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# Determination of imidacloprid in beehive samples by UHPLC-MS/MS

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ABSTRACT

Imidacloprid is a systemic insecticide belonging to the neonicotinoid family. It was the first neonicotinoid introduced in the mid-1990s, and since then, its use has grown rapidly to control pests in a variety of agricultural crops. Several studies have shown that neonicotinoids translocate to the nectar and pollen of treated plants, which represents a potential risk to pollinators. Therefore, an open-field feeding study was carried out. For this purpose, 30 beehives of *Apis mellifera* L. were installed in the same apiary. All colonies were in similar health and population conditions when assays were started. For seven weeks, colonies were fed with sucrose syrup with different concentrations of imidacloprid: 15, 30, 120 and 240 $\mu g\,kg^{-1}$. Thus, the assays were divided into four treatments and a witness (Control) with no added imidacloprid. To check the hives' exposure to imidacloprid and evaluate its distribution, sampling of adult worker bees and larvae was performed before, during and after the whole feeding period (7 weeks). Furthermore, in the 15th week, honey and beeswax (honeycomb) samples were collected from the brood chamber and honey super of all hives. Analytical methodologies for sample preparation based on the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) procedure were optimized and validated. After soaking the bees and honey samples and the extraction using acetonitrile with $MgSO_4$ and NaCl salts, a dispersive solid-phase extraction (d-SPE) step with $MgSO_4$, PSA and C18 was applied. Melted beeswax was subjected to an acetonitrile extraction, followed by freeze-out and d-SPE with PSA and C18. Extracts were evaluated in a UHPLC-MS/MS system. LOQ ($\mu g\,kg^{-1}$) values were 0.25, 0.50 and 1 for honey, bees and beeswax, respectively. Satisfactory recovery performance was achieved with relative standard deviation $\leq 20\%$. Residue concentrations of imidacloprid in samples showed correlation with the doses supplied, indicating exposure of the beehives to the insecticide. Honey stored approximately 60% of the loaded imidacloprid through syrup feeding.

## 1. Introduction

Since 2008, neonicotinoids have become the most important family of insecticides in the global agrichemicals market due to their high target efficacy, versatility in application methods, and declared low risk for non-target organisms and the environment [1]. However, the increase in their use worldwide is correlated with declines and many effects in bee pollinators [2]. Therefore, in 2013, preventively, the European Commission established a precautionary moratorium on the use of three neonicotinoids (clothianidin, thiamethoxam and imidacloprid) on bee-attractive crops, and a final resolution has not currently been adopted [3,4]. Since that moment, numerous studies have been published investigating the effects of sub-lethal dosage of neonicotinoids in bees, and on the occurrence of neonicotinoids in beehive related samples such as pollen, honey, beeswax and other studies has also increased [5]. More recently, EFSA published in February 2018 that a high risk was concluded for various routes of exposure for all the bee groups (honeybees, bumblebees and solitary bees) [6].

Conversely, the use of neonicotinoids is not restricted in Argentina. Five active compounds from this family are registered (acetamiprid, flonicamid, imidacloprid, thiacloprid, and thiamethoxam) for a varied set of crops. Imidacloprid is authorized in various horticultural products, oilseeds and cereals with MRLs in the range of 0.01 to 1 $mg\,kg^{-1}$ [7]. This compound has historical relevance because it was the first compound of the neonicotinoid family with widespread use [8] and is

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the most common neonicotinoid used in South America [9]. Contrary to what was previously mentioned, in this geographical area, there is a lack of data on the effects of these compounds on pollinators and on the environment in general [10]. Moreover, it was reported in a recent review that less attention has been devoted to field studies and the whole-colony behaviour globally, and there is a need to increase research linking the knowledge of the more abundant individual studies [5].

Considering all these aspects, a study was carried out to evaluate the effects on honeybee colonies under field conditions after systematic exposure at sub-lethal levels through feeding with syrups spiked with different doses of imidacloprid. This interdisciplinary study was supported by national and local authorities in order to obtain additional information useful for updating regulations and for improving risk assessment activities. Throughout the experiment, several additional parameters were studied such as population of bees, number of larvae, pollen and honey storage. Moreover, samples of bees, larvae, honey and wax were extracted from beehives, combining the open-field feeding study with laboratory assays, to assess the transfer between in-hive matrixes and to evaluate its possible functional abnormalities.

In the framework of this study, specific analytical methods for determination of imidacloprid in the different beehive matrixes were needed to accomplish reliable and timely measurements of the actual concentrations throughout the complete field experiment. The matrixes under study, whole body bees and bee larvae, as well as honey and beeswax obtained directly from the hives, may be considered difficult and complex matrixes with very different chemical compositions. Insect bodies are rich in lipids, chitin and proteins [11]. Honey is a highly concentrated sugar solution, mostly invert sugar (fructose and glucose) [12], and beeswax is a complex mixture (> 300 compounds) of hydrocarbons, fatty acids, esters, and other substances [13]. In general, it can be said that the analysis of these products is still a challenge, and there is a need to obtain simplified and reliable methodologies. Chromatography coupled with mass spectrometry in different configurations remains the gold standard for the final determination of imidacloprid in many food and environmental matrixes, including beehive products. LC-ESI-MS/MS is often used for its advantages in multiresidue methods that include neonicotinoid family compounds in bee tissue [14], bees and honey [15–17] and beeswax [17,18]. Additionally, GC–MS/MS has been employed with good performance in beehive products [19,20], and more recently, options using combinations of GC and LC have been proposed [11,21,22]. Most of these methodologies require suitable sample preparation or pesticide extraction from each matrix with differentiated procedures. The QuEChERS procedure [23] is one of the most popular sample preparation approaches in the area of pesticide analysis because of its broad scope of applications and its advantages of simplicity and low use of solvents and reagents, among others. In the case of bees, extraction procedures based on QuEChERS strategies with or without modifications are frequently used. Wiest et al. [21] introduced an initial dissolution of honeybees with hexane, and other authors have added triethylamine at the extractant phase to provide basic pH conditions [15,24]. Niell et al., meanwhile, combined dispersive solid-phase extraction (d-SPE) and freeze-out to clean the bee extracts [17]. The improved removal of lipids was achieved by adding novel sorbents for the d-SPE step: Enhanced Matrix Removal-lipid (EMR-lipid); chitin; and a mixture of zirconium oxide and C18 dual-bonded to silica (*Z*-Sep+) [14]. Other authors use conventional SPE cartridges after the extraction with acetonitrile/ethyl acetate [16].

For pesticide analysis in honey, QuEChERS procedures are the most popular choice for sample preparation [25]; however, SPE techniques are also frequently used [26,27]. A study of performance of both QuEChERS and SPE approaches for general pesticide residue analysis of honey showed comparable results [28]. Considering both matrixes, bee and honey, similar conditions in the sample preparation are proposed in some cases, differing only in the use of the determined SPE cartridge for each matrix [16] or the inclusion of a preheating step to treat honey with QuEChERS [15]. Greater modifications in the procedures for each matrix are introduced by other authors [17,21].

The methodologies for beeswax involve a previous melting step including, in some cases, a further dilution with organic solvents followed by an extraction step based on L-L and SPE cleanup [29] or different versions of modified QuEChERS [30].

Considering this analytical background information, the aim of the present work was to develop and validate suitable simplified methodologies for the determination of imidacloprid in each of the complex beehive matrixes. The proposed methods were applied to the analysis of bees, larvae, honey and beeswax from the mentioned field feeding study.

