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Methods

Determination of neonicotinoid residues in nectar by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

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Nectar is a sweet liquid produced by the nectaries of plants. It is the primary source of energy for bees. Melliferous plants visited by pollinators can contain pesticide residues as the result of plant protection treatment or environmental contamination (soil, water or air). Bees can thus come into contact with these residues via the contaminated nectar that they take back to the colony. The laboratory has therefore developed a method for assaying residues of neonicotinoids in nectar to help establish the implication of these insecticides in cases of the weakening of bee colonies.

Principle of the method

The pesticides studied (imidacloprid, clothianidin, acetamiprid, thiacloprid, thiamethoxam and dinotefuran) belong to the neonicotinoid family (**Figure 1**), which are chemical substances used in agriculture (**Table 1**) either for coating seeds or as a foliar spray on crops. They are systemic molecules which can subsequently be found in the plants and the different environmental compartments. It should be noted that these substances have sufficient remanence in soil (Goulson, 2013) for that plants grown the following year even without treatment, including weeds, assimilate them. The nectar secreted by plants can therefore be a good indicator of contamination by these residues (Dively and Kamel, 2012; Stoner and Eitzer, 2012) as it is one of the main vectors for the contamination of foraging bees and their colonies. When a forager returns to its hive, it regurgitates the nectar from its honey stomach into cells in the comb. A bee can transport up to 75 mg of nectar in its honey stomach.

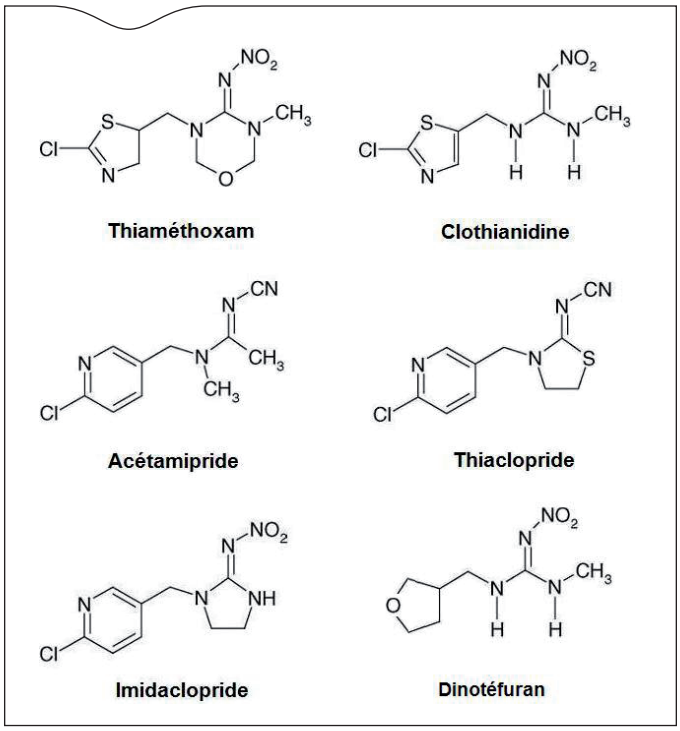

Figure 1: Formulae of the pesticides studied.

When analysed, nectar is found to be a matrix consisting essentially of water and sugars (fructose, glucose and, in much lower quantities, complex sugars such as sucrose). The water content of nectar varies considerably, from 20 to 95%, depending on the species of nectar-producing plant and on environmental, especially meteorological, factors (air humidity, temperature, etc.). The composition in sugars also varies, depending on plant species (Nicolson and Thornburg, 2007). It remains relatively stable for a given species or even for a given family. Depending on the nature and proportions of the sugars, plants can be divided into those where sucrose is dominant in the nectar, those where the quantity of sucrose equals that of glucose and fructose (white clover) and those in which glucose and fructose are dominant (colza) (Kevan and Shuel, 1991). The ratio between glucose and fructose is also usually stable in a given species. For example, in colza, there is a higher level of glucose than of fructose, which can cause a rapid crystallisation.

