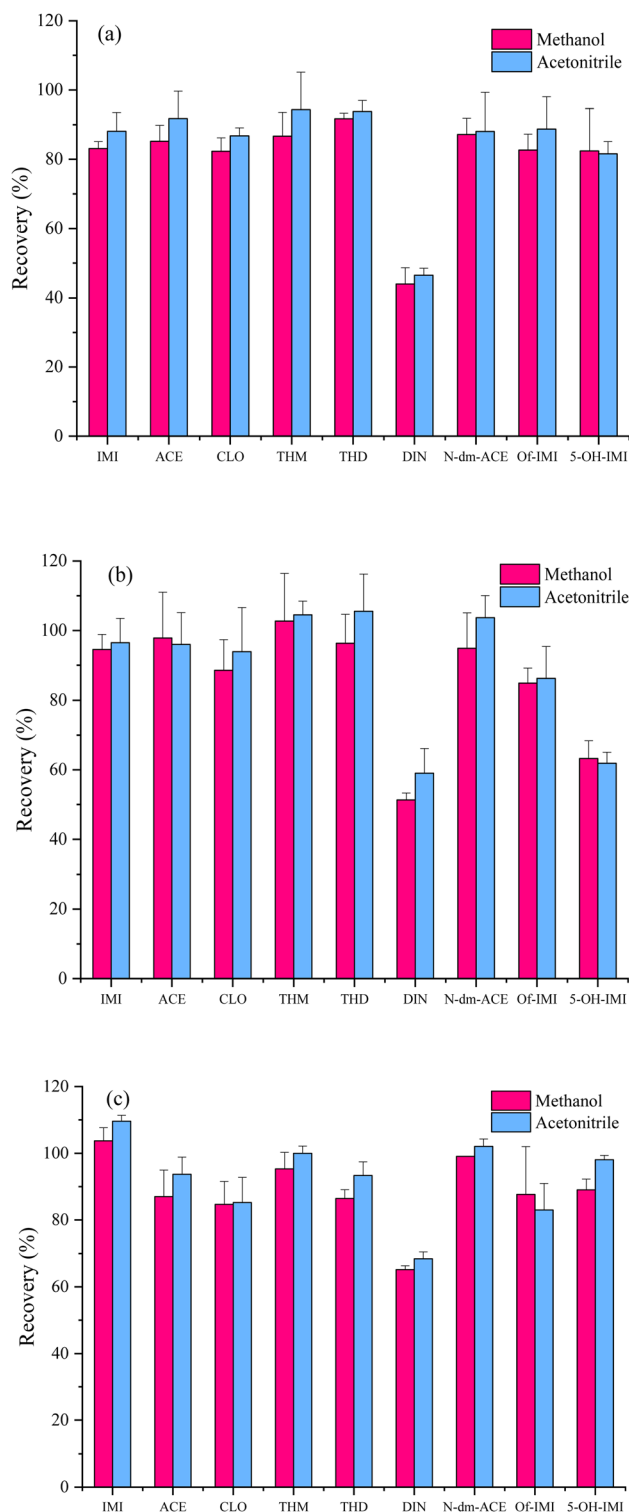

Fig. 3 Effect of elution solvent on the elution efficiency of p-NEOs and m-NEOs in spiked (10 ng mL^{-1}) (a) bovine milk, (b) fetal bovine serum, and (c) pooled urine samples.

allow the molecules to drift apart and escape from the surface of the liquid as gas, compared with the hydrogen bonds between methanol and methanol. Therefore, pure acetonitrile was selected as the optimal elution solvent for sample preparation with an automatic MSPE system.

Table 1 Mean recovery percentages ($n = 5$), linearity, precision ($n = 5$), MDL,[a] MQL[b] and matrix effects of the analytes in spiked samples