## 2. Material and methods

### 2.1. Standards and reagents

Imidacloprid standard (98.9%) was purchased from Sigma-Aldrich (Darmstadt, Germany). Working standard solutions at different concentrations were prepared in acetonitrile and isopropyl alcohol and stored at −10 °C. All solvents, namely, isopropyl alcohol obtained from AppliChem (Darmstadt, Germany) and acetonitrile and water obtained from Sintorgan (Buenos Aires, Argentina), were of HPLC grade. Water was acidified by adding formic acid from Fisher Chemical (98%) (Geel, Belgium). Anhydrous magnesium sulfate ($MgSO_4$) was purchased from AppliChem (Darmstadt, Germany) and sodium chloride (NaCl) was purchased from Carlo Erba Reagents (Val-de-Reuil, France). For the d-SPE step, primary-secondary amine (PSA) was procured from EnviroClean® (Bristol, PA, USA) and Octadecyl-silica (C18) was procured from Selectra® (Bristol, PA, USA). Syringe filters (Econofilter, PTFE, 25 mm × 0.2 μm) were acquired from Agilent Technologies (Santa Clara, CA, USA). For instrumental determination, all the solvents were purchased from Fisher Chemical (Geel, Belgium) and were Optima® grade.

### 2.2. Chromatographic and mass spectrometry conditions

The LC-MS/MS instrument consisted of a Waters Acquity ultra-performance liquid chromatograph (UPLC) (Miliford, MA, USA) coupled to a Waters Micromass TQD (Manchester, England) triple quadrupole mass spectrometer operated in the positive electrospray ionization mode. The UHPLC was equipped with a 100 × 2.1 mm (i.d.), 1.7 μm particle size, Waters Acquity BEH Shield RP18 column (Dublin, Ireland) protected by 5 × 2.1 mm (i.d.) Waters VanGuard guard column (Dublin, Ireland). After optimization of instrumental parameters, the adopted conditions for the determination of the studied compound in each matrix considered are as follows: elution mode involving gradient conditions with a binary mobile phase composed of 0.1% (v/v) formic acid in water/acetonitrile (98:2) (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B) pumped at a flow rate of 0.35 mL/min maintaining the temperature of the column heater at 40 °C. The mobile phase gradient program started at 5% of B up to 0.25 min then increases to 100% at 7 min. Next, a conditioning is performed to equilibrate the column before the next injection. With this purpose B decreases to 75% at 8 min and 40% at 8.5 min. Finally, the mobile phase returned to the initial composition at 9 min and is held for 1 min. The injection volume was 10 μL and sample manager temperature was set at 7 °C.

The MS source temperature was set at 120 °C with nitrogen flow rates of 10 and 400 $L\,h^{-1}$ for the cone and desolvation gases, respectively. The desolvation temperature was 350 °C. Argon was used as the collision gas with a flow of 0.15 mL/min, which produced a pressure of $4 \times 10^{-3}$ mbar in the collision cell. Optimization of cone voltage and collision energy for imidacloprid was achieved by infusing 1 $mg\,L^{-1}$ standards. The ESI interface was operated in positive mode (ESI+) and the mass spectrometer in selected ion monitoring (SIM) with

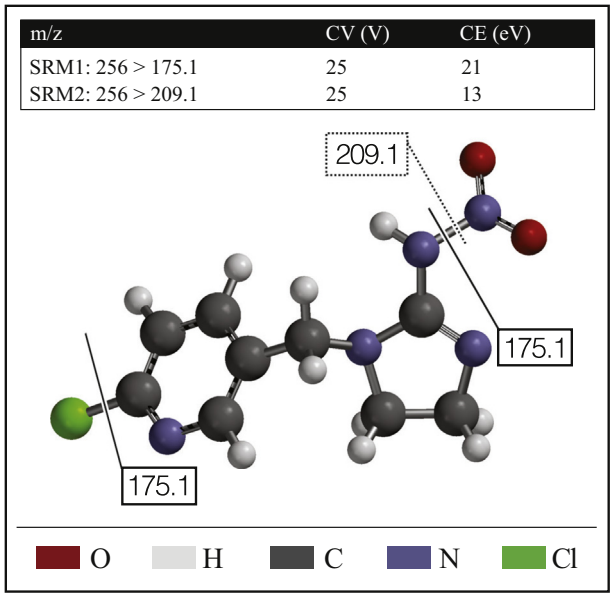


**Fig. 1.** Operational parameters for imidacloprid analysis in beehive samples by UHPLC-ESI-MS/MS. SRM transitions of identification (SRM1 *m/z*) and quantification (SRM2 *m/z*). Capillary Voltage (V), Cone Voltage (V) and Collision Energy (eV).

monitoring of two precursor/products ion transitions. The target ion transition with the highest intensity (primary ion transition) was used for quantitation (*m/z* 256.1 > 175.1), whereas the second target ion transition was used for confirmation (*m/z* 256.1 > 209.1) (Fig. 1). Waters MassLynx software version 4.1 (Manchester, England) was employed for acquisition and data processing.

### 2.3. Beehive samples collection

The open-field feeding study was carried out in the same apiary, where 30 beehives were displayed and submitted to 4 different feeding treatments based of spiked sugar syrups and a Control assay (no imidacloprid added). Each diet treatment (I to IV) corresponded to the supply of a determinate concentration of imidacloprid in sucrose solutions ($\mu g\,kg^{-1}$): 15, 30,120, and 240. The respective doses were fed once a week during a period of seven weeks at the beginning of the honey yield season (September–October 2014). Initially, 15,000 worker bees and a queen were disposed per hive, and a volume of 500 mL of each solution was supplied (0.03 mL/bee). To keep the dose of sucrose solution per bee consistent during the experiment, bee populations were counted weekly and the volume of feeding solutions was adjusted depending on the dynamic of the population. Colonies were inspected weekly to estimate the number of adult bees and number of cells with sealed brood and cells with pollen and honey. Worker honey bee and larvae samples for chemical analysis were collected from two beehives per treatment. Samples of honeycombs from the brood chamber and the honey super of all hives were collected in the 8th week at the end of the experimental feeding period of seven weeks. Once the samples were collected, they were immediately stored at −20 °C to prevent degradation. It has been posited that the effects of both temperature and UV radiation accelerate the decrease of whole-body imidacloprid residues in honeybees [31].

### 2.4. Sample preparation

#### 2.4.1. Bees, larvae and honey

Method development comprised experimental assays with different alternatives of sample preparation suitable for the three matrixes. This was carried out on the basis of a proper laboratory background on the analysis of neonicotinoid pesticide residues in other matrixes, and a suitable approach for multi-residue determination in diverse matrixes was developed [32].

The final adopted procedure is summarized as follows: 5 g of sample homogenized by a glass mortar was weighed into a 50 mL falcon tube, followed by the addition of 10 mL of water containing 2% (v/v) of formic acid. The tube was closed, and the sample was soaked for 1 h. The soaking of dry samples with acid water improves the extraction of acidic pesticides [32]. Then, 10 mL of acetonitrile was added and shaken by vortex for 30 s. The subsequent step was the addition of 4 g of $MgSO_4$ and 2 g of NaCl followed by immediate stirring for 2 min to prevent the formation of crystalline agglomerates during $MgSO_4$ hydration, and then the samples were centrifuged for 5 min at 2500 rpm.

A dispersive-SPE step was then applied in which 2 mL of the organic layer of the sample extracts was transferred to a 15 mL falcon tube containing 300 mg of $MgSO_4$, 300 mg of PSA and 200 mg of C18. Then, the tubes were vigorously shaken for 1 min and centrifuged for 5 min at 2500 rpm. A volume of 1 mL was concentrated to 50 µL under controlled conditions of temperature (50 °C) and $N_2$ stream and reconstituted to the original volume with mobile phase A. Finally, the extracts were filtered through 0.2 µm Phenomenex PTFE filters (Torrance, CA, USA) for the UHPLC-MS/MS analysis.

The same sample preparation process was applied to larvae samples. For honey samples, a similar procedure was also applied, but the previous soak step of 1 h was carried out in a water bath at 50 °C.

