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# Exposure assessment of honeybees through study of hive matrices: analysis of selected pesticide residues in honeybees, beebread, and beeswax from French beehives by LC-MS/MS

**Gaëlle Daniele**[1] · **Barbara Giroud**[1] · **Claire Jabot**[1] · **Emmanuelle Vulliet**[1]

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**Abstract** Apiculture and pollination services are seriously threatened by bee weakening and losses phenomena. In this context, a survey was performed on samples from beehives across French areas during the 2012–2016 growing seasons, primarily taken from symptomatic colonies. A total of 488 honeybees, beebread, and wax were analyzed for the presence of pesticide residues. A total of 13 analytes including neonicotinoids and pyrethroids insecticides together with some of their metabolites and the fungicide boscalid were screened within samples. Methodologies based on efficient modified quick, easy, cheap, effective, rugged, and safe extractions followed by an LC-MS/MS quantification were implemented for each matrix. Thirty-eight percent of the 125 bee samples, 61% of the 87 wax samples, and 77% of the 276 beebread samples contained at least one of the targeted pesticides. Beebread was the most contaminated matrix with an average of two pesticide detections by positive sample and a maximum of seven compounds for a sample. Neonicotinoids and boscalid were the most often detected pesticides, whatever the matrix. The comparison of neonicotinoid detections in samples collected before and after the partial neonicotinoid ban in France displays a decrease in the frequency of detections for contamination levels lower than 1 ng/g in beebread. For higher levels and other matrices, no tendency can be drawn.

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✉ Gaëlle Daniele
gaelle.daniele@isa-lyon.fr

[1] Univ Lyon, CNRS, Université Claude Bernard Lyon 1, Ens de Lyon, Institut des Sciences Analytiques, UMR 5280, 5 rue de la Doua, 69100 Villeurbanne, France

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## Introduction

Due to their foraging activities, pollinators play an essential role in the development cycle of many plant species, crops, and wildflowers. Colony collapse disorder (CCD) and the worldwide observed decline of honeybees have thus serious consequences for the maintenance of biodiversity and for apiculture and food production. Indeed, one third of the world food depends on pollination (Klein et al. 2007).

It is nowadays assumed that pollinator's decline is caused by a combination of several factors, among them pesticides, pathogens, parasites, climate change, the decline in floral abundance and diversity, etc. (Goulson et al. 2015). The contribution of pesticides to the global diminishing of honeybees has received much consideration by the scientific community. This includes pesticides that honeybees may be exposed to during both their foraging activities and within the hive (Mullin et al. 2010). It is now proven that sublethal doses of pesticides induce disruptions of honeybee's learning, memory, navigation and foraging activities, and affect their sensitivity to other stressors (Decourtye et al. 2004; Williamson and Wright 2013; Sánchez-Bayo et al. 2016). Thus, data on pesticide residues in honeybees and beehive products at trace levels are needed for a better risk assessment, as honeybee oral or contact $LD_{50}$ is in the nanogram per gram scale or lower for many pesticides.

The fungicide boscalid is a relatively new-generation compound of the carboxamide family with a broad-spectrum activity, acting by inhibiting fungal respiration. Boscalid is intensively applied in vineyards, oilseed rape and cereals and in fruits and vegetables such as carrots, cabbage, or beans,

mainly during the plant growth and the flowering period. Neonicotinoids and pyrethroids are two of the most widely employed classes of pesticides. Pyrethroids are lipophilic compounds that target ion channels implicated in the function of the nervous and muscular systems. Their main mechanism of action is to interfere with the normal function of voltage-gated sodium channels in the axonal membranes. They are conventionally analyzed by gas chromatography-mass spectrometry (GC-MS). Neonicotinoids exhibit a low volatility and high polarity. They act via a neurologic mechanism in insects, interacting agonistically on the nicotinic acetylcholine receptors of the insect central nervous system. They represent around one third of the worldwide market for insecticides (Bonmatin et al. 2015). They are applied by spraying directly to plant foliage and mainly as seed coating and soil treatments. Given their systemic properties, they can be taken up by the roots or the leaves and circulate through the plant's tissues, including flowers. Thus, they unintentionally contaminate pollen and nectar, having a significant impact on non-targeted species. They may also be transported into the hive, as pollen and nectar are the main food sources for honeybees. So, it is important to measure neonicotinoids at the parts per billion level corresponding to sublethal doses affecting bees (Sandrock et al. 2013; Lundin et al. 2015). The liquid chromatography-tandem mass spectrometry (LC-MS/MS) provides this required sensitivity and is thus generally implemented to monitor such pesticides (Giroud et al. 2013; Yãnez et al. 2013; Gbylik-Sikorska et al. 2015; Jabot et al. 2015; Codling et al. 2016).