	Low spiked level		Medium spiked level		High spiked level			Precision (%)				
	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Linearity (R^2)	Intra-assay	Inter-assay	MDL (ng mL^{-1})	MQL (ng mL^{-1})	Matrix effect (%)
Bovine milk												
DIN	110	14.6	93.9	8.7	108	9.2	0.999	10.7	6.3	0.01	0.03	−7
Of-IMI	103	10.1	110	7.9	101	8.9	0.998	10.6	8.2	0.02	0.05	−58
5-OH-IMI	88.8	10.6	97.4	14.5	81.6	4.4	0.997	4.4	3.7	0.003	0.01	−34
THM	118	3.4	107	3.6	98.7	3.7	0.999	2.7	1.2	0.002	0.005	−12
N-dm-ACE	121	11.7	100	5.8	108	7.3	0.999	5.1	8.6	0.001	0.003	−15
CLO	103	18.0	93.1	9.7	80.8	9.5	0.999	7.7	5.1	0.003	0.01	−50
IMI	102	1.5	112	12.1	94.8	14.9	0.999	4.5	2.2	0.001	0.003	−43
ACE	118	7.2	91.0	18.5	103	5.5	0.999	2.7	5.5	0.001	0.003	−18
THD	99.8	12.7	104	13.2	113	3.1	0.999	9.0	3.9	0.0006	0.002	−51
Fetal bovine serum												
DIN	119	11.4	97.1	9.4	109	3.5	0.996	3.9	7.8	0.02	0.06	−34
Of-IMI	96.0	10.9	81.9	4.7	87.7	16.9	0.999	8.6	5.4	0.02	0.05	−58
5-OH-IMI	81.8	8.4	64.1	4.9	63.8	6.5	0.997	6.5	8.1	0.003	0.01	−38
THM	80.2	2.5	119	9.0	122	5.3	0.996	3.4	2.8	0.003	0.01	−36
N-dm-ACE	88.1	3.6	89.6	5.9	86.8	3.4	0.999	7.0	3.2	0.002	0.005	−35
CLO	107	9.2	84.0	13.2	112	6.6	0.996	14.9	7.6	0.003	0.01	−65
IMI	96.0	3.8	96.1	7.2	101	8.1	0.998	9.9	11.2	0.001	0.003	−58
ACE	91.4	8.3	96.1	7.2	101	8.1	0.999	11.1	13.4	0.002	0.005	−33
THD	89.9	1.8	86.0	3.1	100	6.2	0.998	12.8	7.9	0.0006	0.002	−64
Pooled urine												
DIN	96.6	8.4	93.4	14.1	106	3.1	0.999	5.8	7.1	0.029	0.086	−15
Of-IMI	90.6	9.2	81.2	11.6	88.4	16.3	0.997	7.1	13.4	0.116	0.348	−68
5-OH-IMI	87.9	2.1	85.1	7.5	89.7	3.6	0.996	5.6	4.1	0.058	0.173	−48
THM	86.6	2.8	97.4	5.9	91.0	5.2	0.996	3.9	6.9	0.053	0.159	−47
N-dm-ACE	95.7	4.1	102	4.3	97.2	2.2	0.999	4.1	3.8	0.005	0.015	−34
CLO	92.1	8.4	91.8	12.5	102	8.1	0.998	8.9	9.1	0.037	0.111	−45
IMI	87.3	4.2	92.7	11.2	105	3.8	0.998	8.2	11.1	0.012	0.036	−45
ACE	95.6	4.4	105	2.3	87.1	9.2	0.999	7.2	8.1	0.004	0.012	−38
THD	93.2	5.1	104	3.7	109	3.1	0.999	4.9	5.4	0.006	0.019	−40

[a] Method detection limit (MDL). [b] Method quantitation limit (MQL).

Notably, organic solvents were used only during sorbent bead activation (800 μL of MeOH) and target compound elution (600 μL of acetonitrile). Compared to previous methods used for breast milk, human serum and urine NEO analysis (Table S5†), the newly developed MSPE method required much lower volumes of samples and organic solvent (200 μL and 1.4 mL, respectively), except for the automatic SPE method developed by Nishihama et al.[38] Additionally, the MSPE system was automatic and took less than 30 min to complete the extraction of one batch of samples (simultaneous extraction of 32 samples). In terms of the amount of sorbent used, the sorbent amount used (4 mg) in an MSPE system is one order of magnitude lower than that in a conventional HLB cartridge (30 mg), significantly reducing the costs. Although the previously developed online-SPE methods and automatic SPE have higher efficiency than this MSPE method, they result in increased laboratory costs and reduced accessibility due to the need for instrument modifications and specialized equipment, such as Microlab STAR (Hamilton Company, Reno, NV, USA) and EDR-384SX (BIOTEC Co., Ltd., Tokyo, Japan).[38,39] Moreover, the performances of the previous developed online-SPE methods and automatic SPE method for NEO analysis in serum and breast milk samples need further verification. Therefore, the MSPE method developed in this study enables automatic, rapid, economical, high accessibility and high throughput analysis for multiple types of sample matrices, which could facilitate large-scale epidemiological biomonitoring.