#### 2.4.2. Beeswax

The complex composition of beeswax, characterized by high lipid and hydrocarbon substances content, constitutes a solid matter of high melting point and low solubility in water. It was therefore necessary to experiment with various extraction conditions in order to favour the contact of the matrix components with the extractive solvents to improve the extraction efficiency in terms of recoveries and lowering the limits of determination.

The method used 2 g of warm beeswax that was weighed into a 50 mL falcon tube and incubated at 80 °C until melted. Then, 10 mL of acetonitrile was added to the wax. The tube was closed and stirred by vortex for 15 s. After that, it was placed in the water bath again until the wax melted. Then, the tube was stirred by vortex for 15 s and placed back into the water bath. This procedure was repeated three times. For precipitation of wax, the tube was placed into a freezer (−20 °C) overnight. An aliquot of 2 mL of extracts was transferred to a 15 mL falcon tube containing 300 mg of PSA and 200 mg of C18. The tubes were then shaken for 1 min and centrifuged for 5 min at 4000 rpm at 5 °C.

As in the honey and bee samples, the extracts were brought to controlled dryness, reconstituted with mobile phase A, then centrifuged at 15000 rpm for 10 min and finally filtered through 0.2 µm Phenomenex PTFE filters (Torrance, CA, USA) for the LC-MS/MS analysis.

### 2.5. Method validation

Validation was carried out in bees, honey and wax following the SANTE 11945 guidelines [33] determining selectivity, limits of detection (LOD) and quantification (LOQ) as well as linearity, repeatability and intermediate precision. To assess the selectivity of the method, spiked and non-spiked matrix blanks were injected into UHPLC-MS/MS system. Matrix effects were evaluated in concentration ranges between 0.25 and 200 $\mu g\,kg^{-1}$. The slopes of both the matrix-matched and solvent curves were compared by the ratio matrix effect (ME) (Section 3.2.1) and using Student's *t*-test [34]. Nevertheless, quantifications were performed with matrix-spike curves. The LOD and LOQ were estimated as three and ten times the signal/noise (S/N) ratio, respectively. To evaluate repeatability and intermediate precision, recoveries at two concentrations (LOQ level and 20 x LOQ level) were performed

**Table 1**
Validation parameters of imidacloprid in beehive products.

| Beehive product | LOD ($\mu g\,kg^{-1}$) | LOQ ($\mu g\,kg^{-1}$) | Linear range ($\mu g\,kg^{-1}$) | Recovery rate (%) LOQ level ($n = 5$) ± RSD | Recovery rate (%) 20 x LOQ level (n = 5) ± RSD | ME (%) |
|---|---|---|---|---|---|---|
| Bees | 0.20 | 0.50 | 0.50–200 | 70 ± 20 | 73 ± 8 | −11 |
| Honey | 0.10 | 0.25 | 0.25–200 | 88 ± 12 | 85 ± 4 | −3 |
| Wax | 0.40 | 1.00 | 1.00–200 | 97 ± 16 | 101 ± 4 | −6 |

Method performance and validation: limits of detection (LOD) and quantification (LOQ), range of linearity (7 levels), recoveries (%) and repeatability through relative standard deviation (RSD) and matrix effect (ME).

in quintuplicate. The final figures of merit verified in the validation experiments are described in Table 1.

## 3. Results and discussion

### 3.1. Method optimization

#### 3.1.1. Bees and honey

Our focused analyte imidacloprid (N-[1-[(6-chloropyridin-3-yl)methyl]-4,5-dihydroimidazol-2-yl]nitramide) is a relatively non-volatile, water soluble, polar compound. Its main physicochemical properties are detailed in Table S1 (Supplementary material). This compound is normally included in multiresidue analytical approaches. The QuEChERS method has been successfully applied to a wide variety of matrixes for a great scope of compounds but very scarcely to insect samples [35]. No differences were reported for imidacloprid recoveries when the original version and acetate and citrate buffering alternatives of QuEChERS method were applied to fruit and vegetables [23, 36–38]. In these methods fixing the pH of the extract solution through buffering (pH 4.8 and slightly higher pH 5–5.5 for the acetate and citrate versions respectively) was introduced mainly to avoid hydrolysis of base-sensitive compounds in large scope multi-residue methods. However differences between the three methods were observed in cases of other pH sensitive compounds not originally tested [32]. Moreover buffering is not recommended for lipidic matrixes due to the reduction of PSA retention capacity at such pH resulting in higher amount of co-extractives [39]. For that reason we followed the simpler original version focusing in a single compound of medium polarity such as imidacloprid, introducing some modifications in an attempt to improve recovery of this acidic compound from the basic ground bee tissue sample (pH = 8), also to decrease matrix effects and improve detection limits with this complex matrix [32].

The extraction procedure was optimized on blank bees spiked with imidacloprid in acetonitrile. Considering the acidic characteristics of the analyte, a preliminary soaking treatment with acidified water (2% formic acid) was introduced with good results. The soaking time of 1 h was adequate to complete the solubilization of the analyte and to improve the conditions of the further extraction with pure acetonitrile and the next partition step. This conditioning of sample usually used for dry samples was demonstrated that is also compatible for multiresidue-multiclase methods [32].

For the partition step the ratio between sample and salting-out salts was first adjusted through experimentation. Assays with a sample amount of 5 g and 8 g of $MgSO_4$ and 2 g of NaCl, as well as others with a lesser amount of $MgSO_4$ (4 g) were carried out. No significant differences were found between both alternatives regarding recovery values and matrix effects. Therefore, the last option was adopted as the final ratio. Having in consideration the additional difficulties of mashing and homogenizing composite samples of bee bodies, a medium level of initial sample weight (5 g) compatible with the required detection limits and minor matrix effects was adopted. Regarding the amount of salts 4 g $MgSO_4$ and 2 g of NaCl, following a modified ratio sample/salts, was effective and convenient to economize reagents but also to diminish the loading of inorganic magnesium and sodium salts that compromise the performance and maintenance of the mass spectrometric system. Cleanup is a further important step being the use of solid sorbents in dispersive mode a very effective way to deal with complex organic matrixes. An increase of PSA proportion in the sorbent mix, including the addition of C18 has been proved to be specifically effective for improve the removal of matrix co-extractives from fatty compounds [38]. However it is known that as a polar sorbent, PSA can form hydrogen bonds with polar compounds from the matrix but can also retain polar analytes such as imidacloprid. For that reason in our case a comparison of sample processing with and without the cleanup step using dispersive SPE were performed. In fact in this experiment, higher recovery values were obtained without using the dispersive solid phase extraction with the sorbent $MgSO_4$ + PSA + C18 as cleanup step (Table 2). This was expectable due to the type of matrix and the injection of less processed extracts. However, suppression matrix effects increased notably from the value of 11% for cleanup treated samples up to 45% in no-cleanup samples affecting the sensitivity raising the detection and quantitation limits. A similar signal to noise ratio of 15 (S/N = 15) was achieved at levels of 0.5 $\mu g\,kg^{-1}$ and 1 $\mu g\,kg^{-1}$ with cleanup and without cleanup respectively (Fig. 2).

Therefore validation of the procedure and further processing of samples were performed employing the proposed cleanup alternative in order to detect lower concentrations and preserve the instrument.

The optimized method used to determine imidacloprid in bees was also implemented to treat honey samples with only one modification in the soaking step. Despite being the honey an acidic matrix with pH values ranging 3.4 to 6.1, was compatible with the proposed soaking procedure. But in this case, the temperature employed was raised to 50 °C to deal with its relatively high viscosity at room temperature. This was adequate to liquefy the samples, favouring their homogenization and improving the extraction performance.

