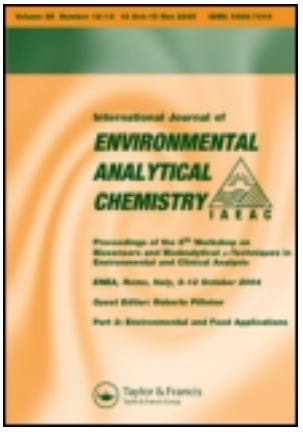

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Validation of a highly sensitive method for the determination of neonicotinoid insecticides residues in honeybees by liquid chromatography with electrospray tandem mass spectrometry

Anne-Claire Martel [a] & Cosette Lair [a]

[a] Agence Française de Sécurité Sanitaire des Aliments (AFSSA) Site de Sophia Antipolis, Laboratoire d'Études et de Recherches sur les Ruminants et les Abeilles, Unité de Pathologie de l'Abeille, 105 route des Chappes, F-06902 Sophia Antipolis Cedex, France

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Agence Française de Sécurité Sanitaire des Aliments (AFSSA) Site de Sophia Antipolis, Laboratoire d'Études et de Recherches sur les Ruminants et les Abeilles, Unité de Pathologie de l'Abeille, 105 route des Chappes, F-06902 Sophia Antipolis Cedex, France

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The validation of a multi-residue method for the determination of five neonicotinoid insecticides (imidacloprid, clothianidin, acetamiprid, thiacloprid and thiamethoxam) in honeybees is described. The method involves the extraction of pesticides using acetonitrile and liquid partitioning with *n*-hexane. One clean-up is then performed on a florisil cartridge (1 g, 6 mL) and the extract is analysed by liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS). The recovery data were obtained by spiking honeybees samples free of pesticides at two concentration levels of the various neonicotinoids. The recoveries were in the range between 93.3 and 104.0% with relative standard deviation (RSD) less than 20%. The limit of quantification (LOQ) was 0.5 ng g^{-1} (corresponding to 0.05 ng bee^{-1}) for all pesticides except for acetamiprid which was 1 ng g^{-1} (corresponding to 0.1 ng bee^{-1}).

Keywords: honeybees; neonicotinoid; pesticides; residues; LC-ESI-MS/MS

1. Introduction

The use of pesticides in agricultural and plant protection practices could cause extensive pollution of the environment and constitutes a potential risk for human health. Honeybees have demonstrated to be excellent bioindicators of the pesticides used in a wide agricultural area [1] and play an important ecological role due to their involvement in pollination of plants. Honeybees come in contact with pesticides in several ways: after beehive treatment against *varroasis* [2] or with contaminated flowering field crops [3]. Worker bees gathering nectar, water and pollen may be directly subjected to the action of pesticides or they may carry pesticide-contaminated pollen back to the hive and expose other honeybees. Because of this, pesticide residues found in bees reflect the type of pesticides applied in the cultivated fields that surround their hives and can be used to evaluate environmental contamination with pesticides.

A number of insecticides registered [4] or forbidden are classified as dangerous or harmful to honeybees [5,6]. The neonicotinoids are a new insecticide class which includes the commercial products imidacloprid, clothianidin, acetamiprid, thiacloprid and thiamethoxam (Figure 1). Neonicotinoid insecticides represent one of the fastest growing

*Corresponding author. Email: ac.martel@afssa.fr

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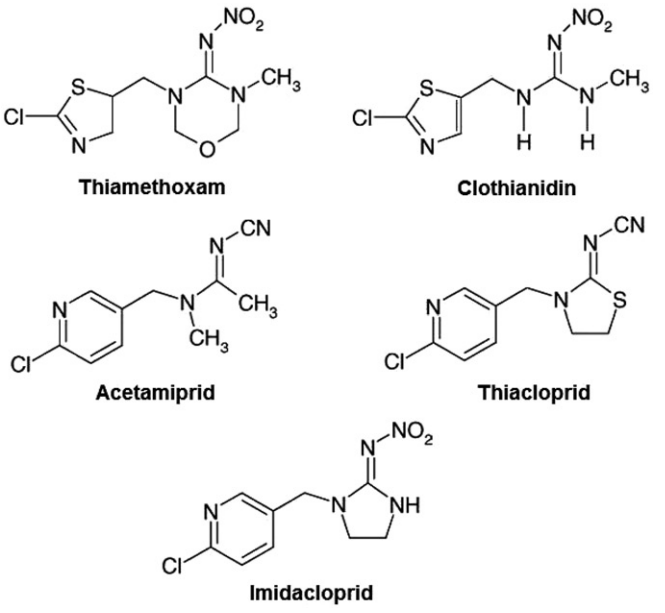

Figure 1. Names and structures of the five neonicotinoids analysed.

