WITHDRAWN: Neonicotinoid Analysis in Sunflower (Helianthus annuus) Honey of Tekirdag Province in Turkey

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Abstract

The widespread neonicotinoid use in agriculture causes environmental pollution, but its toxicity to mammals is more limited. Honeybees can carry pollutants to hives, considered bioindicators of environmental pollution. Forager bees returning neonicotinoids treated sunflower fields cause residue accumulation in the hives, which reason colony-level adverse effects. This study analyzes neonicotinoid residues in sunflower (Helianthus annuus) honey sampled by beekeepers from Tekirdağ province. Honey samples have been subjected to liquid-liquid extraction methods before high-pressure liquid chromatography-mass spectrometry (HPLC-MS/MS). No neonicotinoid residues were found above the maximum residue limit in the sunflower honey samples analyzed.

Introduction

Neonicotinoid-type plant protection products are widely used in the agricultural field, especially for preserving seeds due to their less toxic effect on mammals; also have unintended consequences on non-target organisms like agrarian pollinators. Pollution is also detected in the fields where neonicotinoid-treated seeds are planted and in the application area's surface or public drinking water sources. Residues of neonicotinoids can adversely affect honeybees' intelligence ability variously, as a part of involvement major role in Colony Collapse Disorder. (Fairbrother et al., 2014). The neonicotinoids have been banned in the first Europa due to the adverse environmental effects resulting from a severe decrease in honey bees, insect populations, and bird species and numbers. (EMA, 2013).

Different extraction methods and devices are used to determine neonicotinoid residues in sunflower honey. Liquid-liquid extraction (LLE) (Fidente et al. 2005), solid-phase extraction (SPE) (Martel and Lair 2011, Yáñez et al. 2013), QuEChERS extraction (Zhang et al. 2012, Codling et al. 2016, Mrzlikar et al. 2019), and dispersive liquid-liquid micro-extraction (DLLME) (Jovanov et al. 2013) can be counted among the effective extraction methods used. A high-pressure liquid chromatography diode array detector (HPLC/DAD) (Jovanov et al. 2015), a gas chromatography-mass spectrometer (GC-MS/MS) (Rossi et al. 2005), and liquid chromatography-mass spectrometry (LC-MS/MS) are used for neonicotinoid analyses (Mrzlikar et al. 2019; Valverde et al. 2022).

This study aims to determine neonicotinoids residues in sunflower honey collected from beekeepers around Tekirdağ province. The liquid-liquid extraction (LLE) method was used for the extraction of the honey samples, and quantification was performed by ultra-high pressure liquid chromatography-mass spectrometry (UHPLC-MS/MS).

Materials And Methods

Chemicals and reagents

The following standards for neonicotinoids were used; imidacloprid (99.9%), acetamiprid (99.9%), clothianidin (99.9%), nitenpyram (99.9%), thiacloprid (99.9%), dinotefuran (98.8%) and thiamethoxam (99.6%). The purity of all compounds was greater than 98%. The internal standard of clothianid-d_3 (97%) was obtained from Sigma-Aldrich. Acetonitrile for HPLC was obtained from Fluka. Dichloromethane and glacial acetic acid for analysis were obtained from Merck.

Standards and solutions

Standard stock solution (1 mg mL^{-1} each)

10 mg of each reference standard was weighed into a 10 mL Class A graduated flask. Sufficient methanol was added up to the mark. All stock solutions were stored at -18°C.

S_2 - Working standard solution (10 ng mL^{-1} each)

0.1 mL of each reference standard stock solution was taken and placed in a 10 mL measuring balloon. Sufficient methanol was added up to the mark. The prepared working solutions were stored at 4–6°C.

S_4 - Standard working solution (100 ng mL^{-1} each)

0.1 mL of each S_2-standard working solution was taken and placed in a 10 ml measuring flask. Sufficient methanol was added up to the mark. All standard working solutions were stored at 4–6°C. This prepared solution was used to define reference standards in the MS detector.