To decrease the detection and quantification limits, as well as to minimize the deformation of peaks and improve the resolution to achieve a better separation from co-extractants, the obtained extracts were brought to near dryness and reconstituted by weight with mobile phase A, as the predominant solvent in the liquid chromatography system. Likewise, the reconstitution in this mobile phase (comprised of 98% water) also helps to remove the remaining fat, especially in the most lipidic samples bees and beeswax (as discussed further in Section 2.4).

The better performance achieved in terms of peak shape with narrow width and lower background noise allowing more accurate quantitation and longer column lifetime justified this additional final treatment of extracts (Fig. 2).

The use of pure acetonitrile reduce the co-extraction of lipids

**Table 2**
Recovery values obtained in the assays of sample preparation step for bee samples, employing d-SPE $MgSO_4$ + PSA + C18 and without cleanup.

| | Cleanup | | No-cleanup | |
|---|---|---|---|---|
| Level $\mu g\,kg^{-1}$ | 0.5 | 10 | 1.0 | 20 |
| Recovery ± RSD (n = 5) | 70 ± 20 | 69 ± 10 | 91 ± 16 | 75 ± 6 |

Recoveries spiking bee matrix at two levels of concentration. RSD, relative standard deviation of 5 replicates.

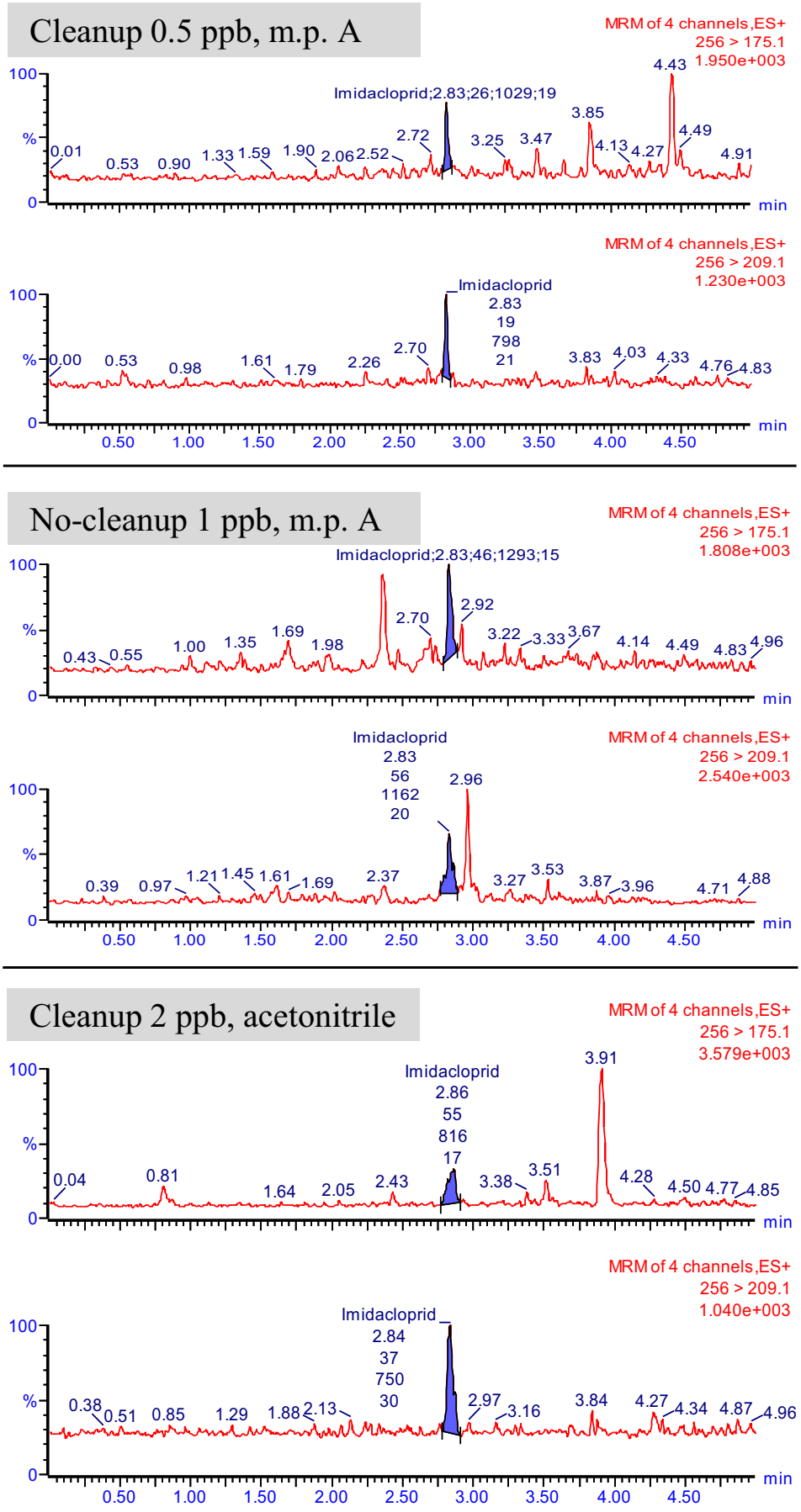


**Fig. 2.** MRM chromatograms of Imidacloprid in bee extracts: 0.5 μg kg$^{-1}$ with cleanup step in mobile phase A (above), 1 μg kg$^{-1}$ without cleanup step in mobile phase A (medium) and 2 μg kg$^{-1}$ with cleanup step in Acetonitrile (below). m.p. A: mobile phase A.

comparing to other solvents and the use of a unique sorbent mix based on an increased ratio of PSA and C18 also enables to minimize co-extractives from fatty matrixes, to levels compatible with acceptable matrix effects, sensitivity losses and need for instrument maintenance. The complete sample preparation procedure as it is presented here, do not use additional non-polar solvents (hexane), buffering salts, and alternative sorbents (diatomaceous material, Alumina, Florisil, *Z*-sep, etc.) (Table S2) implying a simple version of QuEChERS in comparison with other approaches considered from bibliography to solve the analytical complexity of honeybee matrixes.

### 3.1.2. Beeswax

As mentioned previously, beeswax is a complex mixture presented as a solid compact matter at room temperature (melting point 61–66 °C) and is very insoluble in water. Additionally, it is slightly acidic (acid value of 17–24 mg KOH/g). Secreted by bee workers constitute the cell wall structure of the honeybee combs, and has different functions in the beehive activity.

Once free wax is available, a first dissolution step is usually practised using a non-polar solvent aided with a moderate temperature to favour further extraction. For this step in our study, several assays of dissolution of the beeswax with n-hexane and with a mixture of n-hexane and isopropanol at 50 °C were performed [29].

The quantitative extraction of the analyte from this liquid mixture is the critical next step. For that reason, experimentation to test the performance of different extractants was performed. Solvents of high and medium polarity were assayed such as water, acid water and acetonitrile saturated in n-hexane. The extracts were purified by d-SPE with PSA and C18 in all cases. Relatively poor performance was achieved by all the tested procedures with recoveries lower than 70% (Table 3).

The estimation of the final volume of extracts after the addition of the extraction solvents is particularly difficult when isopropanol is used in the initial dissolution of wax because this solvent is quite similarly soluble in both n-hexane and aqueous phase [40]. Therefore, in the subsequent assays, isopropanol was eliminated as a dissolving mixture solvent. However, the good solubility of imidacloprid in this solvent led to the use of isopropanol as a solvent of the spiked standard solutions used for wax matrixes.