Nectar and pollen collected as food resources are brought back to the hive by honeybees thus introducing, if this food is contaminated, the contaminants into the hive. Once collected, the nectar and pollen are stored inside the combs for months, and can induce a transfer of contaminants to wax by diffusion. Beeswax is a complex mixture of mainly highly lipophilic compounds. It is used in the hive by the bees for building the honeycombs. The contamination of this matrix can affect bee colony by transmitting the pollutants stored and accumulated in it to other bee products (Yãnez et al. 2013; Herrera-López et al. 2016) especially as wax is commonly recycled in beehives. Beebread results from the transformation of plant pollen by biochemical processes caused by the enzymes in the saliva and gastric fluid of the honeybee. It is stored in the wax combs and mostly consumed during the winter months for the spawning and survival of honeybees. Thus, it could be one of the causes of wintering colony losses (vanEngelsdorp and Meixner 2010; Codling et al. 2016). Moreover, beebread contains pollens gathered throughout the year by bees; therefore, it could be used as a long-term surveillance matrix.

This paper describes the application of efficient extraction methods previously developed in our lab, followed by a sensitive and selective UHPLC-MS/MS analysis for the detection and quantification of neonicotinoids, pyrethroids, together with some of their metabolites, and boscalid, in three beehive matrices. The list of the targeted pesticides has been established in collaboration with French beekeepers. This study presents the results of the analysis of 488 samples of honeybees, beebread, and beeswax collected during several years, mainly in symptomatic colonies but including samples from apparently healthy colonies. This study has been conducted in order to get an overview of French apiaries' contamination by the selected pesticides.

## Experimental section

### Chemicals and reagents

Ultraperformance liquid chromatography (UPLC)-MS-grade acetonitrile (ACN) and methanol (MeOH) were acquired from Biosolve Chimie (Dieuze, France) as well as ammonium acetate. Heptane, pentane, and dimethylsulfoxide (DMSO) (LC grade), along with acetic acid and formic acid (purity 99%) and triethylamine (TEA), were purchased from Sigma-Aldrich. Ultrapure water was obtained from a water purification device (Milli-Q® Gradient A10) from Merck-Millipore (Saint-Quentin-en-Yvelines, France). Diatomaceous earth sorbent (hydromatrix) was acquired from Agilent Technologies (Massy, France).

Quick, easy, cheap, effective, rugged, and safe (QuEChERS) extract tubes were obtained from Agilent Technologies. The acetate buffer (pH 4.8) packet was composed of 1.5 g of NaOAcetate and 6 g of $MgSO_4$, whereas the citrate buffer (pH 5–5.5) contained 1 g of NaOCitrate, 4 g of $MgSO_4$, 1 g of NaCl, and 0.5 g of disodium citrate sesquihydrate. The primary secondary amine (PSA, containing 150 mg of PSA and 900 mg of $MgSO_4$) and PSA/C18 (which contained 900 mg of $MgSO_4$, 150 mg of PSA, and 150 mg of C18)-dispersive solid-phase extraction (SPE) phases were purchased from Macherey-Nagel (Hoerdt, France).

Analytical standards (≥97.5% purity) of acetamiprid, imidacloprid, thiamethoxam, thiacloprid, clothianidin, bifenthrin, deltamethrin, lambda-cyhalothrin, boscalid, imidacloprid-d4, and cypermethrin-d6, were acquired from Sigma-Aldrich (Saint Quentin Fallavier, France), as well as cypermethrin (purity 92.0%). The standard of 6-chloronicotinic acid (6-CNA) was obtained from Dr. Ehrenstorfer (Augsburg, Germany), the olefin metabolite of imidacloprid (herein named olefin) was synthesized by Orga-Link (Magny les Hameaux, France), and 5-hydroxy-imidacloprid (5-OH) was supplied by UMR 406 INRA UAPV (Avignon, France).

Stock solutions of individual standards were prepared in MeOH at concentrations of 1000 mg/L and stored at −20 °C during 3 months. We verified that all the compounds remain

stable (±5%) over this period. Working solutions were obtained weekly by the appropriate dilution of the stock solutions.

## Sampling

All the samples were obtained thanks to collaborations with French beekeepers belonging either to the Fédération Nationale des Organisations Sanitaires Apicoles Départementales (FNOSAD) or to various Association pour le Développement de l'Apiculture (ADAs) like ADARA for Rhône-Alpes area. Samples were collected all over France for 4 years, as part of different projects between 2012 and 2016. The majority of the samples were collected just after the report of trouble or death observations in the corresponding colonies; nevertheless, samples from apparently healthy colonies were also studied. The samples were immediately frozen after collection and stored at −20 °C until their analysis. A total of 125 bees, 87 wax, and 276 beebread were thus analyzed.