The method for assaying these six insecticides, which are toxic for bees (**Table 2**), is based on extraction by dissolution. The nectar sample obtained is diluted in ultra pure water for injection and analysis by liquid chromatography coupled with tandem mass spectrometry (LC-ESI-MS/MS). This method for multi-residue analysis enables quantification and identification of neonicotinoid residues in the “nectar” matrix. The limit of quantification (LOQ) is 0.3 pg/µl for all the pesticides with the exception of dinotefuran for which the LOQ is 0.6 pg/µl.

Equipment and reagents

The specific equipment consists of (1) a propipette for extracting nectar samples from micro-capillaries; (2) a centrifuge (Centrifuge 5810R, Eppendorf); (3) an HPLC instrument (liquid chromatography) with an autosampler and a column compartment thermostatted (UltiMate 3000, Thermo Scientific) coupled with a TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific) equipped with HESI-II probe (Heated Electrospray Ionization Source).

For the analysis by LC-MS/MS, LC-MS grade methanol and formic acid (98%) were used. The measurement standards were prepared using certified active substances purchased from CIL Cluzeau Info Labo: imidacloprid (98% purity), clothianidin (99.5%), acetamiprid (99%), thiacloprid (99.5%), thiamethoxam

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Table 1: The uses of neonicotinoids in agriculture (AGRITOX, 2013; Index phytosanitaire, 2013; Mitsui Chemicals America, 2013)

Pesticide	Solubility in water (g/l)	Type of application	Crops treated	Commercial brand names
Imidacloprid*	0.613	Treating seeds and plants	Beetroot, oats, wheat, barley, rye	Ferial Gaucho 350 Imprimo Nuprid 70
		Treating aboveground parts	Apricot, peach, plum, rose, forest conifers	Confidor Merit Forest Nuprid 200
Clothianidin*	0.304	Treating aboveground parts	Maize, sorghum, apple	Cheyenne Dantop 50 WG
Acetamiprid	2.95	Treating aboveground parts	Fruit trees *(apricot, citrus, cherry, fig, peach, pear, apple, plum)*, field crops *(potato, oil-bearing crucifers, oats, wheat)*, vegetable crops *(asparagus, aubergine, cabbage, cucumber, courgette, lettuce, parsley, sweet pepper, tomato, beetroot)*, roses, various flower crops, crops grown for seed	Suprême Suprême 20SG Polysect Ultra
Thiacloprid	0.186	Treating aboveground parts	Fruit trees *(apricot, gooseberry, almond, black currant, cherry, chestnut, fig, raspberry and other Rubus, hazel, walnut, olive, peach, pear, apple, plum)*, field crops *(colza, mustard, potato)*, crops grown for seed	Biscaya Calypso Ecail Proteus
		Treating the soil	Ornamental trees and shrubs, various flower crops	Exemptor
Thiamethoxam*	4.1	Treating seed and plants	Beetroot, maize, pea	Cruiser 350 Cruiser FS Cruiser SB
		Treating aboveground parts	Potato, apple, aubergine, cucumber, lettuce, pepper, sweet pepper, tomato, ornamental trees and shrubs, chrysanthemum, various flower crops, rose, all floral species (under glass)	Actara Flagship Pro
		Treating the soil	Ornamental trees and shrubs, various flowering crops (under glass), rose (under glass)	Flagship Pro
Dinotefuran**	39.83	Treating aboveground parts	Rice, cabbage, lettuce, sweet pepper, tomato, cucumber, melon, celery, citrus, apple, peach, potato, cotton	Safari 20SG Safari 2G

* In April 2013, the European Union announced that it would suspend the use of imidacloprid, clothianidin and thiamethoxam on four field crops (maize, colza, sunflower and cotton) for two years, with effect from 1 December.
** Dinotefuran is prohibited in Europe on all crops.

Table 2: Toxicity of the pesticides studied on bees (AGRITOX, 2013, EPA, 2004)

Pesticide	LD_{50} (contact)	LD_{50} (oral)
Imidacloprid	81 ng/bee	3.7 ng/bee
Clothianidin	44.26 ng/bee	3.79 ng/bee
Acetamiprid	8.09 µg/bee	14.53 µg/bee
Thiacloprid	38.82 µg/bee	17.32 µg/bee
Thiamethoxam	24 ng/bee	5 ng/bee
Dinotefuran	47 ng/bee	23 ng/bee

(99%) and dinotefuran (99%). The certified dimethoate-D6 solution (99.8% purity, 100 mg/l in acetone) also came from CIL Cluzeau Info Labo.