Table 1. Toxicity of studied pesticides to honeybees.

Pesticide	LD_{50} (contact)	LD_{50} (oral)
Thiamethoxam	24 ng bee^{-1} [6,9]	5 ng bee^{-1} [9]
Imidacloprid	81 ng bee^{-1} [4]	3.7 ng bee^{-1} [4,6]
Clothianidin	22 ng bee^{-1} [8]	4 ng bee^{-1} [6]
Acetamiprid	8.09 µg bee^{-1} [4,6]	14.53 µg bee^{-1} [4]
Thiacloprid	38.82 µg bee^{-1} [4]	17.32 µg bee^{-1} [4,6]

classes of insecticides introduced to the market since the launch of pyrethroids. Their physicochemical properties make them useful for a wide range of application techniques including foliar, seed treatment, soil drench and stem application. Due to the excellent plant-mobile (systemic) property conferred by the moderate water solubility, the neonicotinoids are active against numerous sucking and biting pest insects, including aphids, leafhoppers, whiteflies, beetles and some *lepidoptera* species as well [7]. They act on the insect nicotinic acetylcholine receptor (nAChR). For honeybees, they are highly toxic (imidacloprid, clothianidin and thiamethoxam) to moderately toxic (acetamiprid and thiacloprid) (Table 1).

This was the main reason for developing a simple, sensitive and reliable method for determining neonicotinoid insecticides in honeybee samples at low concentration levels for studies of environmental pollution. Some methods for the determination of neonicotinoid residues in vegetables have been presented in the literature [10–12], but no method has been published for simultaneous determination of residues of neonicotinoid insecticides in honeybees. A few analytical methods for other classes of pesticides

(pyrethroids, carbamates and organophosphorus) determination in honeybees have been published in the last few years either by GC [13–15] and GC-MS/MS [16] or with LC/MS [17,18] based on liquid/liquid extraction, solid-phase extraction (SPE) and the matrix solid-phase dispersion (MSPD) [19]. In the last few years, a tendency towards the use of more polar pesticides like neonicotinoids rather than non-polar compounds is observed. Coupling of liquid chromatography (LC) with tandem mass spectrometry detection (MS/MS) is becoming one of most powerful techniques for the residue analysis of polar, ionic or low volatility pesticides [20–25]. Furthermore, analytical methodologies employed must be capable of residue measurement at very low levels and must also provide unambiguous evidence to confirm both the identity and the quantity of any residues detected. LC is very effective in separating analytes, while MS allows their identification and confirmation at trace levels.

The aim of the present work is to develop a rapid, sensitive and accurate LC-MS/MS method for determining of multi neonicotinoid residues in honeybees samples using commonly available LC-ESI-MS/MS instrumentation. Finally, the proposed procedure was validated according to the standard European guidelines [26,27].

2. Experimental

2.1 *Materials and chemicals*

An evaporation system TurboVap II (Caliper LifeSciences, Paris, France) equipped with a water bath and Zymarck tubes (200 mL/1 mL) was used. The T25 ultra-turrax blender used for the homogenisation of samples were from Fisher Scientific Labosi (Elancourt, France).

All solvents used (acetone, acetonitrile, *n*-hexane, petroleum ether and dichloromethane) were of ultra pure for pesticides analysis grades (VWR, Strasbourg, France). LC/MS-grade methanol was obtained from VWR. Laboratory-ultra pure water was used. Formic acid (98%) was supplied from Labosi (Elancourt, France). Strata FL-PR florisil cartridges (1 g, 6 mL) used for clean-up were from Phenomenex (Le Pecq, France). Millex PVDF filter (0.45 μm, 13 mm) from Millipore (St Quentin en Yvelines, France) were used.

