Food Analytical Methods
https://doi.org/10.1007/s12161-023-02484-7

Simultaneous Determination of Neonicotinoid Insecticides and Metabolites Residues in Milk and Infant Formula Milk Powder by EMR-Liquid Chromatography-Tandem Mass Spectrometry

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Received: 13 September 2022 / Accepted: 4 May 2023

Abstract
An analytical method based on enhanced matrix removal–lipid liquid chromatography–tandem mass spectrometry (EMR-LC–MS/MS) was developed for the determination of neonicotinoid insecticides and metabolites residues (imidacloprid (IMI) and its metabolites imidacloprid-urea (IMI-U), imidacloprid-olefin (IMI-O), acetamiprid (ACE) and its metabolite N-desmethyl acetamiprid (IM 2–1), dinotefuran (DIN) and its metabolite [1-methy1-3(tetrahydro-3-furylmethy1) urea (DIN-UF), thiacloprid (THIA), thiamethoxam (TMX), clothianidin (CLO, metabolite of thiamethoxam), and flupyradifurone (FLU)) in milk and infant formula milk powder. The residual of neonicotinoid insecticides and their metabolites in samples were exacted by acetonitrile and extraction kits. The quantitative detection was performed by LC–MS/MS with multiple reaction monitoring (MRM) modes under positive ion electrospray ionization (ESI^+). The isotope dilution internal standard or external standard method was used for quantitation. The limits of quantification (LOQs, $S/N = 10$) were 2 μg/kg (IMI, IMI-U, ACE, IM 2–1, DIN-UF, THIA, and TMX) and 5 μg/kg (IMI-O, DIN, CLO, and FLU) for milk; 2 μg/kg (ACE), 15 μg/kg (THIA, IM 2–1, DIN-UF, THIA, and TMX), and 40 μg/kg (IMI-U, IMI-O, DIN, CLO, and FLU) for infant formula milk powder. At three spiked levels of 5 μg/kg, 10 μg/kg, 50 μg/kg (milk), or 40 μg/kg, 80 μg/kg, 400 μg/kg (infant formula milk powder), the recoveries were in the range of 71.7–108.7% and 71.9–107.1%; the relative standard deviations were below 12.6% and 13.9%, respectively. This method was simple, rapid, and accurate to determine the neonicotinoids and their metabolites residues in milk and infant formula milk powder.

Keywords Neonicotinoid · Milk · Infant formula milk powder · LC–MS/MS

Introduction

Milk is an indispensable food in the human diet for its rich nutrition, easy digestion and absorption, good quality, low price, and convenient consumption, and it is also the main raw material for infant formula milk powder. Therefore, its quality and safety (e.g., pesticides or veterinary drugs residues) directly affects the health of consumers and infants. Neonicotinoids (NEOs) have been widely used for controlling pests in crops. They worked by blocking the normal conduction of the central nervous system in insects, leading to paralysis or death (Matsuda et al. 2001; Chagnon et al. 2015). Studies have shown that NEOs can affect animals through the food chain, such as by reducing the population of bees (Lu et al. 2012; Laurino et al. 2013). NEOs have also been detected in the bodies of honeybees and in honey (Kaczynski et al. 2017; Mitchell et al. 2017). Along with the transmission of the food chain, NEOs and their metabolites have been found not only in mammals (Ozsahin et al. 2014; Berheim et al. 2019) but also in human body fluids (e.g., urine, breast milk) (Osaka et al. 2016; Ueyama et al. 2020; Chen et al. 2020). To protect the quality of milk and the health of consumers, the maximum residue limits (MRLs) for some neonicotinoids and their metabolites in milk have been clearly defined in the United States of America (USA), China, European Union (EU), and Japan, as shown in Table 1 (USA. 2014; The National Health Commission of the People's Republic of China 2021; European Union 2022; The Ministry of health, labour and welfare of Japan 2022).

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Published online: 23 May 2023

Table 1 Current of maximum residues limits (MRLs) of neonicotinoid insecticides in the milk of the USA, China, European Union (EU), and Japan

Compound	USA	China	EU	Japan
IMI	100	100	10	20
ACE (sum with IM 2–1)	300[1]	20	200	60[1]
DIN	50	100	100	50
THIA	30	50	50	20
TMX (sum with CLO)	20	50	50	10[2]
CLO	10	20	20	10
FLU	/	700	10	/

[1]The sum of acetamiprid and metabolite IM-2–1 expressed as acetamiprid

[2]The sum of thiamethoxam and metabolite clothianizine expressed as thiamethoxam

Due to the insecticidal properties of NEOs, the determination of NEOs residues is currently centered on plant-origin products such as vegetables, fruits, and grains (Xie et al. 2011; Wang et al. 2012; Vichapong et al. 2013; Pastor-Belda et al. 2016), while animal origin foods are mainly honey, royal jelly, and other bee products (Tanner and Czerwenka 2011; Giroud et al. 2013; Yá˜nez et al. 2013; Hou et al. 2019). The detection methods include high-performance liquid chromatography (HPLC) (Jovanov et al. 2015; Vichapong et al. 2015, 2016), liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Anand et al. 2018; Zhang et al. 2018a, b; Cui et al. 2021), liquid chromatography–high-resolution mass spectrometry (LC-HRMS) (Chen et al. 2020; Zhao et al. 2020), electrochemical detection (ECD) (Papp et al. 2010; Guzsvány et al. 2011; Brycht et al. 2012), capillary liquid chromatography (CLC) (Carbonell-Rozas et al. 2020a, 2021), and micellar electrokinetic chromatography (MEC) (Carbonell-Rozas et al. 2020b). It should be noted that the determination of NEOs residues in other foods of animal origin (e.g., milk, meat, and aquatic products) has been less studied (Alaa et al. 2010; Xiao et al. 2011; Xiao et al. 2013; Craddock et al. 2019). Enhanced matrix removal-lipid (EMR) is a technique that is based on hydrophobic interaction and size exclusion. It can be used to remove fatty acids, phospholipids, triglycerides, and other compounds with long-chain aliphatic functional groups from extracts. However, it does not retain analytes of interest (DeAtley et al. 2015). EMR has been used to determine the presence of NEOs in honeybee and wild boar (*Sus scrofa* L) matrix (Kaczynski et al. 2017, 2021), as well as for the detection of drug or pollutant residues in milk, infant formula powder, and chicken eggs (Zhang et al. 2018a, b, c; Luo et al. 2020; Zhang et al. 2021).