Method validation

Fig. S2–S4† show representative UHPLC-MS/MS chromatograms of standard solution, QC samples and QC samples spiked with known concentrations of NEOs after preparation. No interference peak was found at the retention times of the target analytes, which indicates that the specificity and selectivity of the developed method for each analyte in bovine milk, fetal bovine serum and pooled urine samples were satisfactory. Data on the MDL, MQL, linearity, recovery, precision, accuracy and matrix effect are presented in Table 1. The calibration curves showed good linearity over the concentration range of 0.01–50.0 ng mL^{-1}, with coefficients of determination (R^2) greater than

Table 2 Levels of NEOs in breast milk samples ($n = 10$), human serum samples ($n = 10$) and urine samples ($n = 10$)

Compound	DF[a] (%)	Mean (ng L^{-1})	Median (ng L^{-1})	Min. (ng L^{-1})	Max. (ng L^{-1})
Breast milk					
DIN	0	n.d.[b]	n.d.	n.d.	n.d.
Of-IMI	0	n.d.	n.d.	n.d.	n.d.
5-OH-IMI	0	n.d.	n.d.	n.d.	n.d.
THM	70	55.8	21.9	n.d.	268
N-dm-ACE	100	49.4	46.1	10.4	144
CLO	80	37.3	11.8	n.d.	197
IMI	70	17.5	19.0	n.d.	53.0
ACE	30	0.97	n.d.	n.d.	8.18
THD	0	n.d.	n.d.	n.d.	n.d.
Human serum					
DIN	0	n.d.	n.d.	n.d.	n.d.
Of-IMI	0	n.d.	n.d.	n.d.	n.d.
5-OH-IMI	0	n.d.	n.d.	n.d.	n.d.
THM	50	69.0	16.7	n.d.	286
N-dm-ACE	100	267	101	18.2	1.69×10^3
CLO	40	30.9	n.d.	n.d.	143
IMI	60	9.60	4.43	n.d.	33.1
ACE	100	5.45	4.99	2.50	10.7
THD	0	n.d.	n.d.	n.d.	n.d.
Urine					
DIN	40	314	n.d.	n.d.	2.17×10^3
Of-IMI	90	1.67×10^3	1.52×10^3	n.d.	4.13×10^3
5-OH-IMI	0	n.d.	n.d.	n.d.	n.d.
THM	80	147	96.6	n.d.	422
N-dm-ACE	100	2.77×10^3	937	163	13.1×10^3
CLO	80	1.53×10^3	420	n.d.	11.5×10^3
IMI	80	270	154	n.d.	1.37×10^3
ACE	80	42.0	21.0	n.d.	149
THD	0	n.d.	n.d.	n.d.	n.d.

[a] Detection frequency. [b] Non-detectable.

0.996 for all target compounds in the three matrices. Negative matrix effects were observed for all compounds in bovine milk (−58% to −7%), fetal bovine serum (−65% to −33%) and pooled urine (−68% to −15%), which were lower than the matrix effects reported for the SPE method using four SPE cartridges for NEO analysis in human urine.[35] Therefore, isotope-labeled internal standards that are chemically identical to the target analytes were used to correct for losses during the extraction procedure and compensate for matrix effects. Adequate recoveries, for the three validated levels (0.1 ng mL^{-1}, 1.0 ng mL^{-1} and 10 ng mL^{-1}), were obtained for the target compounds, ranging from 81% to 121% for bovine milk samples, 64% to 122% for fetal bovine serum samples and 81% to 109% for urine samples. The MDL of the target compounds in bovine milk, fetal bovine serum and pooled urine ranged from 0.0006–0.02 ng mL^{-1}, 0.0006–0.02 ng mL^{-1} and 0.004–0.116 ng mL^{-1}, respectively, whereas the MQL ranged from 0.002–0.05 ng mL^{-1}, 0.002–0.06 ng mL^{-1} and 0.012–0.348 ng mL^{-1}, respectively. The MDL and MQL values of NEOs in the developed method were one to three orders of magnitude lower than those in previously developed methods[20,21,27,39,40] and comparable to those reported by Song et al. and Nishihama et al.,[14,38] as shown in Table 3. The intra-day and inter-day

variations were very good (2.7–14.9% and 1.2–13.4%, respectively) for all analytes at all spiked levels in the three studied matrices.

Method application

The validated method was applied to detect and quantify p-NEOs and m-NEOs in 10 breast milk samples, 10 human serum samples and 10 human urine samples. The UHPLC-MS/MS chromatographs of the analytical standards, breast milk sample, serum sample, and urine sample are shown in Fig. S5.† The detection frequencies (DFs) and concentrations of target compounds in the three matrices are presented in Table 2.

The DFs of the target compounds in urine samples were higher than those in breast milk and serum samples. THD and 5-OH-IMI were not detected in any of the samples. DIN and Of-IMI were only detectable in urine samples, with DFs of 40% and 90%, respectively. N-dm-ACE was the most frequently observed compound (100%) in the three matrices, while its parent compound, ACE, was detected in 30%, 100% and 80% of the breast milk, serum and urine samples, respectively. The DFs of other compounds ranged from 70% to 80%, 40% to 60% and 80% to 90% in breast milk, serum and urine samples, respectively.