While trying to improve extraction efficiency, several other assays were performed: i) Considering the fact that the initial dilution with n-hexane did not favour the further partition, the use of this solvent was avoided. Thus, a reduction of dissolving solvent volume (from 15 to 2 mL) did not contribute significantly to improving the efficiency, introducing the need to increase the temperature to melt and dissolve beeswax to 70 °C. Additionally, this concentrated solution-enhanced matrix affects mass spectrometry measurements. ii) Imidacloprid is a compound highly soluble in dimethylsulfoxide (DMSO) (> 200 g/L at 20 °C), a solvent that is insoluble in beeswax [41,42]. For that reason, the inclusion of this solvent in the extractant mixture at a ratio 8:2 (extractant solvent/DMSO) was assayed without significant improvement of recovery. iii) Other assays using an ultrasound bath or introducing a second step in the extraction did not contribute significantly. iv) However, marked improvements of recoveries were observed when the extraction was done with a sequential and vigorous stirring [30].

The final procedure adopted included an increase in the temperature of the initial step to 80 °C to ensure full melting and homogenization of the wax and the elimination of the initial dissolution with n-hexane because the difficulties of working with this solvent at a high temperature. Acetonitrile was chosen as the extractant solvent for its greater affinity with a major number of pesticides than water, and therefore, the method became more versatile to enhancement of the scope of analytes in future applications. The extraction procedure was efficiently achieved by means of mixing the melted wax and the acetonitrile directly (ratio 1:5) at 80 °C and four vortex repetitions of 15 s each. Before the acetonitrile extract was submitted to the dispersive cleanup, it was verified that the wax's fatty components are well separated by a freeze-out (−20 °C) precipitation [30]. A critical aspect in the freeze-out process is to proceed rapidly, maintaining the low temperatures. This direct extraction with acetonitrile avoids any further liquid-liquid partition, and a suitable solution was submitted to the d-SPE with PSA and C18 in order to obtain the final extract measured in the UHPLC-MS/MS system. In this way a procedure with the same general strategy used for the previous beehive matrixes analysed was achieved. The use of acetonitrile with temperature as unique extraction solvent and the PSA and C18 sorbents aided with the freezing out complement led to a good solution to deal with the high fatty material content of this complex matrix. This approach has several differences with other proposals from bibliography to deal with analysis in beeswax for neonicotinoid pesticides.

### 3.1.3. Optimization of chromatographic and detection conditions

The optimization was based on sensitivity (LOQs). Even though, reverse phase systems employing C18 separation columns are widely used in pesticide analyses other alternatives were explored for optimization as the reduction of acetonitrile content in the final extracts. This reduction can be achieved by either dilution of extracts or evaporation and reconstitution in water-rich solutions. In this way, the second option was chosen since lower detection limits are reached. The importance of acetonitrile content reduction lies in both decreasing suppression matrix effects and improving peak shapes of early-eluting compounds as imidacloprid (Fig. 2).

In most studies, mobile phases consist of either methanol or acetonitrile and aqueous solutions (Table S2). Acidic mobile phases were obtained adding formic acid and supporting, in this way, the formation of $[M + H]^+$ adducts in the ESI+ mode.

The following mass system parameters were optimized by direct infusion of 1 mg $L^{-1}$ of imidacloprid solution to achieve highest sensitivity and resolution: precursor ion, cone voltage (CV), products ion, collision energy (CE), desolvation temperature, desolvation gas flow, source temperature, capillary voltage and cone gas flow. The time window was set from 0 to 5 min and the remaining run time was implemented to equilibrate the system.

**Table 3**
Results of assays with different extractant systems using polar and non-polar solvents for the determination of imidacloprid in beeswax.

| Dissolving solvent | n-hexane/isopropanol (4:1) | n-hexane/isopropanol (4:1) | n-hexane | n-hexane | n-hexane | n-hexane |
|---|---|---|---|---|---|---|
| Volume (mL) | 15 | 15 | 15 | 15 | 2 | 2 |
| Temperature (°C) | 50 | 50 | 50 | 50 | 50 | 70 |
| Solvent extractant | water | formic acid in water (2%) | water/acetonitrile[a](4:1) | acetonitrile[a] | formic acid in water (2%)/ acetonitrile[a] (1:1) | formic acid in water (2%)/ acetonitrile[a] (4:1) |
| Volume (mL) | 10 | 10 | 10 | 10 | 10 | 10 |
| Recoveries (%) | 55 ± 14 | 61 ± 7 | 60 ± 11 | 57 ± 6 | 56 ± 6 | 64 ± 8 |

Recovery values at 20 µg $kg^{-1}$ (n = 5).
[a] Acetonitrile was saturated in n-hexane in all cases.

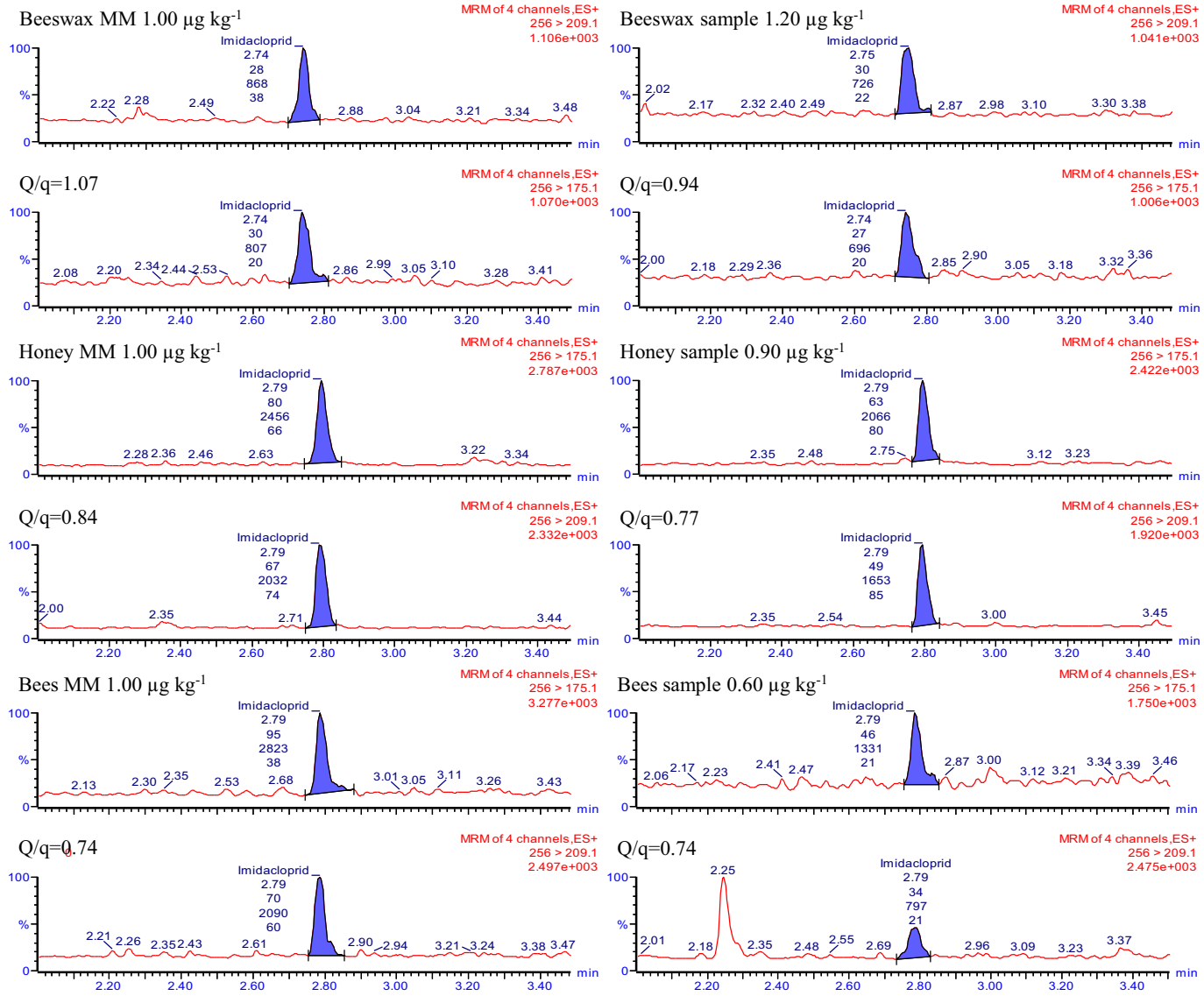


**Fig. 3.** MRM chromatograms of imidacloprid in bee, beeswax and honey samples and matrix-matched responses at LOQ levels of concentration.