Reference standard solution mix for the spike

Spiking solutions were prepared at 10 ng mL^{-1} for clothianidin, dinotefuran, nitenpyram and thiamethoxam, 50 ng mL^{-1} for acetamiprid and imidacloprid, and 200 ng mL^{-1} for thiacloprid, according to the levels of MRL in the honey, respectively. One mL of clothianidin, dinotefuran, nitenpyram and thiamethoxam S_4 working solution, 5 mL of acetamiprid and imidacloprid S_4 working solution and 0.2 mL of thiacloprid S_2 working solution were put into a 10 mL measuring balloon and filled with methanol up to the mark.

Internal standard solution

Clothianidin-d_3 was used as an internal standard at a 10 ng mL^{-1}.

Mobile phase A

Acetonitrile was used as a mobile phase A.

Mobile phase B

Two mL of acetic acid were placed in a 1 L volumetric flask and diluted to the mark with reagent water. The mobile phases were degassed in an ultrasonic bath for 15 min.

Equipment

1. Mutireax (Heidolph Instruments, Germany)
2. Ultrasonic bath
3. Centrifuge
4. UHPLC. Eksigent, ekspert UltraLC 100.
5. Analytical column; Agilent Zorbax Poroshell 120 SB: C182.7 µm 100 x 3.0 mm.
6. AB Sciex 3200 QTRAP, Tandem Quadrapol Mass Spectrometer (MS/MS).
7. Software: Analyst 1.6.1

Collection of samples

During sunflower honey harvest, honey samples were collected from supers at each of the 33 stationary apiaries in July/August of 2015 in the province of Tekirdag (40° 58' 41" N, 27° 30' 42" E).

Extraction of honey samples

The extraction method (Jovanov et al. 2013) was used for the honey samples. Briefly, two grams of each honey sample were weighed into 15 mL polypropylene centrifuge tubes and an internal standard solution (100 µL) was added to the tubes. The mixed standard spiking solutions were added (50, 100, 150 and 200 µL) to control the quality of the samples. 0.5 mL of acetonitril and 2.0 mL of dichloromethan were placed in each tube. The tubes were mixed by vortex for 1 minute, incubated in an ultrasonic bath for 10 minutes, returned to the vortex for 1 minute and centrifuged at 2,500 g for 5 minutes, 6 mL of supernatant was then removed using a pipette and transferred into glass tubes. The organic fraction was evaporated to dryness in a stream of nitrogen at 40 °C within a water bath. Two mL of mobile phase was added onto the dry residue and mixed by vortex for two minutes. The result was filtered into an autosampler vial using a 0.2 µm syringe filter.

Instrumentation

Analyses were performed on AB Sciex 3200 QTRAP brand/model high-pressure liquid chromatography-mass spectrometry equipment controlled by Analyst 1.6.1 software. An Agilent Poroshell 120 SB: C18 2.7 µm 100x3.0 mm column was used for chromatographic separation. Acetonitrile (A) and water acidified with 0.2% acetic acid were used as the mobile phase. The mobile phase linear-gradient flow is shown in Table 1. The flow rate was set at 0.3 mL/min, the injection volume was 10 µL, and the column temperature was 40°C.

Table 1
The linear gradient program

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow rate (mL/min)
00:00	80	20	0.30
01:00	80	20	0.30
01:10	50	50	0.30
03:30	50	50	0.30
03:40	80	20	0.30
06:00	80	20	0.30

Mass spectrometry

The MS/MS parameters and precursor-product ions of each compound were tuned by direct infusion in the multiple reaction monitoring (MRM) mode with a 0.25 mL/min flow rate of the mobile phases A and B (50:50).

The MS/MS detector parameters and precursor-product ions of each referenced standard substance are shown in Table 2. A capillary voltage of 5500 V, nebulizer gas of 7 psi, curtain gas of 30 psi, heater gas of 50 psi, and collision gas of 50 psi were set. The temperature of the TurbolonSprey module was set at 400 °C.