In this work, an LC–MS/MS with EMR method was proposed for the simultaneous determination of NEOs and their metabolites (imidacloprid (IMI), imidacloprid-urea (IMI-U), imidacloprid-olefin (IMI-O), acetamiprid (ACE), N-desmethyl acetamiprid (IM 2–1), dinotefuran (DIN), [1-methy1-3(tetrahydro-3-furylmethy1) urea (DIN-UF), thiacloprid (THIA), thiamethoxam (TMX), clothianidin (CLO), and flupyradifurone (FLU)) (Fig. 1) residues in milk and infant formula milk powder.

Materials and Methods

Chemicals and Reagents

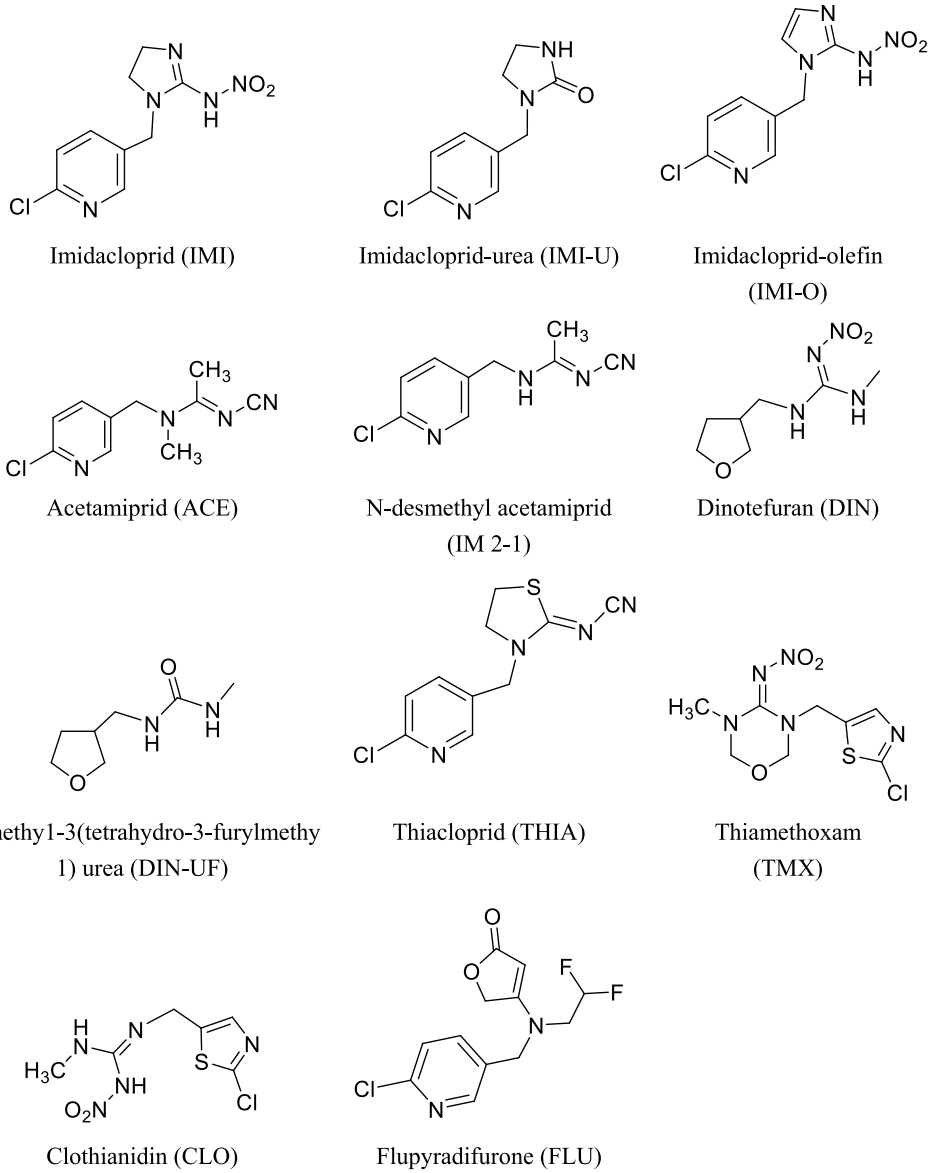
All reagents were analytical grade unless otherwise stated. Imidacloprid (IMI), acetamiprid (ACE), N-desmethyl acetamiprid (IM 2–1), dinotefuran (DIN), thiacloprid (THIA), thiamethoxam (TMX), and clothianidin (CLO), the purity was > 99%, were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Imidacloprid-urea (IMI-U, 99.5%) was purchased from BePure (Beijing, China); imidacloprid-olefin (IMI-O, 96%) was purchased from Toronto Research Chemicals (Toronto, Canada); 1-methy1-3(tetrahydro-3-furylmethy1) urea (DIN-UF, 96%) was purchased from ANPEL (Beijing, China); flupyradifurone (FLU, 99%) was purchased from ChemService (West Chester, PA, USA); imidacloprid-D_4 (IMI-D_4), acetamiprid-D_3 (ACE-D_3), thiacloprid-D_4 (THIA-D_4), thiamethoxam-D_3 (TMX-D_3), and clothianidin-D_3 (CLO-D_3), the purity was > 98%, were purchased from C/D/N Isotopes (Quebec, Canada); dinotefuran-D_3 (DIN-D_3, 97%) was purchased from Toronto Research Chemicals (Toronto, Canada). These standards were dissolved with methanol, diluted, and volume fixed to obtain 10 μg/mL standard stock solutions, which were then diluted to the desired concentrations with methanol as needed.

