Determination of pesticide residues in honey bees, pollen and beeswax: Assessing Pesticide Hazard in Spanish Apiaries

Pau Calatayud Vernich

Tesi Doctoral Internacional

Programa de doctorat 3154 en Química

Determination of pesticide residues in honey bees, pollen and beeswax:

Assessing pesticide hazard in Spanish apiaries

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Determinació de residus de plaguicides en abelles, pol·len i cera d'abella:

Avaluació del perill per plaguicides en apiaris espanyols

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Determinación de residuos de plaguicidas en abejas, polen y cera de abeja:

Evaluación del peligro por plaguicidas en apiarios españoles

Memòria presentada per optar al títol de Doctor per

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Que el graduat *Pau Calatayud Vernich* ha treballat sota la nostra direcció durant més de quatre anys en l'elaboració de la Tesi doctoral amb menció internacional amb el títol **"***DETERMINATION OF PESTICIDE RESIDUES IN HONEY BEES, POLLEN AND BEESWAX: ASSESSING PESTICIDE HAZARD IN SPANISH APIARIES***"**, per la qual cosa autoritzem la seua presentació per optar al títol de Doctor.

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- *Calatayud-Vernich, P.*, Calatayud, F., Simó, E., Suarez-Varela, M.M., Picó, Y. (2016). **Influence of pesticide use in fruit orchards during blooming on honeybee mortality in 4 experimental apiaries.** *Science of the Total Environment* **541**, 33-41. [**JCR (WOS)** IF 4.9 (2016) en l'àrea de Ciències Ambientals 27/251 Q1].
- *Calatayud-Vernich, P.*, Calatayud, F., Simó, E., Picó, Y. (2016). **Efficiency of QuEChERS approach for determining 52 pesticide residues in honey and honey bees**. *Methods X* **3**, 452-458. [**SJR (Scopus)** 0.506 (2016)].
- *Calatayud-Vernich, P.*, Calatayud, F., Simó, E., Picó, Y. (2017). **Occurrence of pesticide residues in Spanish beeswax.** *Science of the Total Environment* **605**, 745-754. [**JCR (WOS)** IF 4.61 (2017) en l'àrea de Ciències Ambientals 27/251 Q1].
- *Calatayud-Vernich, P.*, Calatayud, F., Simó, E., Picó, Y. (2018). **Pesticide residues in honey bees, pollen and beeswax: Assessing beehive exposure.** *Environmental Pollution* **241**, 106-114 [**JCR (WOS) IF** 5.714 (2018) en l'àrea de Ciències Ambientals 25/251 Q1].
- *Calatayud-Vernich, P.*, VanEngelsdorp, D., Picó, Y. (2019). **Beeswax cleaning by solvent extraction of pesticides.** *Methods X* **6**, 980-985. [**SJR (Scopus)** 0.478 (2018)].
- *Calatayud-Vernich, P.*, Calatayud, F., Simó, E., Pascual-Aguilar J.A., Picó, Y. (2019). **A two-year monitoring of pesticide hazard in-hive: High honey bee mortality rates during insecticide poisoning episodes in apiaries located near agricultural Settings.** *Chemosphere* **232**, 471-480. [**JCR (WOS)** IF 5.108 (2018) en l'àrea de Ciències Ambientals 32/251 Q1].

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En tots aquests, Pau Calatayud Vernich ha realitzat la major part o tot el treball experimental, vigilant i supervisant els experiments, així com realitzant l'anàlisi dels resultats i l'elaboració dels manuscrits en directa col·laboració amb nosaltres.