Certified pesticide standards for imidacloprid (98% purity), clothianidin (99.5%), acetamiprid (99%), thiacloprid (99.5%) and thiamethoxam (99%) were from CIL Cluzeau Info Labo (Sainte-Foy-La-Grande, France). The certified solution of dimethoate-D6 (99.8% purity, 100 mg L^{-1} in acetone) was also purchased from CIL Cluzeau Info Labo.

Insecticide stock solutions (1000 mg L^{-1}) of individual pesticide standards were prepared by dissolving 25 mg of each analyte in 25 mL of acetone and kept at −18°C. Under these conditions, standard solutions were demonstrated to be stable for one year. A standard multi-component solution was prepared by diluting each primary standard solution with acetone. This solution was used for spiking honeybees to study the linear dynamic range of the LC-MS/MS and to validate the method. The concentration of the standard working solution was 0.01 mg L^{-1} for all pesticides and 0.02 mg L^{-1} for acetamiprid. This solution was stored during six months under refrigerator conditions (+4–8°C). Matrix calibration standards were prepared by adding to honeybees blank samples before the extraction procedure of these samples appropriate volumes of the standard working solution at four different levels.

The internal standard (IS) stock solution was prepared by diluting the certified solution of dimethoate-D6 with acetone to obtain a concentration of 10 mg L^{-1}. The IS working

solution for monitoring the quality of the extraction procedure was prepared at 100 μg L^{-1} in acetone from the stock solution of dimethoate-D6. These two solutions were stored under refrigerator conditions (+4–8°C) and are stable for at least six months.

2.2 *Preparation of fortified samples*

Samples from untreated honeybees (*Apis mellifica mellifica* species) taken in hives from the experimental apiary located on the site of the laboratory at Sophia-Antipolis (France) were used as control samples and for the fortification experiments. Samples were stored at −18°C until analysis. Aliquots of 2 g of samples were spiked at two levels of each pesticide, each time using the appropriate volume of the working standard stock solution in acetone. Two fortification levels were tested: the first one was chosen to be equal or near to the limit of quantification (LOQ) and for the second one, the concentrations of all compounds were 5 times higher [26]. Five replicates were realised for each level.

Before the homogenisation of samples, 100 μL of IS solution (dimethoate-D6) at 100 μg L^{-1} were added into all samples of honeybees (control and fortified samples).

2.3 *Extraction procedure*

A sample of bees (2 g; about 20 insects) was weighed in a centrifuge tube (50 mL) and 100 μL of the IS working solution were added. The sample was homogenised with 30 mL of acetonitrile using the T25 ultra-turrax blender. The solution was filtrated under vacuum through Whatman filter paper using a Büchner funnel. The extraction was repeated using 30 mL of acetonitrile. The combined extracts were transferred to a separating funnel (500 mL). The vacuum flask was rinsed with 50 mL of *n*-hexane which were transferred to the separating funnel. The separating funnel was shaken vigorously and the filtrate was allowed to separate into two phases. The acetonitrile phase (inferior) was put back in the vacuum flask and the *n*-hexane phase (superior) was discarded. The operation was repeated once on the acetonitrile phase with 50 mL of *n*-hexane. The acetonitrile phase was collected in a Zymarck tube and was evaporated to dryness at about 40°C in TurboVap II under stream of air. The residue was reconstituted with 2 mL of dichloromethane, vortexed to dissolve the residue and was ready for the clean-up.

2.4 *Clean-up*

The SPE florisil column was conditioned with 10 mL of dichloromethane. Two mL of extract were passed through the column and washed with 20 mL petroleum ether/dichloromethane 80 : 20 (v : v). Then, pesticides were eluted by 20 mL acetonitrile/dichloromethane 95 : 5 (v : v). The eluate was collected and evaporated to approximately 0.2 mL at about 40°C in TurboVap II under stream of air. The volume of extract was adjusted to 1 mL with ultra pure water and was vortexed to dissolve the residue. Extracts were filtered through 0.45 μm Millex (PVDF) filter.