## Sample extraction

The extraction procedures, mainly based on modified QuEChERS extractions, were already developed and validated in our lab for the different matrices. They were described in previous papers (Wiest et al. 2011; Giroud et al. 2013; Jabot et al. 2015) and presented briefly here. Boscalid and pyrethroids can be analyzed by both LC and GC; however, the methodologies were developed within the objective of using a unique analytical method for the three families of pesticides; thus, the analysis was performed by LC-MS/MS.

### *Honeybees*

In brief, 5 g of bees was introduced into a 50-mL polypropylene centrifuge tube with 30 g of stainless steel balls. Then 3 mL of ultrapure water, 3 mL of heptane, and 10 mL of ACN with TEA at 2% were added. Next, 200 μL of a solution containing the internal standards (at 100 μg/L) was added and the mixture was vigorously shaken by vortex for 15 s. Then a packet of citrate buffer was added and the tube was immediately manually shaken for 10 s in order to prevent the coagulation of $MgSO_4$ and next vortexed for 20 s to homogenize the sample. Then it was grounded using a Geno/Grinder® (SPEX SamplePrep, Stanmore, UK) 2 min at 1000 spm. Subsequently, the mixture was centrifuged at 5000 rpm for 2 min, at room temperature. An 8 mL aliquot of the supernatant was transferred to a 15-mL tube and frozen for 15 h at −18 °C. Then, 6 mL of the frozen extract was transferred to a 15-mL centrifuge tube containing dispersive PSA/C18 phase and swirled on a vortex mixer for 10 s. Afterward, the extract was centrifuged at room temperature at 5000 rpm for 2 min and 4 mL of the supernatant was transferred to a glass tube. Lastly, the solvent was evaporated to dryness under a gentle stream of $N_2$ at 40 °C. The dry residue was then dissolved in 400 μL of MeOH. Finally, 40 μL of the extract was added to 160 μL of ultrapure water for the UPLC-MS/MS analysis.

### *Wax*

Here, 0.5 g of wax was introduced into a Wheaton tube, with 4 mL of pentane and 250 μL of a solution containing the internal standards at 100 μg/L in MeOH. Due to the complexicity of beeswax matrix, the amount of wax was reduced to 0.5 g in order to lower the matrix interferences. Once dissolved in an ultrasonic bath, the wax was introduced in a 50-mL polypropylene centrifuge tube with 2.4 g of previously grounded diatomaceous earth. After vigorous stirring, the pentane was evaporated under a gentle stream of $N_2$. Then 15 mL of ACN (at 0.1% formic acid) was added and the tube was again vigorously manually shaken and vortexed. After centrifugation (2 min at 5000 rpm, at a temperature of 5 °C), 10 mL of the supernatant was transferred to a new 50-mL tube. The extraction was repeated once with 15 mL of ACN, and this time 15 mL of the supernatant was recovered and pooled with the previous one in the 50-mL tube. Then the tube was let at −20 °C for 15 h. Afterward the extract was centrifuged (2 min at 5000 rpm, at a temperature of 5 °C) and an aliquot of 15 mL was transferred into a 15-mL centrifuge glass tube with 200 μL of DMSO. Next, the tube was vortexed and ACN was evaporated at 40 °C under a gentle stream of $N_2$. Lastly, 1.8 mL of a mixture MeOH/$H_2O$ (20:80, *v*/*v*) was added to the DMSO extract before the UPLC-MS/MS analysis.

### *Beebread*

Briefly, 2 g aliquots of beebread were introduced in a 50-mL polypropylene centrifuge tube with 5 mL of ultrapure water, 5 mL of heptane, and 10 mL of ACN containing TEA at 2%. Then the internal standards (200 μL of a solution at 100 μg/L) were added together with a ceramic bar (Agilent Technologies). The mixture was then vortexed for 15 s and a packet of acetate buffer was added. The tube was immediately manually shaken for 10 s and next vortexed for 20 s to homogenize the sample. Afterward, the mixture was centrifuged at room temperature for 2 min at 5000 rpm. An aliquot of the supernatant (8 mL) was transferred to a 15-mL tube and frozen for 15 h at −18 °C. Then, 6 mL of the frozen extract was transferred to a 15-mL centrifuge tube containing dispersive PSA phase and it was vortexed for 10 s. Next, the extract was centrifuged (2 min at 5000 rpm, at room temperature) and 4 mL of the supernatant was transferred to a glass tube. Finally, the solvent was evaporated to dryness at 40 °C under a gentle stream of $N_2$. The dry residue was dissolved in 400 μL of MeOH. Lastly, the extract was dissolved in MeOH/$H_2O$ (20:80, *v*/*v*) for the UPLC-MS/MS analysis.