Procedure

1. Extraction

Nectar samples were extracted from flowers by capillary action using a micro-capillary tube (5 µl). The samples were then extracted from the micro-capillary tube using a propipette. In a microvial were added ultra pure water, 10 µl of the internal standard (dimethoate-D6) and then, 10 µl of the nectar sample. The nectar sample was then homogenised using a vortex and centrifuged at 500 rpm for five minutes. The volume of the final extract was 100 µl.

2. Measurement

2.1. High-performance liquid chromatography (HPLC)

Chromatographic separation was carried out on a Pursuit PFP (pentafluorophenyl) analytical column 100 x 3 mm (3 µm) (Agilent). The mobile phase consisted of ultra pure water (A) and methanol (B), each solution being acidified with 0.02% of formic acid. The insecticides were separated by gradient elution, with the following protocol: linear gradient from 80% A (at t=0 min) to 0% (at t=13 min), then a linear gradient of 0% A (at t=13 min) to 80% (at t=13.5 min) and holding at 80% A for 4.5 min. The column and autosampler temperature was 25°C, the flow rate was 0.4 ml/min and the injection volume was 15 µl.

2.2. Mass spectrometry

Positive mode electrospray (HESI-II +) was used as the source of ionisation. The divert valve was set to allow the admission of the mobile phase in the source between 2.50 min and 12 min. The mass analyser was a TSQ Vantage triple stage quadrupole and the collision gas was argon. The acquisition mode used was the SRM mode (Selected Reaction Monitoring). Transitions and retention times (indicative only) are given in **Table 3**.

Results and conclusion

For assaying, calibration was performed using a range extracted from the "nectar" matrix (blank and fortified samples). Like blank nectar is not always available, a representative sugars solution of a nectar was prepared for this calibration. This

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Table 3: Transitions of the pesticides studied and retention times (indicative only)

Pesticide	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	Collision energy (V)	S-Lens
Dinotefuran	3.49	203.0	114.1 129.1	13 13	43 43
Thiamethoxam	4.92	292.0	211.0 181.0	13 24	57 57
Imidacloprid	6.09	256.0	209.1 175.1	18 20	65 65
Clothianidin	6.30	250.0	169.1 131.9	15 19	54 54
Dimethoate-D6	6.39	236.0	177.1 131.0	16 22	43 43
Acetamiprid	7.12	223.0	126.0 90.0	21 34	53 53
Thiacloprid	8.03	253.0	126.0 90.0	22 39	71 71

solution with 36% sugars (w/v) was made with a mixture of 6 g of glucose and 3 g of fructose in 25 ml of ultra pure water. The linear range is defined as being the calibration range and has been validated up to 15 pg/µl for each pesticide.

The limits of detection (LOD) and quantification (LOQ) were respectively 0.1 pg/µl and 0.3 pg/µl for imidacloprid, clothianidin, thiacloprid and thiamethoxam. For dinotefuran, LOD and LOQ were respectively 0.2 pg/µl and 0.6 pg/µl (**Figure 2**).

In the absence of reference material, accuracy was estimated by the rate of recovery, determined using a control sample (blank matrix) spiked with analytes assayed at three different concentrations (LOQ, 5LOQ and 10LOQ). For each concentration, three samples of sugars solutions were extracted and analysed. For the method validation, five series of three samples were processed for each spiking level. The mean recoveries obtained were satisfactory as they were between 98.9% and 110.2% at the LOQ, and between 93.0% and 96.6% and between 92.6% and 99.7% for the samples spiked at 5LOQ and 10LOQ respectively (V03-110 Normalisation). The method is repeatable because the relative standard deviation (RSDr) is ≤ 20% for each concentration. The method is also reproducible (RSDR ≤ 22%) for all the pesticides studied (**Table 4**).

It can therefore be stated that this method enables the quantification of residues at very low levels and can thus be applied to samples of nectar extracted directly from flowers (**Figure 3**) or from the honey stomachs of bees in order to monitor the exposure of foragers to environmental contaminants.