Table 2
MS/MS detector parameters and retention times (RT)

Analytes	RT	Precursor ion (m/z)	Product ion (m/z)	DP (volts)	EP (volts)	CEP (volts)	CE (volts)	CXP (volts)
Dinotefuran	2.4	203.08	129.1*	36	8	18	15	4
			114.1	36	8	18	17	4
Nitenpyram	2.8	271.12	225.20*	36	5.5	16	15	4
			56.00	36	5.5	16	49	4
Thiamethoxam	3.0	292.00	211.10*	31	11.5	16	15	4
			132.10	31	11.5	16	25	4
Clothianidin	3.2	250.07	132.00*	41	7.5	14	19	4
			169.10	41	7.5	14	15	4
Clothianidin-d3 (IS)	3.2	253.01	132.00	41	8	14	23	4
Imidacloprid	3.3	256.10	290.10*	36	9	14	19	4
			175.10	36	9	14	21	4
Acetamiprid	3.4	223.07	126.20	41	9	12	27	4
			99.20	41	9	12	47	4
Thiacloprid	3.7	253.06	126.20	46	12	14	29	4
			99.10	46	12	14	53	4

*Confirmative ion

DP: Declustering potential, EP: Entrance Potential, CEP: Cell exit potential, CE: Collision energy, CXP: Collision Cell Exit Potential

Ionization was performed in positive ion mode using the electrospray ionization (ESI) module.

Results

Method validation

The selectivity/sensitivity, linearity, limit of detection (LOD) and limit of quantitation (LOQ), decision limit (CCα), detection capability (CCβ), accuracy, and recovery parameters were calculated for the method validation.

Specificity/selectivity

Blank samples were analyzed by loading different standard substances; no interference was observed in the retention times. It was concluded that the analysis method was suitable for selectivity/sensitivity. The chromatogram obtained from loading at the MRL level is shown in Fig. 1.

Linearity

To determine the linearity of the method, six parallel analyses were performed using four different concentration points at 0.5, 1, 1.5 and 2 MRL levels in accordance with the MRL level in honey. Calibration curves for each standard substance were created. The r^2 value in the calibration curve of each standard item was found to be between 0.9908–0.9984 (Table 3).

Table 3
Summary of LOD, LOQ, CC_α and CC_β

Analyte	Calibration range ($\mu g\ kg^{-1}$)	Linearity	Limit of Detection (LOD)	Limit of Quantification (LOQ)	Decision Limit (CC_α)	Detection Capability (CC_β)	MRL (EU) ($\mu g\ kg^{-1}$)
Dinotefuran	5–20	0.9908	6.25	9.45	10.74	11.50	10
Nitenpyram	5–20	0.9984	6.15	9.77	11.08	12.17	10
Thiametoxam	5–20	0.9963	6.42	9.72	11.48	12.94	10
Clothianidin	5–20	0.9910	5.42	8.70	10.94	11.88	10
Imidacloprid	25–100	0.9956	26.63	36.94	57.18	64.37	50
Acetamiprid	25–100	0.9954	29.18	43.20	56.86	63.73	50
Thiacloprid	100–400	0.9922	102.67	155.34	221.11	242.22	200

Limit of detection (LOD) and limit of quantification (LOQ)

To determine the limit of detection and the limit of quantification, 10 parallel analyses were performed at 0.5 MRL. The results obtained are shown in Table 3.

Decision limit (CC_α) and detection capability (CC_β)

The decision limit (CC_α) and detection capability (CC_β) were calculated using the results obtained from the study linearity and are shown in Table 3.

Accuracy

The accuracy was calculated using the study linearity and recovery results shown in Table 4.

Table 4
Accuracy and recovery of neonicotinoids in honey samples.