### 3.2. Validation

#### 3.2.1. Linearity and matrix effects

The linearity of each procedure was applied to the different matrixes (bees, honey, and beeswax), and calibration experiments were carried out using 7 levels of concentrations and calibration standards prepared in mobile phase A (0.1% formic acid in water/acetonitrile 98:2) and in blank matrix extract (matrix-matched calibration). The results obtained from the regression analysis were considered satisfactory; a correlation coefficient higher than 0.99 and the deviation of back-calculated concentration from true concentration ≤ ± 20% were verified in all cases.

Matrix interferences were studied by comparison of the slopes obtained in the calibration experiment using mobile phase and matrix matched curves. The slope ratios of the corresponding regression lines were obtained using the following formula:

$$ME = [(MM\text{-}MP)/MP]^{*}\ 100.$$

where ME = matrix effect (percent); MM = matrix-matched regression line; and MP = mobile phase regression line.

In the three cases, low negative matrix effects were observed to be consistent with the ion suppression frequently observed in the electrospray ionization process (LC-ESI-MS/MS). The negative ME values were −11%, −3%, and −6% for bees, honey and beeswax, respectively, and may be considered as cases that do not need to be addressed in calibration following the criteria of SANTE [33] (ME < ± 20%).

However, following other, more restrictive criteria for evaluating matrix effects such us the comparison of slopes through *t*-test [34], only honey satisfies the condition of accepting the null hypothesis (no difference between slopes) in a 95% confidence level. With this last criterion in consideration, even though extracts of honey did not present matrix effects, all the quantifications of samples were performed through matrix matched curves.

#### 3.2.2. Accuracy and precision

Accuracy as a combination of precision and trueness was evaluated through a recovery assay, utilizing the complete proposed methodologies and 5 replicates of each matrix spiked with standards at two levels of concentration (LOQ and 20xLOQ) (Table 1). Average recoveries in the two levels were considered satisfactory at the validation stage for honey (85–88%), beeswax (97–101%) and bees (70–73%) with an associate repeatability in terms of the relative standard deviation (RSDr) below 20%. In the case of bees, a lower efficiency in recoveries was

**Table 4**
Imidacloprid concentrations in honey samples.

| | Brood chamber | | | Honey super | | |
|---|---|---|---|---|---|---|
| | Freq. (n+: nt) | Range | Mean | Freq. (n+:nt) | Range | Mean |
| Control | 5:6 | < 0.25–1.18 | 0.38 | 2: 4 | < 0.25–0.33 | 0.26 |
| Treatment I | 6:6 | 0.79–4.05 | 3.43 | 4: 4 | 0.40–6.27 | 2.07 |
| Treatment II | 2:5 | 6.40–7.03 | 6.72 | 3: 3 | 0.39–5.16 | 2.39 |
| Treatment III | 5:5 | 4.24–29.77 | 15.52 | 3: 3 | 3.54–18.72 | 12.78 |
| Treatment IV | 6:6 | 5.70–91.16 | 41.31 | 3: 3 | 17.78–52.87 | 36.60 |

Mean, range of concentration ($\mu g\,kg^{-1}$) and frequency of imidacloprid detected, with nt being the number of samples taken per treatment and n + being the number of positive samples in honey collected from the brood chamber and the honey super of the 30 studied hives.

verified, reflecting the complexity of the matrix as a heterogeneous mix of organic compounds. This matrix showed a systematic differentiated performance, with the greatest variability of recoveries and greater matrix interferences in the mass spectrometry measurement (Table 1). All three methodologies performed adequately through the complete study with recoveries within the accepted criteria of 60–140% (RSDr ≤ 20%) for individual recoveries in routine analysis and meeting the SANTE method performance specifications [33].

### 3.2.3. Determination limits

LOQs were determined by analysing matrix-matched standards at the target levels and checking with the recovery assay at the lowest level considered, meeting the condition of mass spectrometric peak signals with a signal to noise ratio > 10 and the recoveries meeting the acceptability criteria of 70–100% with a RSDr ≤20%. The LOQ level was higher in beeswax (Table 1) than in other matrixes due mainly to the greater initial dilution of wax samples (1:5 sample/extraction solvent), because of its aforementioned complexity at the sample preparation step. For bees and honeybees, the ratio of sample/solvent was equal (1:2), but the low interferences and better performance of the method verified with honey was clearly contributing to the lower LOQ compared to bees. Nevertheless, all the verified LOQs in the range of 0.25–1.00 $\mu g\,kg^{-1}$ were highly satisfactory to fit the purpose of the study and may be considered consistent with other requirements in environmental and food analysis of imidacloprid. Fig. 3 shows chromatograms of matrix-matched solutions at 1 $\mu g\,kg^{-1}$ and real positive samples at low levels of concentration for the three matrixes.

### 3.3. Application of validated methods

The validated methods were applied to collected samples from the field feeding study described in Section 2.3. A field study such as this, which considers the complete colony under beekeeping management practices, is rarely seen in the literature. Many studies measure the effects on individual bees, and it was then suggested that these effects should be linked to the consequences for the whole colony and wider bee populations [5,9]. The distribution mechanisms and the final destination or storage conditions of imidacloprid in the complex structure of in-hive components are still not completely elucidated [5]. Briefly, our results showed that the imidacloprid content in bee's bodies measured after each treatment varied throughout the experiment in concentrations ranging from 0.5 to 30 $\mu g\,kg^{-1}$, highlighting the differences between the treatments. However, no increase in concentration was observed within each treatment over time during the 7 week feeding period. This may be associated with the proper dynamic of colony renewal of sampled adult bees. It should also be considered that parent imidacloprid residues in the complex organic mixture of bee tissues are metabolized and decrease progressively, with an estimated half-life of 7 days in honeybees [43]. Notably, a very low incidence of positives was verified on larvae samples in all treatments, indicating that they behaved as a preserved, pesticide-free population. Wax from honeycombs showed positive measurements in 19 of 28 samples collected from the brood chamber ranging in concentration from 1 to 35.44 $\mu g\,kg^{-1}$, and a minor occurrence was verified in samples collected in the honey super: 8 positive samples from a total of 19 ranging from 1 to 12.39 $\mu g\,kg^{-1}$, in both cases generally correlated with the feeding doses. Findings from a great variety of pesticides in monitoring studies have been reported, which were mostly lipophilic, confirming that beeswax is a component of the beehive susceptible to storing contaminants [20,44]. In our case, it is noteworthy that despite imidacloprid being a water soluble polar compound, it also appeared systematically in wax samples after controlled exposure. However, the detection of imidacloprid and other neonicotinoids in beeswax from beehives has also been reported in the literature [29].