2.5 *High performance liquid chromatography*

The high performance liquid chromatography (HPLC) was performed on a Surveyor HPLC System from ThermoFinnigan (Courtaboeuf, France) equipped with a rheodyne

model number 7739 injector, a 20 µL sample loop, a quaternary LC Pump, a temperature-controlled autosampler and a column oven. The analytical column was a Pursuit PFP (pentafluorophenyl) 100 × 3 mm (3 µm) from Varian (Courtaboeuf, France) which is specifically designed for separation of polar halogenated compounds under standard reversed phase conditions. The mobile phase was water (A) and methanol (B), both acidified with 0.02% formic acid. The insecticides were separated with the following gradient programme: a linear gradient from 80% A at $t = 0$ min to 0% at 13 min; then by a linear gradient from 0% A at $t = 13$ min to 80% to 16 min and maintaining 80% A for 9 min. The column and autosampler temperature was 25°C, the flow-rate was 0.4 mL min^{-1} and the injection volume was 20 µL. The HPLC system was connected to the mass spectrometry (MS). The HPLC eluent was directly introduced into the electrospray ion source only between $t = 4.5$ min and $t = 12$ min using the divert valve.

2.6 *Mass spectrometry operating conditions*

The ESI-MS/MS detection was achieved using TSQ Quantum triple quadrupole mass spectrometer (TSQ Quantum, ThermoFinnigan, USA) equipped with electrospray source interface (ESI) and a motorised divert/inject valve. The ESI source parameters were optimised for all compounds by direct infusion experiments. Flow injection analyses (5 µL) were performed for individual pesticide solutions (1 mg L^{-1}) and for isotope standard in order to obtain the mass spectral data, from which ions were carefully chosen for analysis in the selected reaction monitoring (SRM) mode.

The instrument was operated in positive mode with MS/MS transitions monitored during LC separation in the SRM mode. The operating conditions for ESI were sheath gas (nitrogen) pressure at 40; auxiliary gas (nitrogen) pressure at 20; spray voltage at 4000 V; capillary temperature at 350°C and the collision gas (Argon) at 1 mTorr. Analyte MS/MS transitions and instrument conditions are presented in Table 2. A scan time of 50 ms per transition was used.

The internal standard method of calibration was used for this analysis. One blank sample of honeybees and four honeybees samples spiked with all analytes at different levels

Table 2. Ions monitored under the SRM mode by LC-MS/MS (positive ionization).

Pesticide	Retention time (min)	Precursor ion (m/z)	Monitored ions (m/z)	Collision energy (V)
Thiamethoxam	6.58	291.9	210.9	20
			180.9	31
Imidacloprid	8.29	256.2	208.9	22
			175.0	23
Dimethoate-D6	8.52	236.0	177.1	17
			131.0	32
Clothianidin	8.63	250.0	169.0	18
			131.9	19
Acetamiprid	9.57	222.9	125.9	28
			99.1	43
Thiacloprid	10.70	252.9	126.0	28
			90.1	44

were analysed by LC-ESI-MS/MS in SRM mode followed by detection of the signal of the more abundant ions between $t = 4.5$ min and $t = 12$ min using the divert valve. Calibration curves were obtained by plotting area ratios against concentrations of analytes injected and were used for quantification. Instrument control, data acquisition and integration of the analytes' peaks were performed using Xcalibur and LCquan softwares (ThermoFinnigan).

3. Results and discussion

3.1 *Sample preparation*

The determination of pesticide residues in a complex matrix such as bees requires extraction of residues from the matrix and cleaning of the extracts before chromatographic detection. In the case of honeybee samples, the major problem is the presence of amounts of interfering waxes [28]. The liquid-liquid extraction with *n*-hexane proved sufficient to remove non-polar waxes. Furthermore, a clean-up on the florisil column with solvents of increasing polarity gave eluate free of chromatographic interferences. The selection of solvent systems for SPE purification was based on experiments where the florisil cartridge was rinsed with petroleum ether/dichloromethane (80 : 20) and acetonitrile/dichloromethane (95 : 5). The fractions were first collected and analysed separately under the same chromatographic conditions. All neonicotinoid residues were recovered quantitatively with good repeatability in the second fraction. Then, the first eluate with petroleum ether/dichloromethane (80 : 20) mixture was discarded and the second one with acetonitrile/dichloromethane (95 : 5) was collected for analysis.