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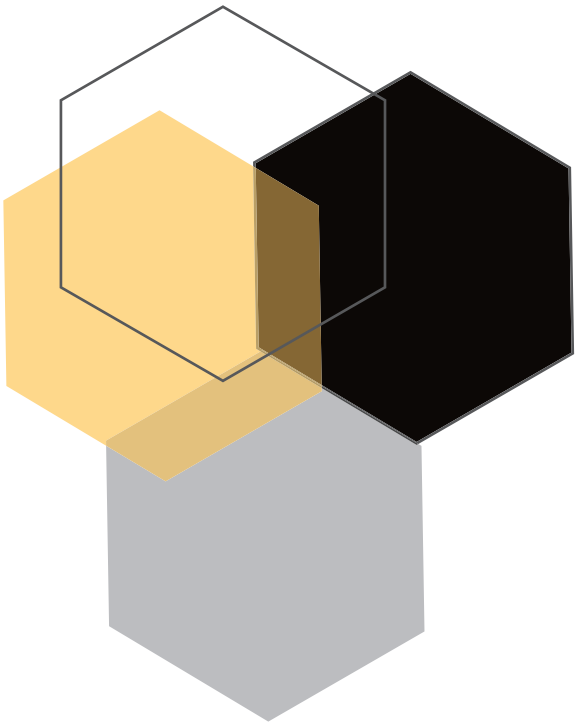

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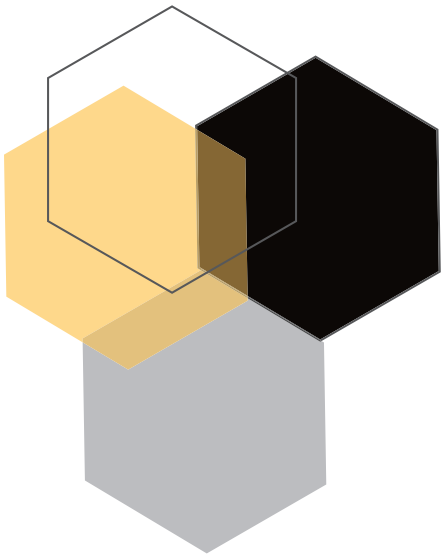
AIM AND STRUCTURE



Pollinator decline is an emerging worldwide problem with serious repercussions on agriculture and environment. Around one third of human food relies on insect pollination, and most of the flowering plants need pollinators to survive. Honey bee (*Apis mellifera L.*) is the main pollinator in environments where anthropogenic pressure has reduced the number of native pollinators, like urban, rural and agricultural areas. Loss of habitat and floral diversity, incidence and globalization of pathogens, and the increasing use of pesticides are the main factors responsible of rise in honey bee colonies mortality and global pollinator loss.

The main cause of beekeeping crisis is parasite *Varroa destructor* and secondary infections associated with the mite. Pesticide contamination and nutritional deficiencies, combined with the parasite, can act synergistically and reduce survival of honey bee colonies. In view of these concerns, the **main aim** of the present thesis is to elucidate the presence and distribution of pesticides in honey bees and beekeeping matrices like beeswax and pollen and to evaluate the consequences on honey bee health.

The primary aim of the thesis was achieved through the following **objectives:**

1. To develop methodology to detect the selected pesticides in different beekeeping matrices by high performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS) with triple quadrupole (QqQ).

2. To validate the extraction and detection methods of pesticides in the studied matrices: honey bees, beeswax and pollen.

3. To establish the distribution of pesticides in apiaries from different Spanish territories.

4. To study the influence of agricultural surroundings of the apiaries on pesticide content in honey bee colonies.

5. To elucidate the potential implication of pesticides on honey bee acute mortality episodes in apiaries located in agricultural areas.

6. To use the hazard quotient (HQ) approach to evaluate the threat that pesticides, from beekeeping and used in plant protection, pose to honey bee.

7. To explore potential methods to eliminate pesticide residues from beeswax.

The present thesis is divided into 6 chapters and presented through six scientific publications.

The **Chapter 1** introduces a serious issue that affects the health of honey bee colonies around the world; the presence of pesticides inside the hive. A review of the previous most relevant articles dealing with this topic, together with the importance of this thesis to stablish the occurrence of pesticides and understand its potential effects on Spanish beekeeping are presented.

The **Chapter 2** show the validation study of the methodology used through a scientific publication. The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) procedure was used for the extraction of the pesticides from samples and HPLC-QqQ-MS/MS for the determination.

• **Article 1.** *Efficiency of QuEChERS approach for determining 52 pesticide residues in honey and honey bees.*

The **Chapter 3** study the pesticide residues in beeswax, pollen and honey bees from 45 different apiaries. The results give a detailed profile about pesticide content in Spanish beehives and allow to evaluate the hazard that pose to honey bee through HQ.