A very interesting behaviour was observed regarding the imidacloprid concentrations in honey samples. Diverse levels were found but closely correlated with the dose supplied in each treatment, ranging between 0.25 and 91.16 $\mu g\,kg^{-1}$ in the brood chamber and lower values ranging from 0.25 to 36.60 $\mu g\,kg^{-1}$ in the honey super, as shown in Table 4. Even in witness control hives, traces of the compound were found, suggesting that the colonies in these cases possibly encountered other sources of contamination or the presence of foreign bees from surrounding colonies. It is notable that up to 60% of the total imidacloprid loaded during the 7 weeks through the supplied syrups was found in the honey. This means that a great proportion of the income to the colony was derived and stored in the produced honey. These findings concern not only the potential harm to the bee's health when using part of the hive honey as its natural food supply but also with the safety of human honey consumption. This observed susceptibility to storing in honey is in accordance with the elevated occurrence of neonicotinoids in global surveys and particularly imidacloprid in South American countries [9]. Tolerances for residue concentrations of this insecticide and other neonicotinoids in honey are not fully established in Argentina or even globally in countries where the insecticide is currently authorized for agricultural use. In general, few regulations exist regarding pesticide residues in honey in comparison with other food products and commodities [24]. Specifically, this compound has established MRLs in the European Regulation (EC) No 396/2005 for honey and other apiculture products at levels of 50 $\mu g\,kg^{-1}$. There is growing evidence of toxicological concern of imidacloprid in the mammalian brain during chronic exposure and the higher affinity of its metabolites versus the parent compound [9]. Additionally, after the recent conclusions of EFSA confirming harm to honeybees [6], we can expect to see modifications in the current status of authorizations and established tolerances in the future.

## 4. Conclusions

Modified QuEChERS methods coupled with UHPLC-MS/MS were developed for the determination of imidacloprid in honeybee hive samples (adult worker bee body, larvae, honey and beeswax) collected from an open field feeding study. After studying the performance of important parameters in the extraction and cleanup stages such as the use of PSA, concentration of extracts, use of freeze-out options and other significant variables, we proposed alternative efficient methodologies that were satisfactorily applied to real samples.

Likewise, the study presents a brief description of the concentrations found in the different components of the beehives under controlled exposure at field conditions, contributing to a better understanding of the distribution and destiny of the loaded contaminant inside the bee colonies under beekeeping management conditions, aspects which are currently poorly elucidated and have sparked global interest for further research.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2018.07.027.

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## Declarations of interest

None.