3.2 *LC-MS/MS determination*

A gradient system (water and methanol, both acidified with 0.02% formic acid) was applied to separate five pesticides as independent peaks. Retention times (t_R) were determined individually and are presented in Table 2. The ratio of the chromatographic retention time of the analyte to that the IS (dimethoate-D6), i.e. the relative retention time of the analyte, should correspond to that of the calibration extract with a tolerance of ±2.5%. The order for injecting the extracts into the analytical instrument is as follows: reagent blank (ultra pure water), the control sample (unfortified sample), the fortified samples used for calibration, the control sample, sample(s) to be determined, a fortified sample and finally, the control sample.

Two transitions were investigated for each pesticide for identification/quantification. The signal-to-noise ratio for each diagnostic ion shall be ≥3 : 1. Figure 2 shows chromatograms of honeybees sample unspiked and spiked at the LOQ (0.1 ng bee^{-1} for acetamiprid and 0.05 ng bee^{-1} for all other pesticides). The LC-MS/MS chromatogram of control honeybees extract shows good baseline stability with no interfering peaks, indicating that the proposed clean-up is suitable for the determination of the target analytes.

Co-eluting, undetected matrix component could inhibit or enhance the analyte signal. Matrix effects can be tested as a ratio of analyte response in matrix-matched standard (analyte was spiked after extraction into the final extract) to its response in solvent [29]. The matrix effect was expressed as the ratio of the mean peak area ($n = 5$) of an analyte and the IS spiked post-extraction to the mean peak area ($n = 5$) of the same analyte and the