### UPLC-MS/MS analysis

A liquid chromatographic system H-Class UPLC from Waters (Saint-Quentin en Yvelines, France) was used for the separation of the 13 pesticides. The chromatographic column was a Kinetex Phenyl-Hexyl (100 × 2.1 mm; 2.6 μm) from Phenomenex (Le Pecq, France). The mobile phases were composed of (A) 0.01% acetic acid with 0.04 mmol/L ammonium acetate in ultrapure water and (B) MeOH. The following gradient was applied: from 5 to 90% (B) for 7 min and then 100% (B) for 2 min. The column was then equilibrated in the initial conditions for 3 min. The flow rate was fixed at 0.4 mL/min, the oven temperature was set at 60 °C, and the injection volume was of 2 μL.

The chromatographic system was coupled to a Xevo TQ-S triple-quadrupole mass spectrometer (Waters) equipped with a StepWave ion guide. Electrospray ionization was conducted in the positive mode with the following optimized parameters: capillary voltage 3200 V, desolvation temperature 450 °C, source temperature 150 °C, nitrogen desolvation, and nebulizer gas flows of 900 and 150 L/h, respectively. The multiple reaction monitoring (MRM) mode was performed, and two transitions were followed for each analyte. The target ion transition with the highest intensity (MRM1) was used for quantitation, whereas the second target ion transition (MRM2) was employed for confirmation. The retention time and the MRM ratios between both target ion transitions were also used as identification parameters, by comparison with analytical standards.

## Results and discussion

The limits of detection (LODs) and of quantification (LOQ) of the targeted pesticides are summarized in Table 1, for each matrix. The lowest LODs and LOQs are generally observed for boscalid and the neonicotinoids, whatever the matrix. Wax leads to the highest LODs and LOQs due to the complexity of this matrix that consequently requires a low matrix amount in order to lower the interferences.

A total of 488 samples have been analyzed, corresponding to 125, 87, and 276 samples of honeybees, wax, and beebread, respectively. Considering whole samples analyzed, every selected pesticide has been detected at least once. In honeybee and beebread samples, we have detected 12 different pesticides and only 6 in wax. This can be partly explained as wax exhibits the highest LODs.

### Honeybees

Within the 125 honeybee samples, we have detected 48 positive samples. We have identified one of the targeted pesticides within these positive samples 80 times. This indicates that 38% of the analyzed honeybee samples are contaminated with at least one targeted pesticides. Moreover, we have detected an average of 1.7 pesticides by positive sample, with a maximum of 5 targeted pesticides in a single sample. Besides the known effects of pesticides on the behavior of honeybees, these compounds act as stressors for the entire colony and thus can impact the development of the colony and honeybee's health (Kasiotis et al. 2014). The only pesticide not detected in the analyzed honeybee samples is the pyrethroid deltamethrin, even if its LOD is quite low, at 0.15 ng/g (Table 1).

The LOQs of the neonicotinoids and of their metabolites are in the same range or lower than published studies focusing on this particular pesticide family in honeybees. Indeed, LOQs ranging from 0.1 to 2.0 ng/g have been published for neonicotinoids and their metabolites in honeybees (Kasiotis et al. 2014; Gbylik-Sikorska et al. 2015).

Boscalid along with the neonicotinoids are the most often detected pesticides in honeybee samples, accounting for around three quarters of the detections (Fig. 1). It is important to notice here that these compounds are as well those with the lowest LODs. The maximum number of detections has been observed for the fungicide boscalid, the neonicotinoid thiacloprid, and imidacloprid with 18, 16, and 11 detections, respectively. Thus boscalid, thiacloprid, and imidacloprid have been detected in 14, 13, and 9% of the honeybee samples, with a maximum concentration of 47.6, 1.6, and 1.7 ng/g, respectively. The two compounds observed at the highest concentrations are boscalid and the metabolite 6-CNA with concentrations up to 47.6 and 12.6 ng/g, respectively. As 6-CNA represents the result of several metabolic pathways, this suggests a high level of contamination by neonicotinoids in some samples.

Nevertheless, the observed concentrations of the targeted pesticides in honeybees are generally low, with 67% of the boscalid and at least 80% of the other pesticides inferior to 1 ng/g. As the LOQs of the pyrethroids are higher (comprised between 0.5 and 5.5 ng/g), 90% of the positive samples containing a pyrethroid display a concentration inferior to its LOQ. The neonicotinoid acetamiprid and the pyrethroid cypermethrin exhibit concentrations ranging from 5 to 10 ng/g, each in one sample.

Moreover, we have detected in the same sample two targeted pesticides in 30%, three in 9%, and four and more in 6% of the analyzed samples. The most frequent "inter-family" combination is boscalid and a neonicotinoid (seven samples), followed by a neonicotinoid and a pyrethroid (four samples). It is important to notice that combinations of pesticides can induce synergistic toxicity for honeybees or their larvae (Schmuck et al. 2003; Thompson et al. 2014; Zhu et al. 2014).