QuEChERS (quick, easy, cheap, effective, rugged, and safe) extraction kits (containing sodium chloride (1.0 g), anhydrous magnesium sulfate (4.0 g), sodium citrate (1.0 g), and sodium hydrogencitrate sesquinydrat (0.5 g)) and Captiva EMR-Lipid purify cartridge (EMR) (6 mL, 600 mg) were purchased from Agilent (Folsom, CA, USA). The Prime HLB solid phase extract (SPE) cartridge (HLB) (6 mL, 500 mg) was purchased from Waters (Milford, Massachusetts, USA). The C_{18} solid phase extract cartridge (C_{18}) (3 mL, 500 mg) was purchased from SUPELCO (Bellefonte, PA, USA). Acetonitrile (ACN), methanol (MeOH), and formic acid were HPLC grade and purchased from Merck (Darmstadt, Germany). Trichloroacetic acid and ammonium acetate were analytical reagents and purchased from Sinopharm chemical reagent Co., Ltd. (Shanghai, China). Water (H_2O) was purified by using a Milli-Q system from Millipore.

Sample Preparation

5.00 g sample (12.5 g infant formula milk powder was diluted with 87.5 g water in advance) into a 50 mL centrifuge tube, adding isotope internal standard (50 μL, 1000 ng/mL), 15 mL of ACN and QuEChERS extraction kit to the tube. The mixture was shaken by vortex for 10 min at room temperature,

Fig. 1 The chemical structures of neonicotinoid and their metabolites



followed by centrifugation for 5 min at 8500 rpm. Transfer the supernatant to a new 50 mL centrifuge tube, and add ACN to bring the volume up to 20 mL, mixing the solution. Take 4 mL of the extract solution and transfer it to a 15 mL centrifuge tube, adding 1 mL of water and mixing. Transfer the solution to an EMR purification cartridge and collect the eluate, then elute the cartridge with 2 mL of ACN:H2O (20:80, vol/vol) and 3 mL of ACN, collecting all of the eluates. Evaporated the eluate to dryness using a rotary evaporator with a water bath at 40 ℃. The dried extract was reconstituted with 2 mL of MeOH:0.15% formic acid solution (10:90, vol/vol), vortex mixed for 60 s. Filter the solution through a 0.2 μm nylon membrane, take 0.1 mL of the filtered solution, and add 0.9 mL of MeOH:0.15% formic acid solution (10:90, vol/vol), mixing using a vortex for 60 s, and then were used for LC–MS/MS analysis.

Analytical Conditions of LC–MS/MS

The LC–MS/MS was carried out on liquid chromatography–tandem mass spectrometer of 1260–6495 (Agilent Technologies, Germany) with an ESI source. A Zorbax Eclipse XDB-C_{18} column (150 mm × 4.6 mm i.d., 5 μm particle, Agilent) was used for chromatographic separation at a flow rate of 0.4 mL/min. The column temperature was held at 40 °C, and the injection volume was 10 μL. Mobile phase A was 0.15% formic acid (with 5 mM ammonium acetate), and mobile phase B was MeOH. The gradient program of mobile phase B was set as follows: 0 min, 10% B; 0–6.0 min, 10–70% B; 6.0–12.0 min, 70% B; 12.0–14.0 min, 70–10% B; 14.0–18.0 min, 10% B.

The mass spectrometer was operated in multiple reaction monitoring (MRM) modes under positive electrospray

ionization (ESI^+). The operational conditions were as follows: capillary voltage, 3000 V; ion source temperature, 150 ℃; drying gas (nitrogen) flow rate, 14 L/min; sheath temperature, 350 ℃; sheath gas (nitrogen) flow rate, 10 L/min. The collision energy of each compound was optimized by flow injection analysis. The transition information and optimized parameters for each compound were listed in Table 2.

Results and Discussion

Optimization of LC–MS/MS Conditions

As shown in Fig. 2, the precursor ions and the major fragment information were monitored in positive by using flow injection for the standard solution of the compound to be measured at a concentration of 1.0 μg/mL. According to the European Union (EU) Directive 2021/808 (European Union 2021), the quantitative confirmation by LC–MS/MS must meet 5 identification points (1 point for chromatographic separation, 1 point for the precursor ion, and 1.5 points for one production). We selected two characteristic ion pairs, in which the ion pair with a high signal-to-noise ratio, good peak shape, and low interference was used as the quantitative ion pair. The ion information as well as the optimized collision energy parameters were shown in Table 2.