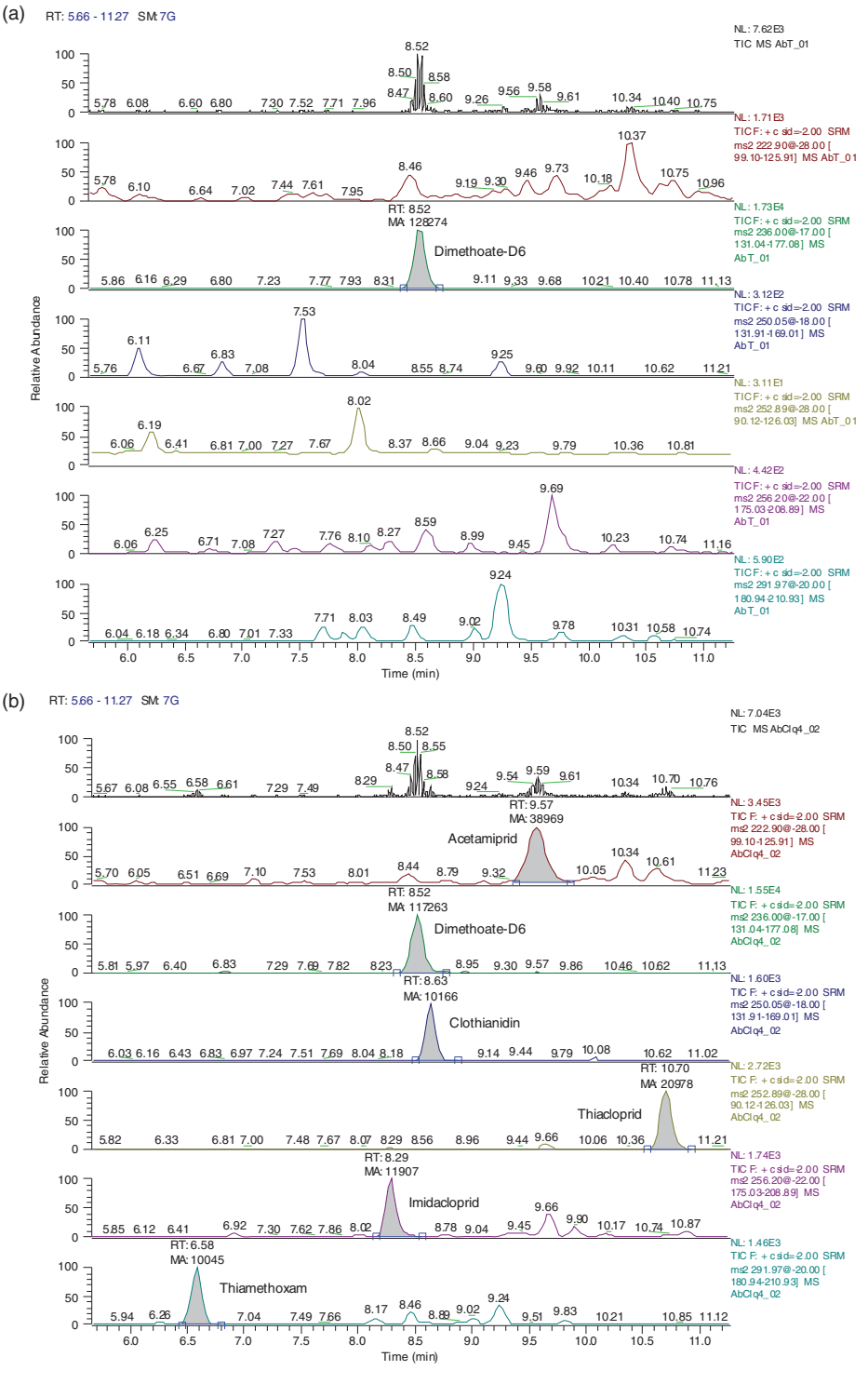

Figure 2. LC-MS/MS chromatograms obtained from (A) blank honeybees sample and (B) fortified honeybees sample at LOQ.

Table 3. Matrix effect and recoveries of the extraction procedure.

	Matrix effect			Recovery (%)		
Pesticide	LOQ	2LOQ	5LOQ	LOQ	2LOQ	5LOQ
Thiamethoxam	99.6	124.3	98.9	103.4	79.1	92.6
Imidacloprid	115.2	119.2	92.7	81.9	75.4	98.8
Clothianidin	107.2	96.0	91.2	82.1	90.5	95.8
Acetamiprid	87.0	86.1	68.9	83.1	99.1	117.7
Thiacloprid	108.3	99.4	85.9	79.0	87.5	106.3

IS standards multiplied by 100. The matrix effect was measured at the LOQ, 2LOQ and 5LOQ and the results revealed variability between the analytes (Table 3). Then, to eliminate a possible effect of the matrix on the response, the quantification of neonicotinoid residues was accomplished by using a calibration curve made from fortified blank samples prepared in the same matrix as the real samples.

3.3 *Method validation*

3.3.1 *Linearity*

The calibration curves were plotted for each pesticide to determine the linearity range and the detection and quantification limits. The linearity of the calibration curves was studied including the origin point that corresponded to the control sample (unfortified honeybee sample) and the LOQ. The curves were constructed from peak area ratios of pesticides to IS versus pesticide concentrations. Good linearity of the response was found for all pesticides in the range 1.6 to 40 $\mu g\,L^{-1}$ for acetamiprid and 0.8 to 20 $\mu g\,L^{-1}$ for all other pesticides, with linear correlation coefficients higher than 0.99.

3.3.2 *Limits of detection and quantification*

The sensitivity was estimated by the limits of quantification. The limits of detection (LODs) and quantification (LOQs) were calculated from the regression data. This approach consists of using the dispersion characteristics of the regression line of the chromatographic peak area ratio against concentration. When the dispersion characteristics have been calculated, the standard deviation of the blank is estimated either by the regression residual standard deviation or by the standard deviation of the intercept. LOD corresponds to the analyte level for which the area is equal to three times the chosen standard deviation and LOQ corresponds to the analyte level for which the area is equal to ten times the chosen standard deviation [27]. The standard deviation chosen to calculate the LODs and LOQs is the residual standard deviation of the regression line for all pesticides in the matrix. The LODs and LOQs were for imidacloprid, clothianidin, thiacloprid and thiamethoxam 0.015 $ng\,bee^{-1}$ (0.15 $ng\,g^{-1}$) and 0.05 $ng\,bee^{-1}$ (0.5 $ng\,g^{-1}$) respectively. The LOD and LOQ were for acetamiprid 0.03 $ng\,bee^{-1}$ (0.3 $ng\,g^{-1}$) and 0.1 $ng\,bee^{-1}$ (1 $ng\,g^{-1}$) respectively. The LODs and LOQs were reported with the "$ng\,bee^{-1}$" unit to allow a direct comparison to the reported LD_{50} values of each pesticide (Table 1). The LOQs calculated were also obtained in practice.

3.3.3 *Specificity*

Blank samples were fortified at different levels with pesticides and were analysed. The specificity was determined from the regression data. The detection was found specific for each pesticide because the slope of each line was found equal to 1 and that the intercept was equal to 0. Furthermore, the checking of each target analyte identity confirmed the absence of interference.