	Added Amount ($\mu g\ kg^{-1}$)	Mean Amount Calculated ($\mu g\ kg^{-1}$)	RSD %	Accuracy %	Recovery %
Dinotefuran	5	5.25	11.56	105.00	84.08
	10	10.86	7.11	108.56	82.57
	15	15.79	6.16	105.29	82.52
	20	18.72	6.92	93.63	78.94
Nitenpyram	5	5.13	7.98	102.75	87.92
	10	10.42	7.27	104.20	92.75
	15	15.71	12.77	104.80	74.75
	20	19.08	8.20	95.30	63.71
Thiametoxam	5	4.98	8.27	99.69	71.36
	10	10.03	9.75	100.25	84.50
	15	15.25	7.27	101.59	78.63
	20	19.79	8.21	98.74	73.02
Clothianidin	5	5.06	11.94	101.34	63.04
	10	10.13	10.94	101.31	73.65
	15	15.18	10.09	101.23	70.72
	20	19.75	6.03	98.81	86.06
Imidacloprid	25	23.55	10.92	94.10	74.68
	50	51.59	6.48	103.21	74.98
	75	77.64	7.26	103.63	77.57
	100	96.70	6.61	96.70	71.26
Acetamiprid	25	23.61	10.34	94.50	103.19
	50	52.36	8.42	104.70	95.83
	75	79.23	9.37	105.53	94.94
	100	94.70	8.56	94.70	93.64
Thiacloprid	100	100.23	7.13	100.23	94.12
	200	209.00	9.30	104.45	92.00

	Added Amount (μg kg^{-1})	Mean Amount Calculated (μg kg^{-1})	RSD %	Accuracy %	Recovery %
	300	318.00	10.60	105.99	92.21
	400	377.88	10.84	94.51	90.56

Recovery

To determine recovery, analysis was performed according to the blank fortified sample at levels 0.5, 1, 1.5, and 2 MRL. The results obtained are shown in Table 4.

According to the sunflower honey samples analysis, the MRL value was not detected for any neonicotinoid residue. The data under the maximum residue levels have not been evaluated. The results of the analysis of the honey samples are shown in Table 5.

Table 5
The results of analysis honey samples

No	Dinotefuran ($\mu g\ kg^{-1}$)	Nitenpyram ($\mu g\ kg^{-1}$)	Thiametoxam ($\mu g\ kg^{-1}$)	Clothianidin ($\mu g\ kg^{-1}$)	Imidacloprid ($\mu g\ kg^{-1}$)	Acetamiprid ($\mu g\ kg^{-1}$)	Thiacloprid ($\mu g\ kg^{-1}$)
1	0,64	< 0	< 0	0,21	1,23	1,95	0,65
2	0,53	< 0	< 0	0,15	1,4	1,97	0,53
3	0,47	< 0	< 0	0,57	1,21	2,07	0,58
4	0,40	< 0	< 0	0,15	1,22	1,95	0,50
5	0,48	< 0	< 0	0,67	1,26	1,95	0,64
6	0,52	< 0	< 0	0,32	1,21	1,93	0,51
7	0,61	< 0	< 0	0,15	1,33	1,94	0,50
8	0,42	< 0	< 0	< 0	1,23	1,94	0,51
9	0,60	< 0	< 0	< 0	1,22	1,95	0,51
10	0,55	< 0	< 0	0,46	1,26	1,97	0,51
11	0,73	< 0	< 0	0,21	1,43	2.00	0,54
12	1,03	< 0	< 0	1,39	1,33	1,99	0,52
13	0,96	< 0	< 0	< 0	1,24	3,64	0,54
14	0,51	< 0	< 0	0,28	1,27	1,98	0,52
15	0,58	< 0	< 0	0,23	1,25	1,94	0,52
16	1,12	< 0	< 0	0,17	1,30	1,95	0,53
17	0,87	< 0	< 0	0,12	1,22	1,95	0,51
18	0,71	< 0	< 0	1,49	1,24	1,96	0,54
19	0,53	< 0	< 0	0,23	1,22	1,96	0,52
20	0,60	< 0	< 0	0,36	1,25	1,99	0,55
21	0,43	< 0	< 0	0,13	1,24	1,95	0,56
22	0,98	< 0	0,07	1,58	1,46	2,06	0,52
23	0,88	< 0	< 0	< 0	1,24	2,16	0,51
24	1,18	< 0	< 0	0,36	1,33	1,98	0,58
25	1,02	< 0	< 0	0,57	1,48	1,99	0,65
26	1,35	< 0	< 0	0,50	1,33	1,98	0,56
27	0,95	< 0	< 0	< 0	1,27	1,96	0,54
28	1,56	< 0	< 0	0,37	1,46	2,06	0,53