Optimization of Liquid Chromatography Conditions

According to references (Hou et al. 2019), the amino column and C_{18} column (4.6 × 100 mm, 5 μm, of both sizes) were selected and compared in four separation systems: MeOH – 0.15% formic acid solution (containing 5 mM ammonium acetate), MeOH – 0.15% formic acid solution, ACN – 0.15% formic acid solution (containing 5 mM ammonium acetate), and ACN – 0.15% formic acid solution. The result showed that the separation effect and the signal intensity of the target compounds were better

Table 2 Mass spectrometry parameters for neonicotinoid insecticides and metabolites analysis

Compound	MRM transitions (m/z)	Collision voltage (V)	Retention time (min)	Internal standard
Imidacloprid (IMI)	256.1/175.1* 256.1/209.1	21 17	9.50	IMI-D_4
Imidacloprid-urea (IMI-U)	212.1/128.1* 212.1/78.1	22 50	9.51	/
Imidacloprid-olefin (IMI-O)	254.1/205.2* 254.1/171.1	18 25	8.98	/
Acetamiprid (ACE)	223.1/126.1* 223.1/56.1	25 18	9.97	ACE-D_3
N-desmethyl acetamiprid (IM 2–1)	209.1/126.1* 209.1/90.1	18 40	10.04	/
Dinotefuran (DIN)	203.1/129.1* 203.1/157.1	10 5	7.81	DIN-D_3
[1-methy1-3(tetrahydro-3-furyl-methy1) urea (DIN-UF)	159.2/102.3* 159.2/67.2	10 20	7.20	/
Thiacloprid (THIA)	253.1/126.1* 253.1/186.1	23 14	10.37	THIA-D_4
Thiamethoxam (TMX)	292.1/211.1* 292.1/132.1	11 28	8.80	TMX-D_3
Clothianidin (CLO)	250.1/169.1* 250.1/132.1	12 20	9.71	CLO-D_3
Flupyradifurone (FLU)	289.1/126.1* 289.1/90.2	28 45	9.93	/
Imidacloprid-D_4 (IMI-D_4)	260.2/179.0	22	9.53	/
Acetamiprid-D_3 (ACE-D_3)	226.1/126.1	24	10.01	/
Dinotefuran-D_3 (DIN-D_3)	206.1/132.0	10	7.84	/
Thiacloprid-D_4 (THIA-D_4)	257.1/126.1	25	10.41	/
Thiamethoxam-D_3 (TMX-D_3)	295.0/214.2	12	8.82	/
Clothianidin-D_3 (CLO-D_3)	253.1/172.1	12	9.71	/

*MS/MS transition used for quantification

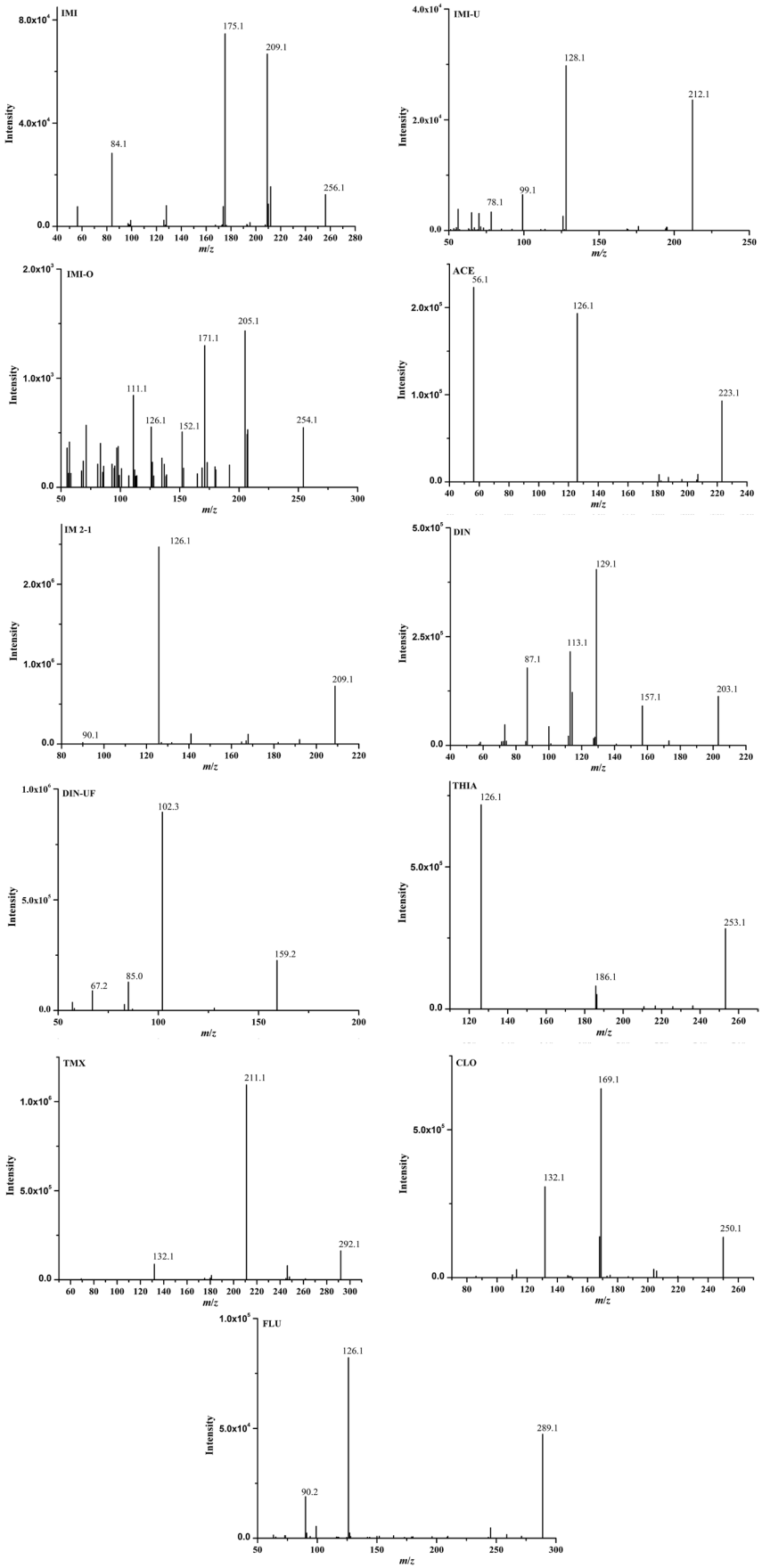


Fig. 2 The ESI–MS/MS product scan spectra of IMI, IMI-U, IMI-O, ACE, IM 2–1, DIN, DIN-UF, THIA, TMX, CLO, and FLU