**Table 1** Recoveries (%) and limits of detection (LOD) and of quantification (LOQ), expressed in nanogram per gram, of each pesticide in the three matrices

| | Beebread | | | Bees | | | Wax | | |
|---|---|---|---|---|---|---|---|---|---|
| | LOD (ng/g) | LOQ (ng/g) | Recovery (%) | LOD (ng/g) | LOQ (ng/g) | Recovery (%) | LOD (ng/g) | LOQ (ng/g) | Recovery (%) |
| | Neonicotinoids | | | | | | | | |
| Acetamiprid | 0.01 | 0.07 | 109 | 0.13 | 0.43 | 103 | 1 | 1 | 92 |
| Imidacloprid | 0.04 | 0.2 | 119 | 0.17 | 0.54 | 108 | 1 | 1 | 92 |
| Thiacloprid | 0.02 | 0.1 | 103 | 0.05 | 0.17 | 108 | 1 | 1 | 96 |
| Thiamethoxam | 0.02 | 0.05 | 93 | 0.06 | 0.18 | 102 | 1 | 1 | 95 |
| | Pyrethroids | | | | | | | | |
| Bifenthrin | 2.5 | 7.5 | 87 | 0.33 | 1.06 | 89 | 5 | 10 | 72 |
| λ-cyhalothrin | 0.83 | 2.6 | 69 | 1.64 | 5.48 | 108 | 40 | 40 | 76 |
| Cypermethrin | 0.04 | 0.1 | 76 | 0.94 | 3.9 | 107 | 10 | 50 | 116 |
| Deltamethrin | 0.1 | 0.18 | 85 | 0.15 | 0.49 | 103 | 5 | 5 | 95 |
| | Carboxamide | | | | | | | | |
| Boscalid | 0.01 | 0.03 | 105 | 0.15 | 0.68 | 111 | 1 | 1 | 102 |
| | Thiamethoxam metabolite | | | | | | | | |
| Clothianidin | 0.08 | 0.16 | 97 | 0.02 | 0.08 | 102 | 1 | 1 | 97 |
| | Imidacloprid metabolites | | | | | | | | |
| 5-OH | 0.25 | 1.25 | 85 | 0.23 | 0.74 | 97 | 2 | 5 | 85 |
| Olefine | 0.75 | 1.5 | 83 | 0.11 | 0.35 | 95 | 5 | 10 | 80 |
| | Imidacloprid, thiamethoxam, and acetamiprid metabolite | | | | | | | | |
| 6-CNA | 12.5 | 40 | 53 | 0.21 | 0.71 | 48 | 5 | 20 | 73 |

## Wax

The LODs and LOQs are higher in wax than in the other matrices, due to the complexity of this matrix, containing many lipophilic compounds. Nevertheless, the LOQs of boscalid and of the neonicotinoids are quite low, at 1 ng/g. These values are lower than those obtained by Yãnez et al. (2013) for neonicotinoid analysis in beeswax, with LOQs comprised between 2.0 and 7.0 ng/g. In their study, Niell et al. (2014) analyzed 38 LC-amenable pesticides in beeswax, among them boscalid and some neonicotinoids, with higher LOQs, 100 and 10 ng/g, respectively.

We have detected at least one of the 13 selected pesticides in 61% of the studied wax samples. Indeed, 53 samples out of the 87 have been contaminated with at least one targeted pesticide. We have observed 66 pesticide detections in all,

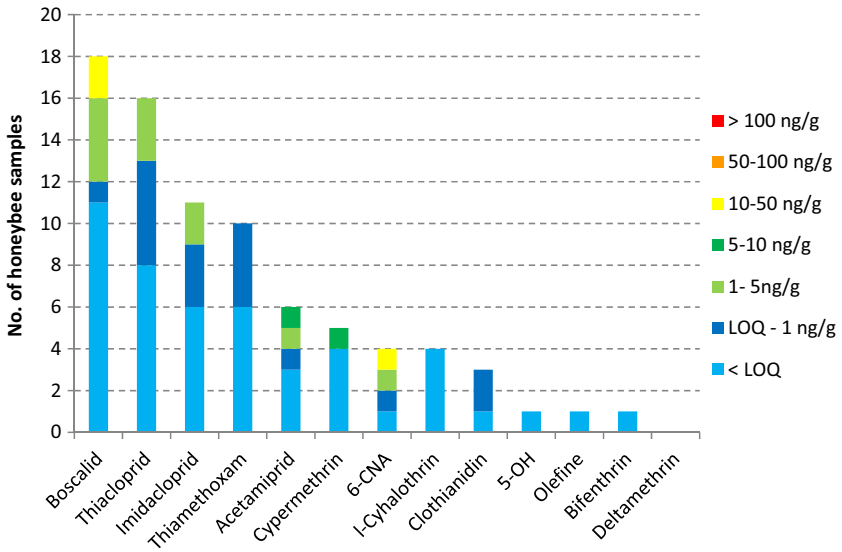


**Fig. 1** Number of detections and measured concentrations (in ng/g) of the 13 targeted pesticides in the honeybee samples. Detection counts are relative to the individual LODs and LOQs mentioned in Table 1