No	Dinotefuran ($\mu g\ kg^{-1}$)	Nitenpyram ($\mu g\ kg^{-1}$)	Thiametoxam ($\mu g\ kg^{-1}$)	Clothianidin ($\mu g\ kg^{-1}$)	Imidacloprid ($\mu g\ kg^{-1}$)	Acetamiprid ($\mu g\ kg^{-1}$)	Thiacloprid ($\mu g\ kg^{-1}$)
29	0,64	< 0	< 0	0,64	1,23	1,93	0,51
30	0,89	< 0	< 0	0,11	1,25	3,39	0,52
31	0,55	< 0	< 0	0,68	1,44	1,95	0,51
32	0,81	< 0	1	0,81	1,78	2,01	0,53
33	0,48	< 0	< 0	0,51	1,24	2,46	0,51

Discussion

Honey, which must comply with EU standard norms, must be reliable regarding food safety and sustainable colony health. High pesticide concentrations can cause high mortality in bees, loss of colonies, and honey production unsuitable for food safety. For this reason, pesticides in food have become a severe health and safety checkpoint worldwide, and demands for detecting chemicals that may pose an environmental risk have increased in recent years.

Pollination, usually performed by various bees, is an important ecosystem service for the world's flowering plants and croplands.

Honeybees, *Apis mellifera* L., provide pollination services to more than a hundred different crops worldwide. During blooming sessions, migratory beekeepers visit sunflower fields with more than two million pollinator colonies to get sunflower honey. The importance of sunflower honey in the global honey trade is different; its flavor is not strong dominantly. Therefore it blends well with other honey types, and it is one of the most suitable and economic honey for commercial blending. Sunflower honey is preferred to produce honey wine made from non-dominant aromatic honey, as in Spain and France.

Sunflower (Helianthus annuus), the world's fourth most used edible oil source, was produced at approximately 19.5 million tons/liter in 2020-2021, but the 2021-2022 price still increased by 50 percent. Global sunflower seed yield is affected negatively due to increased parasitic pests and decreased pollinators. Neonicotinoid pesticides have been used as an alternative to increasing oilseed yield by reducing parasite populations. Commercial beekeepers move millions of honeybee colonies to sunflower fields to get sunflower honey during the blooming sessions. The neonicotinoids contaminate the colonies via sunflower nectar and pollen during bee foraging. So, sunflower oil production related to pollinators' activity differs among countries. (Nderitu et al.;, 2008). Successful results have been obtained in analyzing multiple residues of antibiotics and pesticides in honey using liquid chromatography-mass spectrometry (LC-MS/MS). Previous studies published about the confirmation method and validation of the residues of neonicotinoids in honey are summarised below.

When sunflower and corn were planted on soils containing 2–18 µg kg-1 still imidacloprid due to previous treatments, imidacloprid was not detected in pollen and nectar (Schmuck et al. 2001).

In Austria, acetonitrile extraction and dispersive solid-phase extraction (QuEChERS type) were used in Tanner and Czerwenka's analytical method to detect neonicotinoid residues in honey. Residues of acetamiprid, thiacloprid, and thiamethoxam were detected in Austrian honey samples; however, no sample exceeded the maximum residue limits.

Flower honey samples contained more neonicotinoid residues than forest honey samples (Tanner and Czerwenka 2011).

Ligor et al. developed a method using QuEChERS extraction and UHPLC/UV to determine neonicotinoid residues in honey samples. The method was applied to honey collected from Poland and other countries, including Turkey. In 19 honey samples, neonicotinoid residues were detected above the LOQ[.1] . No neonicotinoid residues were detected in the Turkish sample (Ligor et al. 2020).