• **Article 2.** *Pesticide residues in honey bees, pollen and beeswax: Assessing beehive exposure.*

In **Chapter 4** honey bee mortality together with pesticide residues in honey bees, beeswax and pollen were monitored in experimental apiaries located in different environments. Both scientific publications in this chapter contribute to comprehend the influence of the environment on the presence of dangerous pesticides in samples and the sudden honey bee mortality changes.

• **Article 3.** *Influence of pesticide use in fruit orchards during blooming on honey bee mortality in 4 experimental apiaries.*

• **Article 4.** *A two-year monitoring of pesticide Hazard in-hive: High honey bee mortality rates during insecticide poisoning episodes in apiaries located near agricultural Settings.*

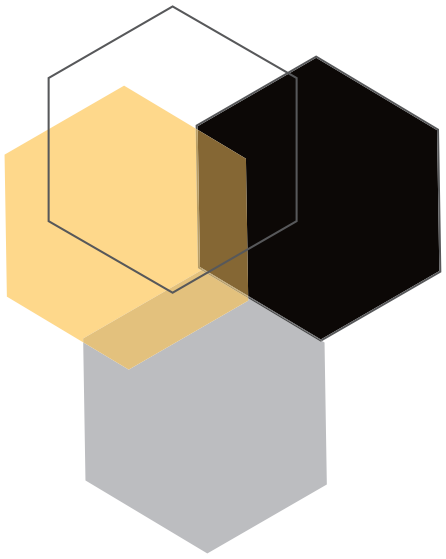
The **Chapter 5** compare the pesticide content among different sources of beeswax used in beekeeping: beeswax cappings, foundation, old combs and virgin beeswax. Furthermore, a preliminary study carried out during the research stay in the University of Maryland (United States of America) about beeswax cleaning by solvent extraction of pesticides is presented. This chapter contains two scientific publications:

• **Article 5.** *Ocurrence of pesticide residues in Spanish beeswax.*

• **Article 6.** *Beeswax cleaning by solvent extraction of pesticides.*

The **Chapter 6** is a detailed summary of the main results of the **chapters 2, 3, 4 and 5** and their discussion. Finally, main **conclusions** reached during the development of the thesis are presented. The **annex** section contains a glossary with the basic words used in beekeeping.

OBJECTIUS I ESTRUCTURA



Un problema emergent a nivell mundial és la progressiva disminució d'insectes pol·linitzadors i la seva greu repercussió sobre l'agricultura i el medi ambient. Al voltant d'un terç de l'alimentació humana depèn de la pol·linització entomòfila, i la majoria de plantes amb flor necessiten dels pol·linitzadors per a la seva supervivència. L'abella de mel (*Apis mellifera* L.) és el pol·linitzador majoritari en ambients on la pressió antròpica ha disminuït la presència de pol·linitzadors natius, com àrees urbanes, rurals i agrícoles. La pèrdua d'habitat, la reducció de la diversitat floral, la incidència i globalització de patògens i l'increment en l'ús dels plaguicides, són els principals causants de l'augment de la mortalitat anual de colmenes i de la disminució generalitzada de pol·linitzadors.

La principal causa de la crisi de l'apicultura a nivell mundial és l'àcar paràsit *Varroa destructor* i les infeccions secundàries induïdes per aquest. La contaminació per plaguicides i els dèficits nutricionals poden formar junt al paràsit, un complex multifactorial que de forma sinèrgica compromet la supervivència de les colònies d'abelles. En aquest context s'emmarca **l'objectiu global** d'aquesta tesi, esbrinar la presència i distribució de plaguicides en

les abelles, la cera i el pol·len i avaluar les conseqüències d'aquesta presència sobre la salut de les colònies d'abelles mel·líferes.

Per tal d'aconseguir l'objectiu principal, s'estableixen els **objectius específics** següents:

1. Desenvolupar la metodologia per detectar els plaguicides seleccionats a les diferents matrius de l'apicultura mitjançant cromatografia líquida d'alta eficàcia (HPLC) i espectrometria de masses en tàndem (MS/MS) amb triple quadrupol (QqQ).