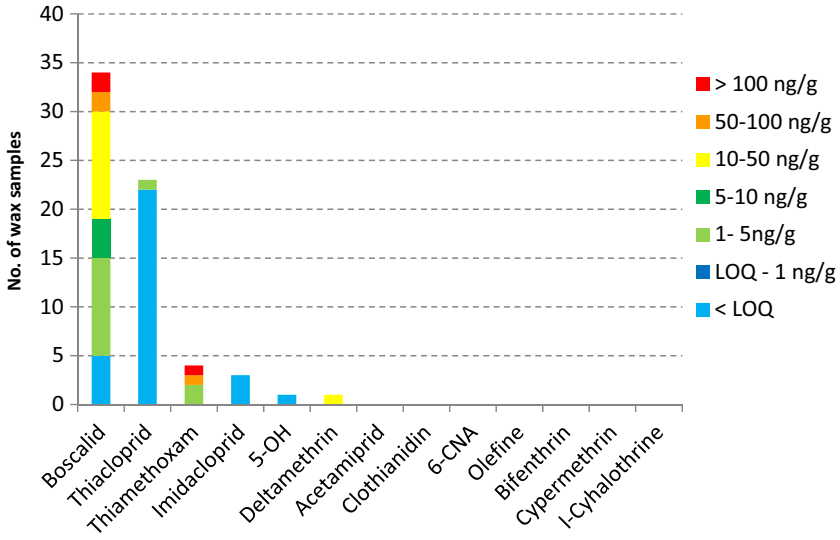


**Fig. 2** Number of detections and measured concentrations (in ng/g) of the 13 targeted pesticides in the wax samples. Detection counts are relative to the individual LODs and LOQs mentioned in Table 1

corresponding to an average of 1.2 pesticides by positive sample with a maximum of 3 pesticides.

Due to its particular physicochemical properties, wax stores the pesticides to which the hive is in contact and the pesticide residues can be accumulated in it. As beeswax is commonly recycled in new hives, wax can be a long-term contamination source for honeybees and this contamination can also be transmitted to other bee products.

The neonicotinoids and boscalid are the most present classes of pesticides in wax samples (Fig. 2). Surprisingly we expected to detect many pyrethroid pesticides as they are lipidic compounds, but these molecules have not been detected so often in our samples due perhaps to their high LODs or to their metabolization. We have only quantified deltamethrin in one sample, at a concentration of 28.3 ng/g. It is worth noting here that pyrethroids are more toxic to bees by contact than by oral exposition as they are hydrophobic substances (Sanchez-Bayo and Goka 2014).

The most frequent pesticides are again boscalid and thiacloprid, with 34 and 23 detections, respectively. They have been detected in 39 and 26% of the wax samples, with concentrations up to 302.3 and 3.4 ng/g, respectively. Only 15% of the samples contaminated with boscalid display concentrations inferior or equal to 1 ng/g. In their study conducted in North America, Mullin et al. (2010) obtained similar results with the detection of boscalid in 10% of their wax samples, with concentration up to 388 ng/g. Neonicotinoids have also been detected in 4 to 6% of the honeybee wax comb samples analyzed by Herrera-López et al. (2016), with concentrations up to 4.0, 5.1, and 10.4 ng/g for acetamiprid, imidacloprid, and thiacloprid, respectively. In their study, Yãnez et al. (2013) analyzed 30 beeswax samples, collected near orchards, for neonicotinoids. They found three compounds with decreasing frequency of detection: thiamethoxam, acetamiprid, and imidacloprid, with concentrations up to 153, 61, and 39 ng/g, respectively.

Generally the observed concentrations are higher in wax. This can be explained as wax is often reused within the hive for several years and can thus accumulate pesticides. Two samples exhibit high concentrations of thiamethoxam, at 50.9 and 106.5 ng/g. It is worth noting that thiamethoxam is highly toxic for bees (topical $LD_{50}$ 0.02 μg/bee) (Sanchez-Bayo and Goka 2014). A large part (44%) of wax samples contaminated with boscalid contains this fungicide at concentrations higher than 10 ng/g, and two samples exhibit concentrations even greater than 100 ng/g. Although boscalid is not toxic to bees ($LD_{50}$ >200 μg/bee), it poses a risk as it is found with a high frequency and at relatively high concentrations (Sanchez-Bayo and Goka 2014).