Table 3 Method performances of the most commonly used analyses of NEOs and m-NEOs in human biological matrices

Chemicals	Sample type	Volume (μL)	Clean-up method	Apparatus	MDL	Recovery (%)	ME (%)	Ref.
6 NEOs 3 m-NEOs	Serum Breast milk Urine	200	Automatic MSPE (HLB sorbent (30–50 μm) from Agile Bio)	UHPLC-MS/MS (Ascentis Express C18, 100 mm × 4.6 mm × 2.7 μm)	0.0006–0.02 0.0006–0.01 0.006–0.116	64–122 81–121 81–109	−65 to −33 −58 to −7 −68 to −15	This study
6 NEOs 4 m-NEOs	Urine	3000	LLE (ethyl acetate)	HPLC-MS/MS (Zorbax SB-C18, 100 mm × 2.1 mm × 3.5 μm)	0.015–0.03	71–107	0.6–1.6	14
8 NEOs 3 m-NEOs	Serum Urine	1000	SPE (Extrelut® NT 3 cartridges)	HPLC-MS/MS (Ascentis® C18, 150 mm × 2.1 mm × 3 μm)	0.1–0.2 0.1–1	80.9–101.8 91.9–106	0–34 −31.5 to 6	20
4 NEOs	Breast milk	5000	QuEChERS (50 mg PSA, 150 mg MgSO$_4$)	HPLC-MS/MS (Symmetry C18, 100 mm × 2.1 mm × 5 μm)	0.3–0.6	85–98	n.a.	21
7 NEOs 4 m-NEOs	Urine	500	SPE (Bond Elut Plexa 60 mg, 3 mL)	UHPLC-MS/MS (Betasil C18, 100 mm × 2.1 mm × 5 μm)	0.003–0.015	83.7–119	−82 to −58	35
9 NEOs 4 m-NEOs	Urine	100	SPE (ISOLUTE® HYDRO DME+ 400 mg plate)	UHPLC-MS/MS (ACQUITY UPLC HSS T3, 100 mm × 2.1 mm × 1.8 μm)	0.0013–0.52	89.7–103	n.a.	38
7 NEOs 9 m-NEOs	Urine	300	Online-SPE (TurboFlow Cyclone-P, 50 × 0.5 μm)	HPLC-MS/MS (Kinetex Phenyl-Hexyl, 150 mm × 3 mm × 2.6 μm)	0.02–0.64	89–112	n.a.	39
8 NEOs	Breast milk	1000	QuEChERS (100 mg PSA, 100 mg C18, 150 mg of MgSO$_4$)	UHPLC-MS/MS (RRHD Eclipse Plus C18, 50 mm × 2.1 mm × 3 μm)	0.05–0.18	74.3–105.9	−35.6 to 1.2	40

Among the detectable NEOs, the highest concentration in breast milk samples was that of THM (mean: 55.8 ng L^{-1}), followed by N-dm-ACE (mean: 49.4 ng L^{-1}) and CLO (mean: 37.3 ng L^{-1}). In serum samples, the NEO with the highest concentration was N-dm-ACE (mean: 267 ng L^{-1}), followed by THM (mean: 69.0 ng L^{-1}) and CLO (mean: 30.9 ng L^{-1}). N-dm-ACE also had the highest concentration among the NEOs in urine samples (mean: 2.77×10^3 ng L^{-1}), followed by Of-IMI (mean: 1.67×10^3 ng L^{-1}) and CLO (mean: 1.53×10^3 ng L^{-1}). ACE had the lowest concentrations of all the NEOs in all three matrices, with mean values of 0.97 ng L^{-1} in breast milk, 5.45 ng L^{-1} in serum and 42.0 ng L^{-1} in urine samples. Notably, the concentrations of NEOs in urine samples were one to two orders of magnitudes higher than those in breast milk and serum samples. This may suggest that most NEOs that enter or are metabolized in the human body can be eliminated through urine. Moreover, the different distribution patterns of NEOs in the three human matrices may indicate matrix-specific accumulation of NEOs in the human body. Further studies with a larger sample size are needed to elucidate the levels and distribution characteristics of NEOs in the human body.

Conclusion

To summarize, we developed a sample preparation method using a MSPE system to simultaneously determine NEOs and their metabolites in human fluid samples. Optimization of the method showed that 4 mg of HLB packing magnetic sorbent, pure water and acetonitrile were the optimal sorbent amount, washing solvent and elution solvent, respectively. The method showed excellent sensitivity, recovery, repeatability, reproducibility and precision for human biomonitoring of NEOs in breast milk, serum and urine samples. Compared to the conventional HLB cartridge, the newly developed MSPE method required significantly smaller volumes of sample (200 μL less) and organic solvent (1.4 mL less) and had a much lower processing time (less than 30 min for a batch of 32 samples) and cost (lower organic solvent and sorbent amount used). Therefore, MSPE offers advantages of automation, rapidity, low cost and high throughput for large-scale epidemiological biomonitoring.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article or its ESI.†

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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