2. Validar els mètodes per a l'extracció i detecció dels plaguicides de les matrius estudiades: abelles, cera i pol·len.

3. Conèixer la distribució dels plaguicides en apiaris situats en diferents territoris de l'estat espanyol.

4. Estudiar la influència de l'entorn agrari dels apiaris sobre el contingut de plaguicides en les colònies d'abelles.

5. Aclarir la possible implicació de plaguicides en episodis de mortalitat aguda a apiaris situats en entorns agrícoles.

6. Avaluar mitjançant el coeficient de perillositat (HQ) l'amenaça que representen per a les abelles cadascuna de les fonts de plaguicides contaminants, acaricides d'ús apícola i fitosanitaris.

7. Explorar mètodes potencials per a eliminar els plaguicides presents en la cera d'abella.

Aquesta tesi doctoral es presenta estructurada en sis capítols, dins dels quals es troba recopilada tota la informació científica generada mitjançant sis publicacions científiques.

En el **Capítol 1** s'introdueix la problemàtica que afecta a la salut de les colònies d'abelles de tot el món; la presència de plaguicides a l'interior de les colmenes. Es realitza un anàlisi dels treballs previs més rellevants que han estudiat aquesta problemàtica i s'exposa la importància del contingut d'aquesta tesi per conèixer la presència i les possibles repercussions dels plaguicides en l'apicultura espanyola.

El **Capítol 2** mostra l'estudi de validació i els motius d'elecció de la metodologia emprada mitjançant una publicació científica. Es va utilitzar el mètode QuEChERS (acrònim de l'anglès de *Quick, Easy, Cheap, Effective, Rugged* i *Safe*) per a l'extracció de plaguicides de les mostres i l'HPLC-QqQ-MS/MS per a la seva detecció.

• **Article 1.** *Efficiency of QuEChERS approach for determining 52 pesticide residues in honey and honey bees.*

El **Capítol 3** estudia el contingut de plaguicides en la cera, pol·len i abelles procedents de 45 apiaris distints. Els resultats donen una visió completa de la distribució dels plaguicides a l'interior de les colmenes a

nivell nacional i permeten establir el perill que representen per a l'abella mitjançant l'HQ.

• **Article 2.** *Pesticide residues in honey bees, pollen and beeswax: Assessing beehive exposure.*

El **Capítol 4** es centra en el seguiment de la presència de plaguicides en abelles, cera i pol·len, juntament amb el registre de la mortalitat d'abelles en apiaris experimentals situats en entorns de diferent naturalesa. Les dos publicacions científiques d'aquest capítol permeten comprendre la influència de l'entorn sobre la perillositat dels plaguicides trobats a les mostres i sobre els canvis bruscs de la mortalitat d'abelles:

• **Article 3.** *Influence of pesticide use in fruit orchards during blooming on honey bee mortality in 4 experimental apiaries.*

• **Article 4.** *A two-year monitoring of pesticide Hazard in-hive: High honey bee mortality rates during insecticide poisoning episodes in apiaries located near agricultural Settings.*

El **Capítol 5** compara el contingut de plaguicides de les diferents fonts de cera d'abella utilitzades a l'apicultura: cera d'opercle, cera de làmines estampades, cera de quadres i cera verge. Seguidament, i com a resultat de l'estada doctoral en la *University of Maryland* (Estats Units), es presenta un estudi preliminar de neteja de la cera mitjançant l'extracció dels plaguicides amb dissolvents. Aquest capítol està compost per dos publicacions científiques:

• **Article 5.** *Ocurrence of pesticide residues in Spanish beeswax.*

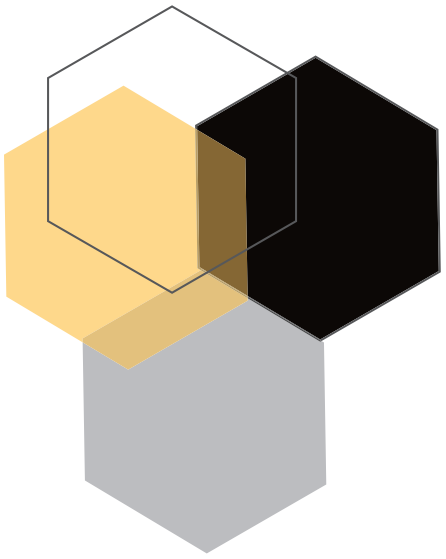
• **Article 6.** *Beeswax cleaning by solvent extraction of pesticides.*

El **Capítol 6** és un resum general dels principals resultats dels **capítols 2, 3, 4 i 5** i la seva discussió. Finalment, es presenten les **conclusions** de la investigació realitzada durant la present tesi doctoral. En l'apartat de **l'annex** trobem un glossari amb un recull del vocabulari bàsic utilitzat en l'apicultura.