Residue analyzes were carried out in honey bees sampled from sunflower grown from seeds treated with thiamethoxam or clothianidin. Residue concentrations in bee bread and adult bee samples were in the range of 0.10–2.89 ng g^{-1} and 0.05–0.12 ng g^{-1} (clothianidin); they were detected at the levels of 0.10–0.37 ng g^{-1} and 0.01–0.05 ng g^{-1} (thiamethoxam), respectively (Hernando et al. 2018).

A 3-year field study was conducted in France from 2002 to 2005 to examine pesticide residues found in colonies and honeybee (Apis mellifera L.) colony health by Chauzat et al. No pesticide residues were detected in 12.7% of the sampling periods. It was reported that no statistical relationship was found between colony mortality and pesticide residues (Chauzat et al. 2009). Imidacloprid and 6-chloronicotinic acid residues were frequently detected in pollen, honey and honeybee samples.

Mrzlikar et al. developed a reliable analytical method using two extraction techniques (SPE, QuEChERS) and LC-MS/MS (SRM) for five neonicotinoids in 51 honey samples collected between 2014 and 2016. Despite being banned in the country in 2011, residues of acetamiprid and thiacloprid were detected in low contamination (Mrzlikar et al. 2019).

An average of 8.2 ng g^{-1} clothianidin and 17.2 ng g^{-1} thiamethoxam were detected in 68% and 75% of honey samples, respectively, from hives located 30 km from Saskatchewan City in Canada. Moreover, clothianidin was found in > 50% of bee and pollen samples. Imidacloprid was detected in ~30% of honey samples (Codling et al. 2016).

The pollen, nectar, and leaves were collected to measure the residual levels of neonicotinoids using different application doses during flowering in a cotton crop especially treated with imidacloprid and thiamethoxam. Imidacloprid residues were detected between 1.61 and 64.58 ngg-1 in pollen samples and up to 1.769 ngg-1 in nectar samples. Thiamethoxam residues reached 14,521 ngg-1 in pollen samples and 4,285 ngg-1 in nectar samples. The study results provide insight into the transfer of both components to planting areas by seed treatment and the potential exposure of bees and other pollinators to systemic insecticides (Jiang et al. 2018).

A study conducted in North America from 2007 to 2008 examined the effects of pesticides on the health of bee colonies. 1% of 208 wax samples, 17.7% of 350 pollen samples and 0.0% of 140 honey samples were detected as having imidacloprid residues (Mullin et al. 2010).

Residues of neonicotinoids were investigated in honey, pollen and bee samples sampled in Greece between 2011 and 2013, while any residue did not detect in the honey samples. But 0.7–14.7 ngg clothianidin in bee samples in 2011, 6.1–69.04 ngg in pollen samples, and 2.7–39.9 ngg was detected in 2012 bee samples and 308.3–1273 ngg clothianidin in pollen samples (Kasiotis et al. 2014).

The neonicotinoid products restricted in European Union were not found in honey samples sampled in Tekirdağ on the European side of Turkey.

Conclusion

According to the analysis results of 33 sunflower honey samples collected from Tekirdağ province are free of any neonicotinoid residues exceeding the maximum residue limits were detected. It was understood that in further studies, more honey samples should be analyzed as well as other hive products.

Declarations

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Competing Interests

Nurullah OZDEMIR and Muatafa Necati MUZ declare they have no financial interests.

Author Contributions

All authors contributed to the study conception and design.

All authors contributed to the material preparation and sample collection.

Data collection and analysis were performed by Nurullah OZDEMIR

The first draft of the manuscript was written by Nurullah OZDEMIR

All authors commented on previous versions of the manuscript.

All authors read and approved the final manuscript.