on the C_{18} column than on the amino column, the signal response of THIA being more than 10 times higher, and IMI-U, ACE, IM 2–1, and DIN-UF being 3 to 6 times higher. For the C_{18} column under different conditions, the result showed that the signal response of each compound was significantly higher when MeOH was in the organic phase, and the mobile phase contained ammonium acetate; therefore, MeOH – 0.15% formic acid solution (containing 5 mM ammonium acetate) was finally used as the mobile phase for separation experimenting. Under this separation system, the MRM chromatograms of LC–MS/MS of milk spiked with each compound (spiked level: 5 μg/kg) were shown in Fig. 3.

Optimization of Extraction

The extraction process parameters were optimized to obtain the optimal extraction efficiency and remove proteins and lipids from milk or infant formula milk powder to reduce the matrix effects. The sample was extracted with several different solvents, such as MeOH, ACN, and trichloroacetic acid solution (50%); among the MeOH and ACN experiments, extraction kits (containing sodium chloride, anhydrous magnesium sulfate, sodium citrate, and sodium hydrogencitrate sesquinydrat) were added to improve the efficiency of extraction and cleanup. The results were shown in Fig. 4; when the trichloroacetic acid solution was added, the recoveries of IMI (61.1%) and IMI-U (63.4%) were lower than 65%; the recovery of DIN (157.8%, MS/MS transition of 203.1/129.1) would have significant matrix effects in the test. When MeOH was used as an extracted solution and cleanup with EMR, the recoveries of IMI-U (62.2%), ACE (46.9%), IM 2–1 (39.4%), DIN UF (54.2%), THIA (40.5%), and CLO (58.1%) were all lower than 60%, and when ACE was used as an extracted solution, the recoveries of each compound were higher than 80%. Therefore, ACN was used to extract and precipitate protein directly.

Optimization of Cleanup Method

The cleanup procedure was optimized by a spiked standard solution. Both C_{18}, HLB, and EMR were compared; the results were shown in Fig. 5. When C_{18} cartridges were eluted with acetonitrile directly, the recoveries of DIN (46.9%) and DIN-UF (39.8%) were lower than 50%. For HLB cartridges, the washing solution of different proportions of ACN/H_2O 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, and 70:30 (vol/vol) was studied; the results showed that DIN-UF and DIN could be eluted separately when the ACN/H_2O was 10:90 and 20:80. The recovery of each compound was higher than 85% when ACN eluted directly. When the EMR cartridges were eluted with ACN/H_2O (80:20) and ACN, the recovery of each compound was also higher than 85%. Compared to the full scan plots of HLB and EMR cartridges solution, EMR showed lower baseline noise. Therefore the cleanup procedure was finally performed with EMR cartridges.