CHAPTER 1: INTRODUCTION

01

INTRODUCTION



Pollination is the transfer of pollen from the male to the female part of plants. Gymnosperms pollen is predominantly dispersed by air, whereas most of angiosperms need pollinators to complete their life cycle (**Ollerton et al., 2011**). The majority of these pollinators are insects that play a crucial role in the environment, and besides provide a key service that guarantee an optimal crop production (**Fijen et al., 2018**). A 35 % of fruit, vegetable and seed global production depends directly on pollinators (**Klein et al., 2007**). In Europe, the 84 % of crop species rely directly on bees (**Gallai et al., 2009**). Furthermore, bee pollination increases yield of many crops like raspberries, oilseed rape, avocado (**Andrikopoulos and Cane, 2018; Peña and Carabalí, 2018;**

Perrot et al., 2018). Although managed pollinators, principally honey bees (*Apis mellifera* L.), are the main source of pollinators in agricultural settings, wild pollinators play an important role complementing honey bee pollination services (**Arathi et al., 2019**). Pollination dependence of agriculture is increasing (**Aizen and Lawrence, 2009**), and simultaneously, pollinators are disappearing across the globe (**Sanchez-Bayo and Wickhuys, 2019**). Habitat loss and conversion to intensive agriculture or urbanization, the indiscriminate use of synthetic pesticides and pathogens, are the main causes involved in global reduction of wild bees and other pollinators (**Goulson et al., 2015**). Although beekeepers mitigate the effects on managed pollinators, honey bee colonies have also been experiencing concerning loss rates worldwide (**Kulhanek et al., 2017; Brodschneider et al., 2018**). The main drivers of this steady decline appears to be a combined interaction between the effects of varroa mite (*Varroa destructor)* and secondary infections associated with the parasite (**Le Conte et al., 2010**; **Wells et al., 2016**; **Benaets et al., 2017**), nutritional deficiencies (**Tritschler et al., 2017; Annoscia et al., 2017**) and the exposure of honey bees to different pesticides (**Porrini et al., 2016; Traynor et al., 2016**).

Honey bees patrol extensive areas when foraging for nectar and pollen. These foraging flights expose them to compounds applied to crops like insecticides, fungicides, nematicides and herbicides through different routes (Figure 1). The ingestion of pollen and nectar from treated crops, and weeds and bushes at field margins contaminated by spray drifts, are the most common exposure of honey bees to pesticides (**Long and Krupke, 2016; McArt et al., 2017**). Sprayings of pesticides can also fall directly on forager bees with fatal consequences due to high concentrations found in droplets (**Sánchez-Bayo and Goka, 2016**). Bees and other pollinators can also ingest hazardous doses of pesticides when drinking from ponds and puddles of agricultural areas (**Samson-Robert et al., 2014**). Because of this, honey bees have been used as bioindicator of pesticides in agro-environments (**Niell et al., 2017**). Honey bees are also exposed to pesticides applied inside the hive against varroa mite. This parasite is the most important cause of honey bee colony losses (**Rosenkranz et al., 2010; Barroso et al., 2019**), and since its worldwide spread, beekeepers have used a wide variety of compounds and formulations inside the hives. As a result, honey bees are exposed to cocktails of pesticides inside and outside their colonies. Considering honey bees as crucial pollinators in farmlands, it is important to measure such exposure in order to be able to understand its repercussion on them.