A combination of two and three pesticides has been detected in 21 and 2% of the positive samples, respectively. The most frequent inter-family combination is boscalid together with at least one neonicotinoid (12 samples). Some studies have proven that neonicotinoids' toxicity can be intensified when they are in presence of other agrochemicals, with an increase of toxicity that can reach factors of 1.52 to 1141 depending on their combination with certain fungicides (Iwasa et al. 2004; van der Sluijs et al. 2013).

## Beebread

Beebread is the most contaminated matrix. Indeed we have detected at least one of the 13 selected pesticides in 77% of the 276 beebread samples that corresponds to 213 positive samples. We have observed a total of 415 pesticide detections, corresponding to an average of about 2 pesticides by positive sample, with a maximum of 7 of the selected pesticides in a single sample. In their study, Simon-Delso et al. (2014) have found 39 pesticide residues during the analysis of 108 beebread samples resulting in a weaker contamination. However,

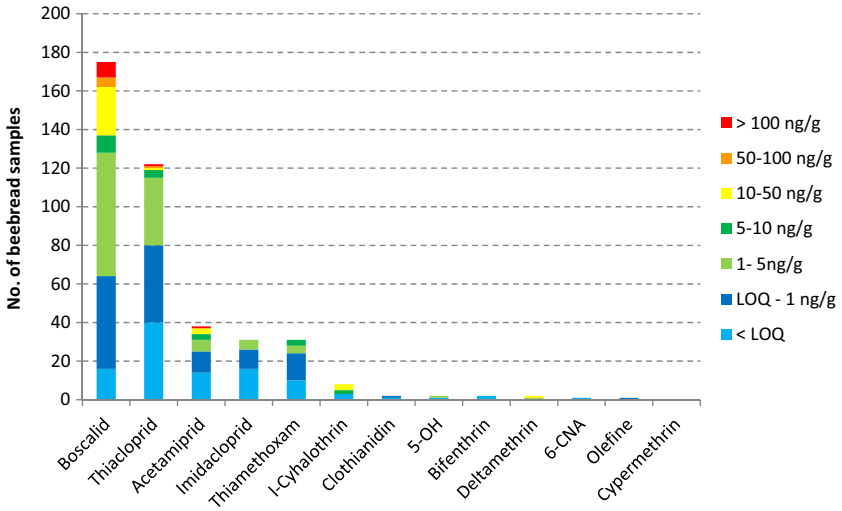


**Fig. 3** Number of detections and measured concentrations (in ng/g) of the 13 targeted pesticides in the beebread samples. Detection counts are relative to the individual LODs and LOQs mentioned in Table 1

their LOQs (100 ng/g in a multiresidue analysis) were higher than those obtained here for the targeted compounds.

The most often detected pesticides are again boscalid and thiacloprid (Fig. 3), followed by the other targeted neonicotinoids, acetamiprid, imidacloprid, and thiamethoxam. However, it is worth noting that these compounds exhibit the lowest LODs. Cypermethrin was the only pesticide not detected in this matrix, even with a very low LOD at 0.04 ng/g. Boscalid and thiacloprid have been detected 175 and 122 times, with concentrations up to 733 and 177 ng/g, respectively. Same results have been observed in the study of Simon-Delso et al. (2014) where the most frequent fungicide detected was boscalid. It is worth noting here that both compounds can pose a risk to bees even with their quite low toxicity (oral $LD_{50}$s 17.3 and >166 μg/bee for thiacloprid and boscalid, respectively), but as their frequency of detection is important (Agritox database; Sanchez-Bayo and Goka 2014).

The observed concentrations are generally quite high with only 70, 42, and 36% of the neonicotinoids, pyrethroids, and boscalid present at concentrations lower than or equal to 1 ng/g, respectively. Fifty percent of the samples contaminated with pyrethroids and 27% with boscalid exhibit concentrations higher than 5 ng/g. Deltamethrin and λ-cyhalothrin have been quantified at a maximum concentration of 25.9 and 27.5 ng/g, respectively. Both compounds exhibit a high acute toxicity to honeybees, with $DL_{50}$s of 1.5 and 38 ng/bee, respectively (Agritox database). The neonicotinoid acetamiprid has been quantified in one sample up to 171.4 ng/g.

We have detected two pesticides in 41%, three pesticides in 14%, and four or more in 7% of the positive samples. The most frequent inter-family combination (103 positive samples) corresponds to boscalid and a neonicotinoid. Six samples are contaminated by boscalid in combination with at least one pyrethroid and one neonicotinoid. It is noteworthy that mixtures of pesticide residues may result in synergistic toxicity to honeybees (Schmuck et al. 2003; Iwasa et al. 2004; van der Sluijs et al. 2013; Thompson et al. 2014; Zhu et al. 2014).