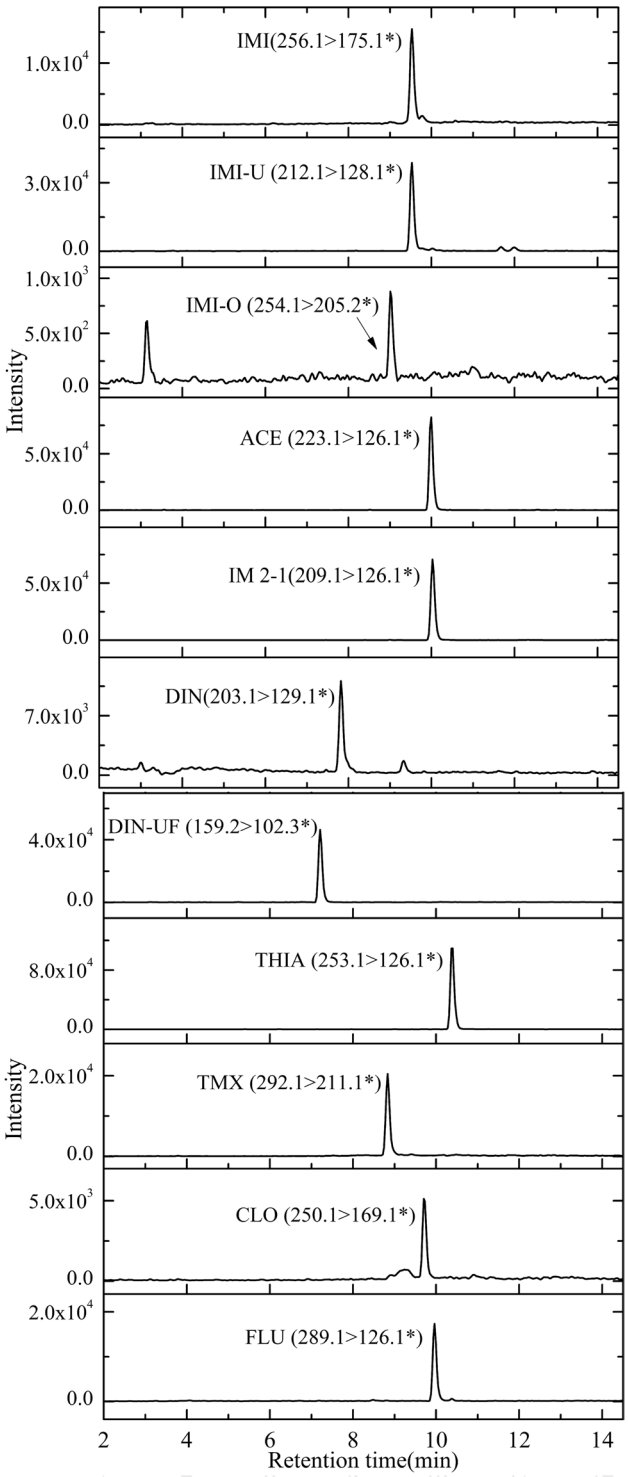


Fig. 3 The LC–MS/MS MRM chromatograms of milk spiked with each compound (5 μg/kg)

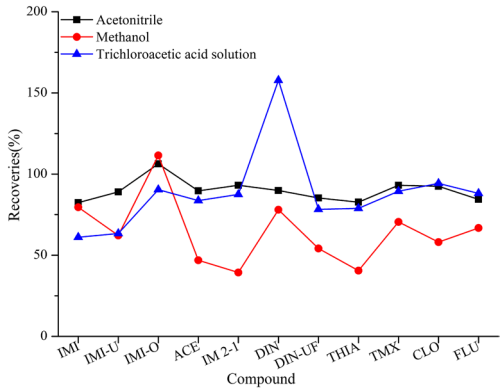


Fig. 4 The extraction efficiencies of neonicotinoids and their metabolites from milk by different solutions

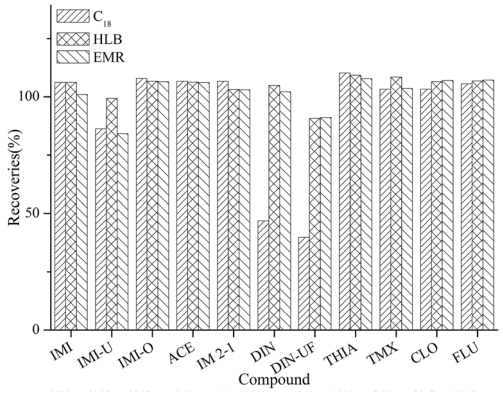


Fig. 5 The recoveries of neonicotinoids and their metabolites by different cleanup cartridges

Method Validation Results

Matrix Effect Evaluation

Matrix effects were evaluated by a modified version of the equation described by Stahnke et al. (2012). Matrix effects (%) = 100% × [peak area (postextracted spiked sample) − peak area (solvent standard)]/peak area (solvent standard), where peak area (postextracted spiked sample) is the analyte spiked into extracted matrix after the extraction and cleanup procedure. The peak area of the solvent standard is the same concentration of analyte in the solvent solution, and this solvent solution was the reconstitution solution used for the postextracted spiked sample. So, the negative result indicates suppression, and the positive result indicates enhancement of the analyte signal in the postextracted spiked sample. The results were shown in Table 3; IMI-O has a significant matrix enhancement effect in milk or infant formula milk powder with matrix effects higher than 50%, while the matrix effects of other compounds were lower than 10%. In order to reduce the matrix effects, the experiment finally used the addition of isotope-labeled internal standards and matrix-matched solution calibration curves for quantitative determination.

Calibration and Sensitivities

To overcome the matrix effects, the matrix-matched calibration standards with concentration levels of 0 μg/kg, 5 μg/kg, 10 μg/kg, 50 μg/kg, and 100 μg/kg (milk) and 0 μg/kg, 40 μg/kg, 80 μg/kg, 400 μg/kg, and 800 μg/kg (infant formula milk powder) for each compound, the isotope-labeled dilution internal standard method (IMI, ACE, DIN, THIA, TMX, and CLO) and external standard method (IMI-U, IMI-O, IM 2–1, DIN-UF, and FLU) were used for quantification of the analytes. The regression equation and correlation coefficients (r^2) values were shown in Table 4; the r^2 of each calibration curve

Table 3 The matrix effects of milk and infant formula milk powder for each compound

Compound	MRM transitions (m/z)	Peak area (postextracted spiked sample) [1]	Peak area (solvent standard) [1]	Matrix effects (%)[1]	Peak area (postextracted spiked sample)[2]	Peak area (solvent standard)[2]	Matrix effects (%)[2]
IMI	256/208.9	288,444	271,016	6.43	278,485	255,607	8.95
IMI-U	212.1/128.1	1,361,556	1,359,134	0.18	1,285,101	1,234,254	4.12
IMI-O	254.07/205.2	29,921	18,013	66.11	24,702	15,816	56.18
ACE	223.1/126.1	2,620,999	2,664,830	−1.64	2,469,238	2,387,290	3.43
IM 2–1	209.1/126.1	2,480,338	2,504,185	−0.95	2,394,719	2,262,880	5.83
DIN	202.9/156.8	273,374	285,817	−4.35	264,514	254,337	4.00
DIN-UF	159/102	1,658,649	1,708,271	−2.90	1,549,229	1,588,718	−2.49
THIA	253.1/186.1	298,119	277,619	7.38	273,144	255,907	6.74
TMX	292/211	703,086	682,405	3.03	648,811	641,277	1.17
CLO	250/169.1	176,952	184,232	−3.95	178,078	179,467	−0.77
FLU	288.8/125.8	623,655	599,083	4.10	588,194	585,452	0.47