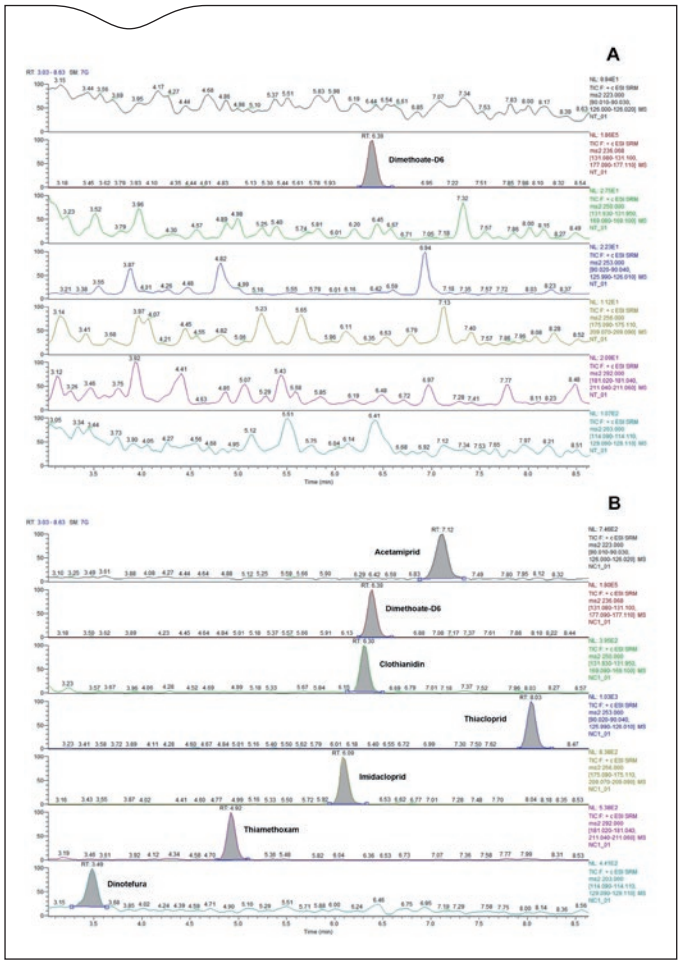

Figure 2: Chromatograms obtained by LC-MS/MS for (A) the blank sample (36% sugar solution) and for (B) the sample fortified with pesticides at the LOQ.

Table 4: Validation data of the method (AFNOR Normalisation V03-110)

Pesticide	Representative sugars solution of a nectar														
	1st fortification level (n=3, repeated 5 times)					2nd fortification level (n=3, repeated 5 times)					3rd fortification level (n=3, repeated 5 times)				
	C (pg/µl)	Mean recoveries (%)	RSD_r (%)	RSD_R (%)	Uncertainty (%)	C (pg/µl)	Mean recoveries (%)	RSD_r (%)	RSD_R (%)	Uncertainty (%)	C (pg/µl)	Mean recoveries (%)	RSD_r (%)	RSD_R (%)	Uncertainty (%)
Imidacloprid	0.3	106.7	8.6	12.5	26.6	1.5	95.8	6.6	8.2	17.4	3.0	98.3	4.5	6.6	14.0
Clothianidin	0.3	99.8	7.5	11.5	24.6	1.5	95.2	6.7	8.4	17.7	3.0	97.3	5.1	5.9	12.4
Acetamiprid	0.3	106.8	7.3	9.0	18.9	1.5	96.5	6.1	7.0	14.7	3.0	98.7	5.2	6.8	14.5
Thiacloprid	0.3	110.2	6.4	9.2	19.5	1.5	96.6	6.0	7.4	15.6	3.0	99.7	5.2	6.9	14.7
Thiamethoxam	0.3	98.9	9.4	16.3	35.1	1.5	93.0	7.4	10.4	22.2	3.0	92.6	5.5	9.6	20.7
Dinotefuran	0.6	105.5	7.4	14.0	30.2	3.0	93.5	6.5	9.7	20.7	6.0	94.7	5.1	7.0	15.0