**Table 2** Comparison (number and concentrations in ng/g) of imidacloprid, clothianidin, and thiamethoxam residues observed in the honeybee, wax, and beebread samples, before and after their restriction in 2013

| | | Honeybees | Beeswax | Beebread | | | |
|---|---|---|---|---|---|---|---|
| | No. of samples | ≤2013<br>63 | ≥2014<br>62 | ≤2013<br>56 | ≥2014<br>31 | ≤2013<br>66 | ≥2014<br>210 |
| Imidacloprid | <LOQ | 3 | 3 | 3 | – | 9 | 8 |
| | –1 ng/g | 1 | 2 | – | – | 6 | 4 |
| | 1–5 ng/g | 1 | 1 | – | – | 1 | 4 |
| Clothianidin | <LOQ | 1 | – | – | – | – | 1 |
| | LOQ–1 ng/g | 2 | – | – | – | 1 | – |
| Thiamethoxam | <LOQ | 6 | – | – | – | 9 | 1 |
| | LOQ–1 ng/g | 4 | – | – | – | 12 | 2 |
| | 1–5 ng/g | – | – | – | 2 | 2 | 2 |
| | 5–10 ng/g | – | – | – | – | 1 | 2 |
| | 50–100 ng/g | – | – | – | 1 | – | – |
| | >100 ng/g | – | – | – | 1 | – | – |

### Impact of the European Union's amendment

In 2013, the European Union has adopted a regulation restricting the use and sale of seeds treated with formulations containing one of the three neonicotinoids: imidacloprid, clothianidin, and thiamethoxam for bee attractive plants and cereals (EC Regulation 2013). However, these temporary restrictions concern seed coating and other agrochemical formulations are yet authorized. We have thus compared the results of the analysis of samples collected before the amendment and after 2013 (Table 2).

It has to be noticed that the use of clothianidin as a pesticide was already banned in France before 2013; nevertheless, it is a metabolite of thiamethoxam. This can explain the low frequency of detection of this compound regardless of the matrix. However, this compound has been detected three times in honeybee samples before 2013 and no longer after the restriction.

The detection of imidacloprid in our honeybee samples is similar before and after 2013, whereas thiamethoxam has no longer been detected in the honeybee samples collected after 2013. On the same manner, imidacloprid has been detected in three beeswax samples collected before 2013, but no longer after the restriction. On the contrary, thiamethoxam has only been quantified in wax samples collected after 2013, with two on the four samples at concentrations higher than 50 ng/g. Concerning beebread samples, the frequencies of detection of imidacloprid and thiamethoxam have decreased after the restriction, for the lowest levels of contamination (lower than 1 ng/g). Indeed, before the restriction, 23 and 32% of the analyzed beebread samples have been contaminated with imidacloprid and thiamethoxam, respectively, compared to 6 and 1.4% after 2013. The frequency of detection for higher levels of contamination is almost the same before and after the restriction, with around 2% of beebread sample contaminated with imidacloprid or thiamethoxam. Since the European moratorium, we can thus observe a reduction of the frequency of detection of low levels of neonicotinoid residues, especially in beebread. Nevertheless, there is concern that these compounds can act as stressors and trigger deleterious effects for the entire honeybee colony at sublethal levels (Sanchez-Bayo and Goka 2014; Sandrock et al. 2014; Charreton et al. 2015).

In France within the framework of the law on the biodiversity, the use of all the neonicotinoids is going to be totally banned from 2018. It will be interesting to further analyze such beehive samples until this interdiction and some years after in order to evaluate the impact of the law on the neonicotinoid residues observed in beehives.

## Conclusions

Sensitive analytical methods, previously developed, have been applied in this study to detect and quantify 13 pesticides belonging to the neonicotinoid and pyrethroid families and some of their metabolites, together with the fungicide boscalid in beehive matrices. Samples of honeybees, wax, and beebread have been provided by beekeepers located in different French areas. These samples have been mainly collected after disorder's appearance or honeybees' death in the corresponding apiaries. A total of 488 samples collected between 2012 and 2016 have been analyzed. In the samples provided, the most frequent pesticides are boscalid and the neonicotinoids, particularly thiacloprid, whatever the matrix. Nevertheless, these pesticides exhibit the lowest LODs. Beebread is the matrix the most contaminated, with 77% of positive samples. Wax is the matrix with the highest concentrations of pesticides, up to 302.3 and 106.5 ng/g for boscalid and thiamethoxam, respectively. These results indicate the high contribution of field pesticides (farmer applied) to bee exposure within the hive itself. The comparison of the contamination observed before and after the restriction of neonicotinoid use in France shows a decrease in the frequency of detection of these molecules mainly at low levels (inferior to 1 ng/g), especially in beebread.

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