[1]Milk

[2]Infant formula milk powder

Table 4 Regression equations, correlation coefficient, recoveries, and repeatability of the neonicotinoids and their metabolites in the milk and infant formula milk powder samples spiked at 3 concentrations ($n=6$)

Compound	Regression equations	Correlation coefficients (r^2)	Spiked level (μg/kg)	Recoveries[1] (%)	RSD[1,2] (%)	Recoveries[3] (%)	RSD[3] (%)
IMI	$y=0.0226*x-2.85*10^{-7}$	0.9996[4]	5.0	88.3–100.6	7.67	80.1–96.7	7.7
			10	84.4–102.3	6.54	85.1–97.3	4.9
			50	93.9–105.8	4.25	93.4–106.8	4.6
	$y=0.00282*x-3.28*10^{-8}$	0.9993[5]	40	86.1–100.6	6.12	78.9–98.3	7.8
			80	85.6–96.8	5.05	81.8–99.4	7.3
			400	92.0–105.6	4.59	92.8–101.0	3.0
IMI-U	$y=75{,}116.786*x-1.13$	0.9979[4]	5.0	72.5–85.8	7.5	72.8–87.7	7.1
			10	73.2–81.5	4.12	73.3–81.3	4.2
			50	73.1–88.2	7.91	74.7–93.8	9.4
	$y=8562.828*x-0.122$	0.9984[5]	40	72.1–90.4	9.65	71.9–79.0	3.5
			80	74.4–92.2	8.17	72.0–77.4	2.5
			400	75.9–87.1	5.26	73.2–80.0	3.4
IMI-O	$y=1655.740*x-0.0267$	0.9981[4]	5.0	71.8–77.3	3.21	74.4–94.3	10.4
			10	73.3–86.7	6.12	72.1–79.7	4.4
			50	72.5–97.0	11.14	72.6–83.2	5.4
	$y=156.120*x-0.00210$	0.9992[5]	40	72.0–86.6	6.43	73.1–101.6	13.9
			80	73.5–99.6	12.15	73.3–92.1	9.2
			400	73.1–87.9	7.3	72.2–87.9	9.2
ACE	$y=0.0233*x-3.58*10^{-7}$	0.9970[4]	5.0	78.1–83.7	2.52	82.4–87.8	2.8
			10	82.6–92.4	4.66	86.3–91.0	2.0
			50	81.7–93.6	4.83	89.5–100.0	4.0
	$y=0.00286*x-4.34*10^{-8}$	0.9978[5]	40	94.3–87.1	2.89	86.3–90.5	1.9
			80	88.9–95.9	4.25	84.7–91.8	2.8
			400	89.1–100.4	4.18	87.9–94.2	2.6
IM 2–1	$y=135{,}240.478*x-2.013$	0.9979[4]	5.0	74.4–93.2	8.56	73.0–91.0	9.3
			10	74.6–86.3	6.81	73.3–87.1	7.4
			50	75.5–101.7	11.98	77.8–98.7	9.5
	$y=16{,}307.202*x-0.239$	0.9978[5]	40	74.8–81.0	3.04	74.9–82.9	3.7
			80	79.2–85.8	2.98	78.9–84.9	2.9
			400	80.3–90.6	5.53	80.5–86.3	3.0
DIN	$y=0.0133*x-1.99*10^{-7}$	0.9977[4]	5.0	89.5–97.7	3.91	76.8–96.4	7.6
			10	91.4–103.5	4.58	85.7–102.9	6.1
			50	92.7–105.5	4.75	95.3–105.8	6.1
	$y=0.00164*x-2.34*10^{-8}$	0.9988[5]	40	81.1–101.8	9.35	90.0–106.6	6.5
			80	75.4–96.5	4.98	91.0–95.8	1.9
			400	94.2–102.2	3.12	97.1–104.5	3.3
DIN-UF	$y=89{,}833.547*x-1.30$	0.9982[4]	5.0	71.8–84.5	6.55	73.2–84.0	5.8
			10	72.5–77.0	2.32	72.9–78.7	3.0
			50	71.7–83.2	6.38	72.4–92.2	10.6
	$y=10{,}501.528*x-0.142$	0.9987[5]	40	73.7–86.4	6.06	72.0–85.6	6.6
			80	72.3–82.7	6.35	71.9–77.3	3.1
			400	75.3–83.7	4.65	75.1–82.5	3.1
THIA	$y=0.00172*x-2.40*10^{-8}$	0.9982[4]	5.0	83.1–89.8	2.89	76.4–86.4	5.1
			10	87.7–96.8	3.63	86.8–92.9	2.6
			50	93.8–106.2	5.26	93.7–105.7	4.0
	$y=2.12*x-2.87*10^{-9}$	0.9994[5]	40	80.1–91.4	4.49	85.4–87.7	1.2
			80	84.4–92.5	3.41	83.7–94.8	4.8
			400	92.4–98.4	2.55	92.5–98.6	2.3

Table 4 (continued)