C: pesticide concentration, RSD_r: repeatability, RSD_R: reproducibility

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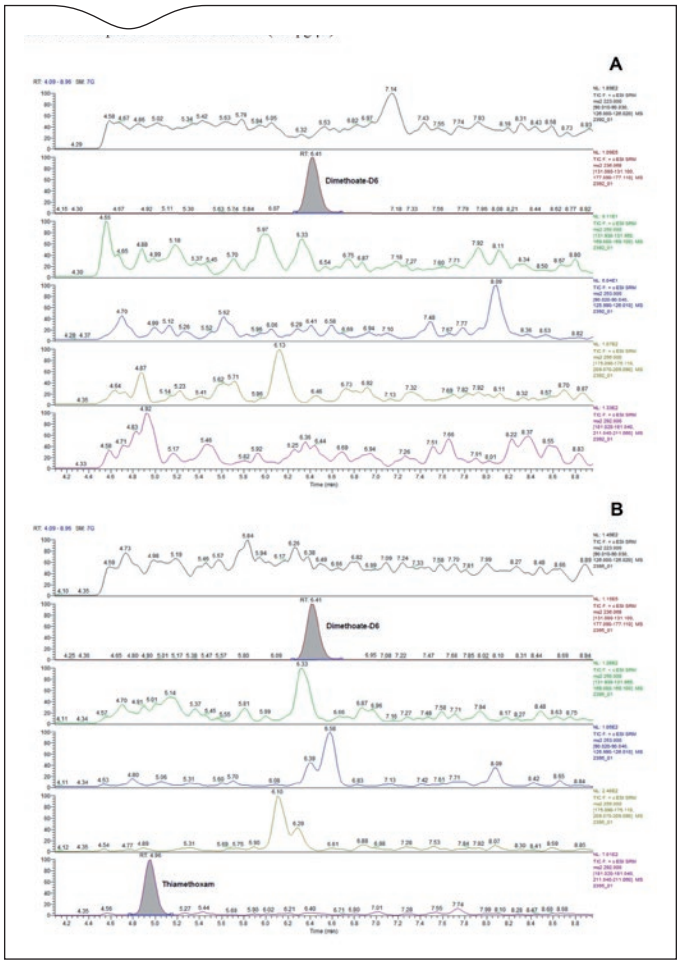

Figure 3: Chromatograms obtained by LC-MS/MS for (A) a blank sample of nectar of colza and for (B) a nectar of colza positive in thiamethoxam (0.5 pg/µl).

Acknowledgements

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References

AGRITOX, 2013, *Base de données sur les substances actives phytopharmaceutiques*. [consulted in 2013] http://www.agritox.anses.fr/php/fiches.php

Index phytosanitaire, 2013. *ACTA*, 49ème édition, 984 p.

Mitsui Chemicals America, Inc.: Dinotefuran Field Performance [consulted in 2013] http://www.mitsuichemicals.com/dinotefuran-field-performance.htm

Goulson D., 2013. An overview of the environmental risks posed by neonicotinoid insecticides. *Journal of Applied Ecology*, 50(4): 977-987.

Dively GP, Kamel A., 2012. Insecticide residues in pollen and nectar of a cucurbit crop and their potential exposure to pollinators. *Journal of Agricultural and Food Chemistry*, 60(18): 4449-56.

Stoner KA, Eitzer BD., 2012. Movement of soil-applied imidacloprid and thiamethoxam into nectar and pollen of squash (*Cucurbita pepo*), *PLoS ONE*, 7(6): e39114.

Nicolson SW, Thornburg RW. Nectar Chemistry. 2007 [consulted in 2013] http://www.bb.iastate.edu/~thorn/www/Publications/pdfFiles/12_Nectar_Chemistry(Proof).pdf

Kevan PG, Lee H, Shuel RW., 1991. Sugar ratios in nectars of varieties of canola (*Brassica napus*), *Journal of Apicultural Research*, 30(2): 99-102.

EPA, Pesticide Fact Sheet: Name of Chemical: Dinotefuran - Reason for Issuance: Conditional Registration - Year Issued: September 2004, United States Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances (7501C). [consulted in 2013] http://www.epa.gov/opp00001/chem_search/reg_actions/registration/fs_PC-044312_01-Sep-04.pdf

French normalisation. *Analyse des produits agricoles et alimentaires : protocole de caractérisation en vue de la validation d'une méthode d'analyse quantitative par construction du profil d'exactitude,* AFNOR V03-110 Standard (May 2010).