SHIMADZU

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Ultra-sensitive and rapid assay of neonicotinoids, fipronil and some metabolites in honey by UHPLC-MS/MS.

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1. Introduction

Neonicotinoids are a class of insecticides widely used to protect fields as well as fruits and vegetables.
Recently the use of this compounds became very controversial as they were pointed as one cause of the honeybees colony collapse disorder. Since pollination is essential for agriculture, extensive studies have been conducted to evaluate the impact of neonicotinoids on bee health. Following this the EFSA limited the use of thiamethoxam, clothianidin and imidacloprid. Fipronil, a pesticide from a different chemical class, has been also banned by EFSA for maize seed treatment due to its high risk for honeybee health.
In order to better understand the effect of these compounds on bees and their contamination in pollen and honey, a highly sensitive assay method was necessary.

2. Methods and Materials

Standards and Reagents

All analytical standards were provided by Sigma-Aldrich. Acetamiprid, chlothianidin, thiamethoxam, imidacloprid, thiacloprid, dinotefuran, nitenpyram and fipronil were selected as target compounds. Acetamiprid-n-desmethyl and fipronil sulfone were selected as metabolites (see figure 1). Thiamethoxam-d3, imidacloprid-d4 and chlothianidin-d3 were used as internal standards.
Solvents (including water and mobile phase additives) were of ULC/MS quality (Biosolve).

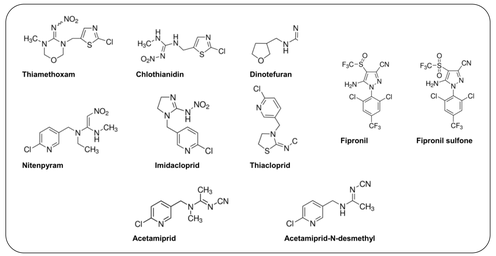


Figure 1 Structure of the target compounds

Sample Preparation

Compound extraction was performed using a QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method with an additional dispersive Solid Phase Extraction (dSPE) step.
5 g of honey (±1%) were weighted in a 50 mL polypropylene tube. 5 µL of internal standard solution at 5µg/mL of each compound in acetonitrile was added on honey and let dry for 10 minutes. 10 mL of ultra pure water were added and the samples were homogenized by vortex mixing for 1 minute. 10 mL of acetonitrile were then added followed by vortex mixing for 1 minute.
Salts mix (4g MgSO4, 1g Sodium Citrate, 0.5g Sodium Citrate sesquihydrate, 1g NaCl ; Biotage Q0020-15V) were added. After manual shaking, samples were centrifuged at 3000g for 5 minutes at 10ºC.
Supernatant (6 mL) was transferred into a 15 mL tube containing 1200 mg of MgSO4, 400 mg PSA and 400 mg C18 (Biotage Q0050-15V). After centrifugation at 3000 g and 10ºC for 5 minutes the supernatant was transferred into inert glass vial for analysis (Shimadzu LabTotal 227-34001-01).

UHPLC-MS/MS Conditions

Analysis were performed using a Nexera X2 UHPLC system coupled with LCMS-8060 with Heated ESI in positive ionization.
Mobile phase composition was optimized to generate the highest sensitivity. Ion source parameters (gas flows, temperatures) were also optimized using the Interface Setting Support Software (Shimadzu Corp.). All parameters are shown in tables 1 and 2.

Table 1 UHPLC conditions

Parameter	Value
System	Shimadzu Nexera X2
Column	ACE SuperC18 100 x 2.1mm 2µm
Column Temperature	30°C
Mobile phases	A: Water + 0.05% ammonia B: Methanol + 0.05% ammonia
Flow rate	0.6 mL/min
Gradient	5% B to 100%B in 3 min. 100%B to 5% in 0.1min. Total run time 4 min.
Injection volume	2 µL (POISe mode with 10 µL of water)

Table 2 MS/MS conditions

Parameter	Value				
System	Shimadzu LCMS-8060				
Ionization mode	Positive and negative HESI (polarity switching 5 msec)				
Acquisition mode	MRM				
MRM transitions	Name	Polarity	MRM Quan	MRM Qual	ISTD Group
	Acetamiprid	+	223.1 > 126.0	223.1 > 56.1	2
	Acetamiprid-N-desmethyl	+	209.1 > 126.0	211.1 > 128.0	2
	Clothianidin	+	250.1 > 169.1	250.1 > 132.0	3
	Dinotefuran	+	203 .0 > 114.0	203.0 87.0	1
	Fipronil	-	435.0 330.0	435.0 > 250.0	3
	Fipronil sulfone	-	451.0 > 415.0	451.0 > 282.0	3
	Imidacloprid	+	256.1 > 175.1	258.1 > 211.1	2
	Nitenpyram	+	271.0 > 126.0	271.0 > 225.0	3
	Thiacloprid	+	253.1 > 126	253.1 > 90.1	1
	Thiamethoxam	+	292.1 > 211.1	292.1 > 181.1	1
	Thiamethoxam-D3	+	295.1 > 214.05	---	1
	Imidacloprid-D4	+	260.1 > 179.1	---	2
	Clothianidin-D3	+	253.1 > 132.05	---	3
Dwell time	3 to 34 msec depending upon the number of concomitant transitions to ensure to have at least 30 points per peak (max total loop time 140 msec).				
Pause time	1 msec.				
Quadrupole resolution	Q1: Unit	Q3: Unit			
Temperature	HESI: 400°C	DL: 200°C	Heater block: 400°C		
Gas flow	Interface: 10 L/min	Nebulizer: 3 L/min	Drying: 5 L/min		