This introduction discusses the main problems and challenges of pesticide determination in honey bees and hive products, and provide a broad coverage of the extraction techniques, clean-up procedures and instrumental analysis of these analyses (**Barganska et al., 2018**). Here we present the most relevant studies performed in the last decade that have evidenced the widespread presence of pesticide residues in honey bee colonies around the world. This preface also discusses the impact of pesticide exposure on honey bee health and highlight the importance of the present thesis assessing the honey bee colonies exposure to pesticides and measuring its potential repercussion, as a whole (**Benuszak et al., 2017**).

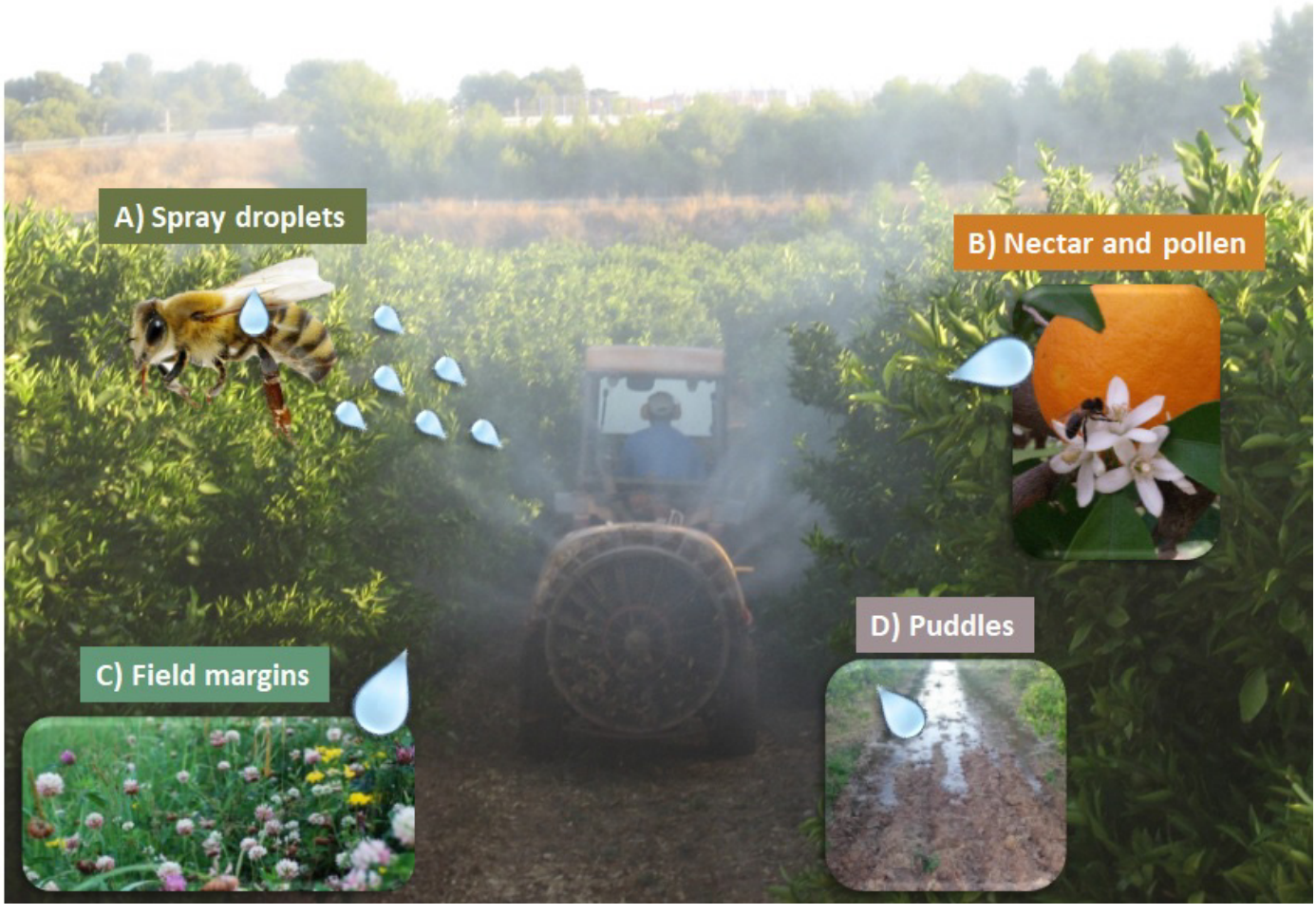
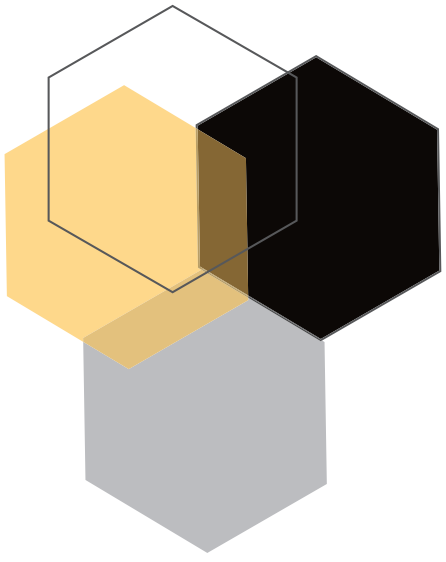