3.3.4 *Recovery*

Recovery experiments, concerning the five pesticides, were carried out, in five repetitions, at two fortification levels, by adding an appropriate volume of the standard mixed solution of pesticide in acetone into blank matrix of bees samples. The samples were analysed according to the proposed method. Blank sample matrix (without analytes) was analysed at the same time with spiked samples. Samples were spiked just before analysis (set 1). The pesticides were extracted and cleaned up by solid-phase extraction as described in the Experimental Section above and the sample solutions were analysed by LC-MS/MS under the optimal conditions described in the Experimental Section. Each analyte was spiked at two different concentrations (LOQ and 5LOQ) and five repetitions were carried out for each fortification level. Mean recoveries of pesticides (Table 4) added at the LOQ level were between 93.3 and 102.7%, while the corresponding values at the higher fortification

Table 4. Recoveries, repeatability (RSD_r) and reproducibility (RSD_R) of the insecticides at 2 levels of fortification for honeybees samples (15 samples per evaluated level of fortification).

	Honeybees							
	1st fortification level ($n = 5$, repeated 3 times)				2nd fortification level ($n = 5$, repeated 3 times)			
Pesticide	C ($ng\,g^{-1}$)	Mean recoveries (%)	RSD_r (%)	RSD_R (%)	C ($ng\,g^{-1}$)	Mean recoveries (%)	RSD_r (%)	RSD_R (%)
Thiamethoxam	0.5	94.7	18.8 11.9 10.6	17.9	2.5	102.2	12.5 9.7 4.1	8.9
Imidacloprid	0.5	93.3	18.0 19.4 9.0	18.3	2.5	95.0	7.5 3.5 8.9	9.8
Clothianidin	0.5	97.8	7.4 9.1 13.1	13.7	2.5	94.7	8.8 8.0 7.3	12.3
Acetamiprid	1	100.8	8.7 7.5 12.0	12.3	5	104.0	4.0 5.2 2.8	4.6
Thiacloprid	0.5	102.7	12.3 9.1 10.3	10.3	2.5	96.1	6.0 6.3 5.2	5.7

Note: C, Pesticide concentration.

level were between 94.7 and 104.0%. Therefore, the calculated values indicate a good accuracy because mean recovery values at each fortification level are within the range 70–120% [27].

A supplementary test was realised at different levels to determine the 'true' recovery value that is not affected by the matrix. This recovery from the extraction procedure was evaluated for each pesticide by comparing the mean peak areas of analyte and IS obtained in set 1 to those in set 2. In set 2, the analytes and the IS were spiked after extraction into honeybees extracts, whereas in set 1, the analytes and the IS were spiked into honeybees before extraction. Results obtained for three different levels tested (LOQ, 2LOQ and 5LOQ) are presented in Table 3 and were similar to the values above. Recoveries from the extraction procedure were ranged between 70 and 120%. The recovery of analytes is measured with each batch of analyses to control extraction procedure of each routine analysis.

3.3.5 *Precision*

The precision of the method was determined by repeatability and reproducibility studies, expressed by the relative standard deviation (RSD). The repeatability RSD_r (intra-assay precision) was measured by comparing standard deviation of the recovery percentages of spiked honeybees samples run the same day. The reproducibility RSD_R (as between-day precision) was determined by analysing spiked honeybees samples for three alternate days. Replicate ($n = 5$ for each concentration level) samples were all run and the RSD value was calculated for each insecticide. The method was found to be precise (RSD_r and $RSD_R \leq 20\%$) for all compounds studied at both spiking levels (Table 4).

4. Conclusion

A LC-MS/MS multi-residue method is reported for routine determination of the 5 most important, toxic insecticide neonicotinoid residues for bees. This method used an extraction and clean-up followed by liquid chromatography with mass spectrometry detection. Solid phase extraction with florisil cartridge yields high recovery rates for almost all compounds studied.

The main advantages of the method described are: the more important neonicotinoid insecticides can be determined simultaneously, highly sensitive and rapid method. These results indicate that the present method can be applied on samples of bees to routine analysis for monitoring the environmental pollution and the cases of declines and mortalities of colonies. Furthermore, this method allows the determination at levels lower than the LD_{50} of each pesticide.

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