Ethics approval

The approval of research ethics committees was not required to achieve the objectives of this study because animal food (honey) was used in the study.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

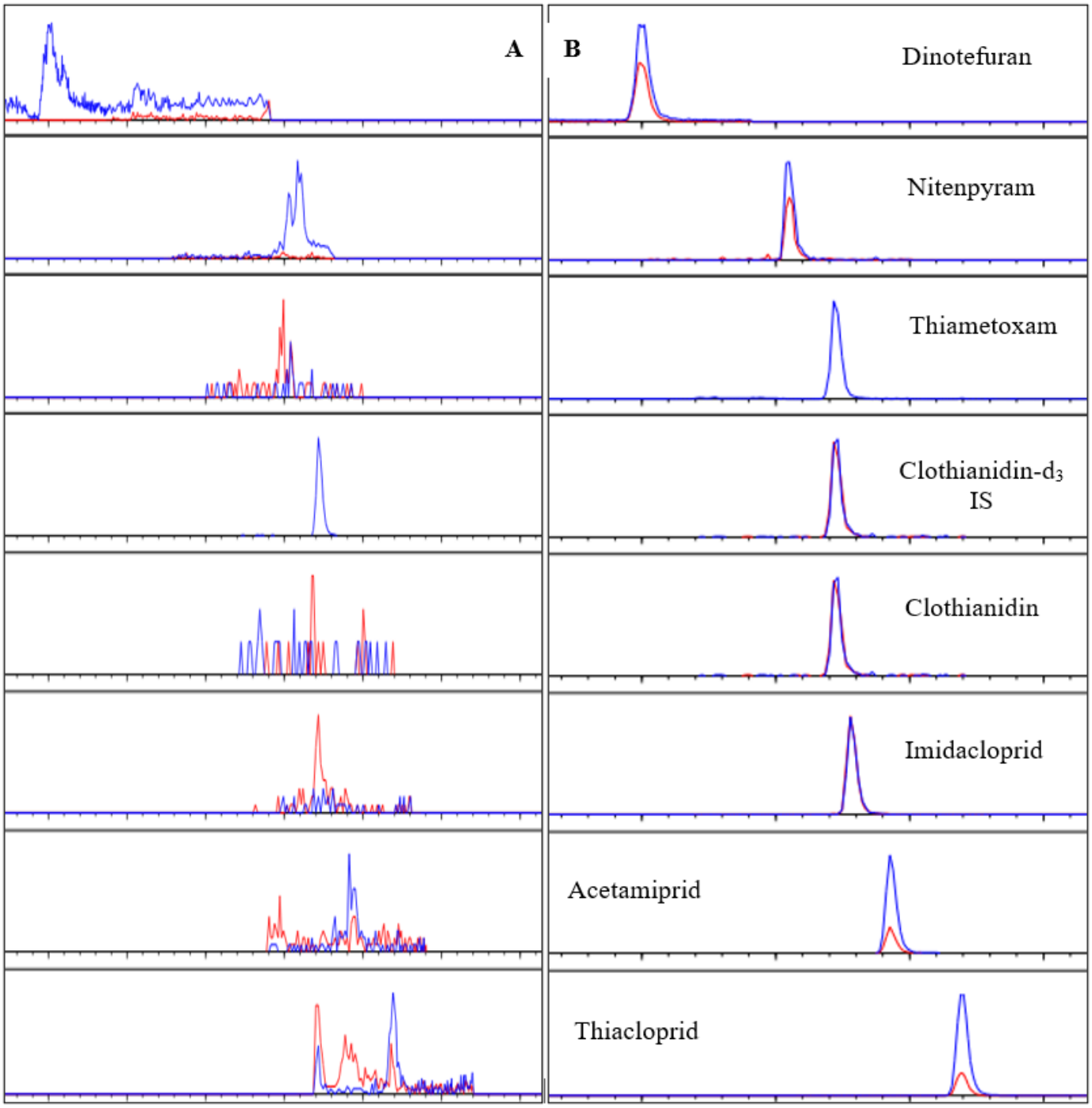

Figure 1

Chromatograms of blank honey samples (A) and fortified spiked honey samples (B).