## References

[1] P. Jeschke, R. Nauen, M. Schindler, A. Elbert, Overview of the status and global strategy for neonicotinoids, J. Agric. Food Chem. 59 (2011) 2897–2908, https://doi.org/10.1021/jf101303g.
[2] F. Sánchez-Bayo, D. Goulson, F. Pennacchio, F. Nazzi, K. Goka, N. Desneux, Are bee diseases linked to pesticides? A brief review, Environ. Int. 89–90 (2016) 7–11, https://doi.org/10.1016/j.envint.2016.01.009.
[3] European Food Safety Authority, Conclusion on the Peer Review of the Pesticide Risk Assessment for Bees for the Active Substance Imidacloprid, EFSA J. 11 (1) (2013) 3068, https://doi.org/10.2903/j.efsa.2013.3068.
[4] D. Auteri, M. Arena, S. Barmaz, A. Ippolito, A. Linguadoca, T. Molnar, R. Sharp, C. Szentes, B. Vagenende, A. Verani, Neonicotinoids and bees: the case of the European regulatory risk assessment, Sci. Total Environ. 579 (2017) 966–971, https://doi.org/10.1016/j.scitotenv.2016.10.158.
[5] J. Benuszak, M. Laurent, M.P. Chauzat, The exposure of honey bees (*Apis mellifera*; Hymenoptera: Apidae) to pesticides: room for improvement in research, Sci. Total Environ. (2017), https://doi.org/10.1016/j.scitotenv.2017.02.062.
[6] European Food Safety Authority, Conclusion on the peer review of the pesticide risk assessment for bees for the active substance imidacloprid considering the uses as seed treatments and granules, EFSA J. 16 (2) (2018) 5178, https://doi.org/10.2903/j.efsa.2018.5178.
[7] SENASA, National Food Safety and Quality Service, Resolution N 934, Maximum Residues Limits for Agricultural Products, http://www.senasa.gob.ar/resolucion-9342010-productos-agropecuarios, (2010) , Accessed date: 20 December 2017.
[8] I. Laycock, K.C. Cotterell, T.A. O'Shea-Wheller, J.E. Cresswell, Effects of the neonicotinoid pesticide thiamethoxam at field-realistic levels on microcolonies of *Bombus terrestris* worker bumble bees, Ecotoxicol. Environ. Saf. 100 (2014) 153–158, https://doi.org/10.1016/j.ecoenv.2013.10.027.
[9] E.A.D. Mitchell, B. Mulhauser, M. Mulot, A. Mutabazi, G. Glauser, A. Aebi, A worldwide survey of neonicotinoids in honey, Science 358 (2017) 109–111, https://doi.org/10.1126/science.aan3684.
[10] O. Lundin, M. Rundlöf, H.G. Smith, I. Fries, R. Bommarco, Neonicotinoid insecticides and their impacts on bees: a systematic review of research approaches and identification of knowledge gaps, PLoS One 10 (8) (2015) 1–17, https://doi.org/10.1371/journal.pone.0136928.
[11] T. Kiljanek, A. Niewiadowska, S. Semeniuk, M. Gaweł, M. Borzęcka, A. Posyniak, Multi-residue method for the determination of pesticides and pesticide metabolites in honeybees by liquid and gas chromatography coupled with tandem mass spectrometry-Honeybee poisoning incidents, J. Chromatogr. A 1435 (2016) 100–114, https://doi.org/10.1016/j.chroma.2016.01.045.
[12] M. Baroni, C. Arrua, M.L. Nores, P. Fayé, M.P. Díaz, G.A. Chiabrando, D.A. Wunderlin, Composition of honey from Córdoba (Argentina): Assessment of North/South provenance by chemometrics, Food Chem. 114 (2009) 727–733, https://doi.org/10.1016/j.foodchem.2008.10.018.
[13] F. Fratini, G. Cilia, B. Turchi, A. Felicioli, Beeswax: a minireview of its antimicrobial activity and its application in medicine, Asian Pac J Trop Med 9 (9) (2016) 839–843, https://doi.org/10.1016/j.apjtm.2016.07.003.
[14] P. Kaczynski, I. Hrynko, B. Lozowicka, Evolution of novel sorbents for effective clean-up of honeybee matrix in highly toxic insecticide LC/MS/MS analysis, Ecotoxicol. Environ. Saf. 139 (2017) 124–131, https://doi.org/10.1016/j.ecoenv.2017.01.033.
[15] K.M. Kasiotis, C. Anagnostopoulos, P. Anastasiadou, K. Machera, Pesticide residues in honeybees, honey and bee pollen by LC-MS/MS screening: Reported death incidents in honeybees, Sci. Total Environ. 485–486 (2014) 633–642, https://doi.org/10.1016/j.scitotenv.2014.03.042.
[16] M. Gbylik-Sikorska, T. Sniegocki, A. Posyniak, Determination of neonicotinoid insecticides and their metabolites in honey bee and honey by liquid chromatography tandem mass spectrometry, J. Chromatogr. B 990 (2015) 132–140, https://doi.org/10.1016/j.jchromb.2015.03.016.
[17] S. Niell, F. Jesús, C. Pérez, Y. Mendoza, R. Díaz, J. Franco, V. Cesio, H. Heinzen, QuEChERS adaptability for the analysis of pesticide residues in beehive products seeking the development of an agroecosystem sustainability monitor, J. Agric. Food Chem. 63 (2015) 4484–4492, https://doi.org/10.1021/acs.jafc.5b00795.
[18] S. Herrera López, A. Lozano, A. Sosa, M.D. Hernando, A.R. Fernández-Alba, Screening of pesticide residues in honeybee wax comb by LC-ESI-MS/MS. A pilot study, Chemosphere 163 (2016) 44–53, https://doi.org/10.1016/j.chemosphere.2016.07.008.
[19] M.D. Gil García, S. Uclés Duque, A.B. Lozano Fernández, A. Sosa, A.R. Fernández-Alba, Multiresidue method for trace pesticide analysis in honeybee wax comb by GC-QqQ-MS, Talanta 163 (2017) 54–64, https://doi.org/10.1016/j.talanta.2016.10.083.
[20] M.P. Chauzat, J.P. Faucon, Pesticide residues in beeswax samples collected from honey bee colonies (*Apis mellifera* L.) in France, Pest Manag. Sci. 63 (2007) 1100–1106, https://doi.org/10.1002/ps.1451.
[21] L. Wiest, A. Buleté, B. Giroud, C. Fratta, S. Amic, O. Lambert, H. Pouliquen, C. Arnaudguilhem, Multi-residue analysis of 80 environmental contaminants in honeys, honeybees and pollens by one extraction procedure followed by liquid and gas chromatography coupled with mass spectrometric detection, J. Chromatogr. A 1218 (2011) 5743–5756, https://doi.org/10.1016/j.chroma.2011.06.079.
[22] J. Ravoet, W. Reybroeck, D.C. de Graaf, Pesticides for apicultural and/or agricultural application found in Belgian honey bee wax combs, Bull. Environ. Contam. Toxicol. 94 (2015) 543–548, https://doi.org/10.1007/s00128-015-1511-y.
[23] M. Anastassiades, S.J. Lehotay, D. Štajnbaher, F.J. Schenck, Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce, J. AOAC Int. 86 (2) (2003).
[24] A. Kamel, Refined methodology for the determination of neonicotinoid pesticides and their metabolites in honey bees and bee products by liquid chromatography-tandem mass spectrometry (LC-MS/MS), J. Agric. Food Chem. 58 (2010) 5926–5931, https://doi.org/10.1021/jf904120n.
[25] P. Amaral Souza Tette, L. Rocha Guidi, M.B. Abreu Glória, C. Fernandes, Pesticides in honey: a review on chromatographic analytical methods, Talanta 149 (2016) 124–141, https://doi.org/10.1016/j.talanta.2015.11.045.
[26] L. Sánchez-Hernández, D. Hernández-Domínguez, M.T. Martín, M.J. Nozal, M. Higes, J.L. Bernal Yagüe, Residues of neonicotinoids and their metabolites in honey and pollen from sunflower and maize seed dressing crops, J. Chromatogr. A 1428 (2016) 220–227, https://doi.org/10.1016/j.chroma.2015.10.066.
[27] A. Jones, G. Turnbull, Neonicotinoid concentrations in UK honey from 2013, Pest Manag. Sci. 72 (2016) 1897–1900, https://doi.org/10.1002/ps.4227.
[28] P. Calatayud-Vernich, F. Calatayud, E. Simó, Y. Picó, Efficiency of QuEChERS approach for determining 52 pesticide residues in honey and honey bees, MethodsX 3 (2016) 452–458, https://doi.org/10.1016/j.mex.2016.05.005.
[29] K. Yáñez, J.L. Bernal, M. Nozal, M. Martín, J. Bernal, Determination of seven neonicotinoid insecticides in beeswax by liquid chromatography coupled to electrospray-mass spectrometry using a fused-core column, J. Chromatogr. A 1285 (2013) 110–117, https://doi.org/10.1016/j.chroma.2013.02.032.
[30] S. Niell, V. Cesio, J. Hepperle, D. Doerk, L. Kirsch, D. Kolberg, E. Scherbaum, M. Anastassiades, H. Heinzen, QuEChERS-based method for the multiresidue analysis of pesticides in beeswax by LC-MS/MS and GC × GC-TOF, J. Agric. Food Chem. 62 (17) (2014) 3675–3683, https://doi.org/10.1021/jf405771t.
[31] M. Schott, G. Bischoff, G. Eichner, A. Vilcinskas, R. Büchler, M.D. Meixner, A. Brandt, Temporal dynamics of whole body residues of the neonicotinoid insecticide imidacloprid in live or dead honeybees, Nature (July 2017) 1–10, https://doi.org/10.1038/s41598-017-06259-z.
[32] O. Lacina, M. Zachariasova, J. Urbanova, M. Vaclavikova, T. Cajka, J. Hajslova, Critical assessment of extraction methods for the simultaneous determination of pesticide residues and mycotoxins in fruits, cereals, spices and oil seeds employing ultra-high performance liquid chromatography-tandem mass spectrometry, J. Chromatogr. A 1262 (2012) 8–18, https://doi.org/10.1016/j.chroma.2012.08.097.
[33] European Commission Health, Directorate-General for Health and Food Safety, Guidance document on analytical quality control and method validation procedures for pesticide residues analysis in food and feed, SANTE 11945 (2015).
[34] D.L. Massart, B.G.M. Vandeginste, L.M.C. Buydens, S. De Jong, P.J. Lewi, J. Smeyers-Verbeke, Handbook of chemometrics and qualimetrics: part A. Chapter 8 — straight line regression and calibration, Data Handl. Sci. Techn. 20 (1998) 208–210, https://doi.org/10.1016/S0922-3487(97)80038-X.
[35] L. Haroune, R. Cassoulet, M.P. Lafontaine, M. Bélisle, D. Garant, F. Pelletier, H. Cabana, J.P. Bellenger, Liquid chromatography-tandem mass spectrometry determination for multiclass pesticides from insect samples by microwave-assisted solvent extraction followed by a salt-out effect and micro-dispersion purification, Anal. Chim. Acta 891 (2015) 160–170, https://doi.org/10.1016/j.aca.2015.07.031.
[36] AOAC Official Method 2007.01, Pesticide residues in foods by acetonitrile extraction and partitioning with magnesium sulfate, J. AOAC Int. 90 (2) (2007) 485–520.
[37] CEN Standard Method EN 15662, Foods of plant origin — determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE - QuEChERS method, Brit. Stand. (2008).
[38] S.J. Lehotay, K. Ae Son, H. Kwon, U. Koesukwiwat, W. Fu, K. Mastovska, E. Hoh, L. Natchanun, Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables, J. Chromatogr. A 1217 (2010) 2548–2560, https://doi.org/10.1016/j.chroma.2010.01.044.
[39] K. Mastovska, K.J. Dorweiler, S.J. Lehotay, J.S. Wegscheid, K.A. Szpylka, Pesticide multiresidue analysis in cereal grains using modified QuEChERS method combined with automated direct sample introduction GC-TOFMS and UPLC-MS/MS techniques, J. Agric. Food Chem. 58 (10) (2010) 5959–5972, https://doi.org/10.1021/jf9029892.
[40] A. Skrzecz, D. Shaw, A. Maczynski, IUPAC-NIST solubility data series 69. Ternary

alcohol-hydrocarbon-water systems, J. Phys. Chem. Ref. Data 28 (4) (1999) 1146, https://doi.org/10.1063/1.556052 (accessed 12 February 2018).
[41] U. Banasiak, Imidacloprid (206), (2000), p. 688 http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation02/IMIDA_EVjjb.pdf , Accessed date: 15 February 2018.
[42] Gaylord Chemical Company, Dimethyl Sulfoxide, Solubility Data, 2 https://es.scribd.com/document/159511575/Dmso-Dimetil-Sulfuxido, (2007) (accessed Feb 2018).
[43] F. Sánchez-Bayo, L. Belzunces, J.M. Bonmatin, Lethal and sublethal effects, and incomplete clearance of ingested imidacloprid in honey bees (*Apis mellifera*), Ecotoxicology 26 (2017) 1199–1206, https://doi.org/10.1007/s10646-017-1845-9.
[44] M. Perugini, S.M.R. Tulini, D. Zezza, S. Fenucci, A. Conte, M. Amorena, Occurrence of agrochemical residues in beeswax samples collected in Italy during 2013–2015, Sci. Total Environ. 625 (2018) 470–476, https://doi.org/10.1016/j.scitotenv.2017.12.321.