Compound	Regression equations	Correlation coefficients (r^2)	Spiked level (μg/kg)	Recoveries[1] (%)	RSD[1,2] (%)	Recoveries[3] (%)	RSD[3] (%)
TMX	$y=0.0164*x-1.93*10^{-7}$	0.9991[4]	5.0	84.4–92.8	4.07	78.9–98.8	7.9
			10	89.6–92.4	2.79	87.6–99.1	4.4
			50	102.3–106.9	1.94	100.9–105.8	2.1
	$y=0.00212*x-2.68*10^{-8}$	0.9982[5]	40	82.0–95.7	5.5	86.6–95.5	4.2
			80	86.8–99.8	5.23	84.4–99.9	5.6
			400	89.7–105.1	5.42	96.3–107.1	3.7
CLO	$y=0.0259*x-3.40*10^{-7}$	0.9992[4]	5.0	73.7–89.1	7.58	76.2–90.5	7.6
			10	82.0–96.0	5.77	88.0–94.9	2.6
			50	96.4–104.9	2.8	97.0–104.1	2.6
	$y=0.00318*x-4.23*10^{-8}$	0.9994[5]	40	86.1–94.2	3.36	79.0–94.4	7.4
			80	82.9–93.4	4.46	81.7–93.1	4.9
			400	98.9–104.1	2.19	99.8–104.2	1.8
FLU	$y=32{,}474.611*x-0.423$	0.9993[4]	5.0	76.7–99.8	8.81	76.0–108.7	12.6
			10	7.21–84.9	6.72	73.8–98.8	10.3
			50	84.3–107.9	9.86	80.4–98.5	10.1
	$y=3868.787*x-0.0541$	0.9981[5]	40	77.8–80.8	1.65	78.0–81.1	1.5
			80	77.7–82.6	2.5	77.8–85.9	4.0
			400	82.6–91.9	3.6	82.7–91.0	3.3

[1]Intra-day

[2]*RSD*, relative standard deviation

[3]Inter-day

[4]Milk

[5]Infant formula milk powder

was more than 0.995. On the basis of $S/N=10$, the limits of quantification (LOQ) of the analytes were 2 μg/kg (IMI, IMI-U, ACE, IM 2–1, DIN UF, THIA, TMX) and 5 μg/kg (IMI-O, DIN, CLO, FLU) for milk, 2 μg/kg (ACE), 15 μg/kg (IMI, IM 2–1, DIN UF, THIA, TMX), and 40 μg/kg (IMI-U, IMI-O, DIN, CLO, and FLU) for infant formula milk powder.

Assay Specificity

The specificity was evaluated by the analysis of 20 blank milks and 20 infant formula milk powers. No interfering peaks from endogenous compounds were found in the retention time of the target analyte for samples.

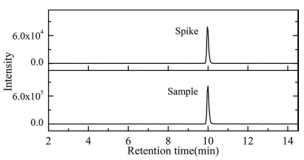


Fig. 6 The LC–MS/MS MRM chromatograms of ACE in spiked milk (5 μg/kg) and real sample

Accuracy and Precision

The method accuracy was evaluated by analyzing a series of spiked samples following the developed method. Three different concentrations (high, medium, and low) of the standard target compounds were spiked into the "blank" samples and then treated following the optimized experimental procedure, analyzed by LC–MS/MS. The recoveries were 71.7–108.7% (milk) and 71.9–107.1% (infant formula milk powder). To evaluate the precision of this method, both intra-day and inter-day repeatability were examined by determination of milk and infant formula milk powder sample at three different concentrations. Good stability and satisfactory repeatability were achieved, the relative standard deviations (RSDs) values of milk were below 11.9% and 12.6%, infant formula milk powder was below 12.2% and 13.9% for intra-day and inter-day analyses, respectively.

Determination of Real Samples

15 milk and 21 infant formula milk powder samples were purchased from local supermarkets and analyzed by the validated method (before analysis, infant formula milk powder

was at a dilution ratio of 1:8 with warm water and then cooled to room temperature). The results were shown in Fig. 6; one milk sample contained residues of ACE (45.4 μg/kg), and no neonicotinoids and their metabolites were detected in any of the infant formula milk powder samples.

Conclusions

In the present study, the method of simultaneous determination of seven neonicotinoids and four metabolites in milk and infant formula milk powder samples by EMR-LC–MS/MS was established. Both isotope-labeled internal standards and matrix-matched calibration standards were used to alleviate the matrix effects. Good recoveries (71.7–108.7%) and precision (RSDs below 13.9%) were obtained; the results indicate that the developed method was simple and rapid and the LOQ meets the current requirements of the maximum residue limits of relevant compounds in the USA, China, EU, and Japan, which can be applied for the simultaneous determination of neonicotinoids and their metabolites in milk or infant formula milk powder.

Author Contribution Jianbo Hou designed and implemented the experimental program and wrote the main manuscript text. Wen Xie participated in designing the experimental program and wrote the main manuscript text. Yan Qian purchased chemicals and reagents for this experiment, determined the parameters of LC–MS/MS and maintenance of experimental equipment, and prepared Figs. 1 and 2. Wenhua Zhang purchased experimental samples (milk and infant formula milk powder) and prepared Figs. 3, 4, and 5. Yingzhu Shi carried out pretreatment experiments and parameter determination and prepared Tables 2 and 3. Wei Song organized the maximum residue limits (MRLs) and analyzed the background and prepared Table 1. Chengjie Lou collected and analyzed experimental validation data and prepared Table 4.

Funding This work was supported by the National Program on Key Research Project of China (2019YFE0103900) as well as the European Union's Horizon 2020 Research and Innovation Program under Grant Agreement No 861917-SAFFI.

Data Availability All data generated or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to Participate All authors consented to participate.

Consent for Publication All authors reviewed the manuscript and consented to publication.

Conflict of Interest Jianbo Hou declares that he has no conflict of interest. Wen Xie declares that she has no conflict of interest. Yan Qian declares that she has no conflict of interest. Wenhua Zhang declares that he has no conflict of interest. Yingzhu Shi declares that she has no conflict of interest. Wei Song declares that he has no conflict of interest. Chengjie Lou declares that he has no conflict of interest.

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