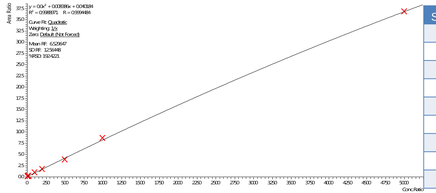
3. Results

Calibration

Calibration curves were prepared in acetonitrile to obtain final concentrations ranging from 0.5 pg/mL (1 fg on column) to 5 ng/mL. These concentrations corresponds to 1 ppt and 10 ppb in honey, respectively. For each compound, the lower limit of quantification was selected to give an accuracy between 80-120% (see table 3). A typical calibration curve is shown in figure 2.

Table 3 limits of quantification

Compound	LOQ (µg/kg) / (pg on column)	Compound	LOQ (µg/kg) / pg on column
Acetamiprid	0.005 / 10	Fipronil sulfone	0.001 / 2
Acetamiprid-N-desmethyl	0.005 / 10	Imidacloprid	0.020 / 40
Chlothianidin	0.020 / 40	Nitenpyram	0.020 / 40
Dinotefuran	0.010 / 20	Thiacloprid	0.005 / 10
Fipronil	0.001 / 2	Thiamethoxam	0.005 / 10



Standard (µg/kg)	Accuracy (%)
0.005	106
0.010	97.2
0.020	95.6
0.100	107
0.200	98.4
0.500	91.5
1.000	104
5.000	99.9
10.000	100

Figure 2 Calibration curve of Acetamiprid

Recovery

An “all-flowers” honey from the local supermarket was extracted with or without spike at 50 ppt. A blank extract (no honey) was prepared to evaluate losses or non specific interactions. Results are presented in Table 4.
No loss due to non specific interactions was observed. Calculated recoveries are within acceptance values 70-120% from EU SANTE/11945/2015.

Table 4 Recovery results

Compound	Blank recovery	Honey recovery	Compound	Blank recovery	Honey recovery
Acetamiprid	101%	78.8%	Fipronil sulfone	101%	74.2%
Acetamiprid-N-desmethyl	100%	93.4%	Imidacloprid	100%	83.2%
Chlothianidin	99.5%	70.6%	Nitenpyram	102%	87.0%
Dinotefuran	100%	76.6%	Thiacloprid	99.2%	82.2%
Fipronil	99.0%	78.1%	Thiamethoxam	100%	75.6%

Real Sample Analysis

Nine honey samples purchased at the local supermarket were assayed as unknowns. Thanks to the very high sensitivity reached, even low concentrations of neonicotinoids were quantified. Results are presented in table 5.

Table 5 Honey sample results (concentrations in µg/kg)

Honey	Acetamiprid	Clothianidin	Imidacloprid	Thiacloprid	Thiamethoxam
Provence creamy	---	---	0.20	---	0.010
Italy creamy	0.15	---	0.17	---	---
Pyrenees liquid	0.38	---	0.043	0.020	---
French-Spanish creamy	0.27	---	0.047	0.020	---
Thyme liquid	---	---	---	---	---
Lemon tree creamy	1.7	---	0.15	0.033	---
Orange tree liquid	1.2	---	0.62	---	---
Flowers creamy	0.14	---	0.055	0.39	---
Flowers liquid	0.34	---	0.11	0.010	---
Honey	**Dinotefuran**	**Nitenpyram**	**Acetamiprid-N-desmethyl**	**Fipronil**	**Fipronil sulfone**
Provence creamy	---	0.052	0.005	---	---
Italy creamy	---	0.040	---	----	---
Pyrenees liquid	---	---	0.015	0.004	---
French-Spanish creamy	---	0.032	---	---	---
Thyme liquid	---	---	---	---	---
Lemon tree creamy	---	---	0.020	---	---
Orange tree liquid	---	0.024	0.018	---	---
Flowers cremy	---	---	0.016	---	---
Flowers liquid	---	---	0.006	---	---

Chromatograms

Calibration standard at the lowest limit of quantification (see table 3 for concentration values).

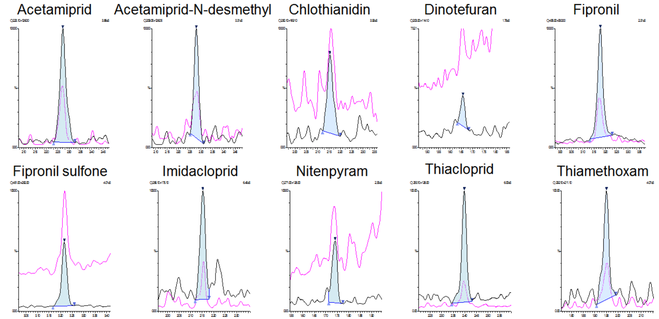


Figure 3 Standard chromatogram at the LOQ

4. Conclusions

A method for ultra sensitive assay of neonicotinoids in honey was set up. The sample preparation was simple but provided excellent recoveries, whatever is the honey type. The injection mode used prevented the use of tedious evaporation/reconstitution or dilution steps. The sensitivity obtained enabled assay in real samples at very low levels far under the regulated residue levels. This method can be a very efficient support tool to better understand the impact of neonicotinoids on honey bee colonies.