Figure 1. Routes of exposure of forager bees to pesticides used in plant protection.

02

ANALYTICAL TECHNIQUES FOR THE DETERMINATION OF PESTICIDES IN HONEY BEES AND BEEKEEPING MATRICES



The determination of pesticide in honeybees and hive products is complicated for several reasons that have to be considered: the relatively low concentrations of pesticides in some cases (e.g.: live honey bees); the complexity of the different hive products and honey bees; the variety of pesticides that can be present in the same sample. The relatively low concentrations of pesticides make it necessary to use exhaustive extraction procedures capable of concentrating the analyte together with very sensitive determination techniques. The complexity and diversity of the beehive samples makes it necessary to optimize the solvents used in the extraction to reduce as much as possible the co-extraction of matrix compounds as well as to design an extensive clean-up that reduces them. The large amount of pesticides with different physicochemical properties that can be present in the samples must be added to the previous factors because

complicate the selectivity of the extraction process. These aspects condition the analytical methodology to be used, and require an in-depth study.

Multiresidue methods (MRMs) for the screening, identification and quantitation of pesticides require a high sensitivity and reliability. The LC-MS/MS is the preferred technique over GC-MS in terms of wider scope, sensitivity and selectivity for most of pesticide classes (**Alder et al., 2006**). The LC-MS/MS with the triple quadrupole (LC-QqQ-MS/MS) is a rugged technique that can cover the majority of the challenges involved in that task (**Fernández-Alba and García-Reyes, 2008**) (Table 1). However, approaches using mass spectrometry with QqQ are restricted to a limited number of pesticides that must be predefined in advance and misses unknown and non-target compounds that were not included at the beginning of the analyses. Although liquid and gas chromatography with time-of-flight mass spectrometry (LC/GC-TOF-MS) provide lower sensitivity than QqQ instruments, it can perform screenings for compounds beyond the targeted list (**Barganska et al., 2018**). This technique can be used alone or as a complementary approach in order to give a more detailed profile of the pesticide content in bees and beekeeping products.

Prior to the determination of the pesticides, it is necessary an efficient extraction procedure as comprehensive as the equipment used in the identification. **Anastassiades et al.** (**2003**) developed a quick, easy, cheap, effective, rugged, and safe (QuEChERS) multiresidue method using acetonitrile partitioning and "Dispersive Solid-Phase Extraction" for the determination of polar and non-polar pesticides. The simplicity of the method in terms of equipment, the minimal amounts of solvent used, the capability of the procedure to be applied to fatty and complex matrices, together with the potentiality of being used as previous step in LC-GC pesticide analysis, explain the rapid expansion of this methodology in pesticide monitoring programs (Table 1).

Table 1. Analytical methodologies used to determine pesticide residues in honey bees and hive products.

Matrix	Pesticide residues	Extraction approach	Determination	Validation parameters*	Reference
Beeswax	Neonicotinoids, Pyrethroids and others (n= 13)	dSPE	LC-(ESI)-QqQ-MS/MS	R: 72 - 116 % LOD and LOQ: 1 - 40	Jabot et al., 2015
Honey bees, pollen and beeswax	Organophosphates, pyrethroids and others (n= 11)	SLE + dSPE	GC-(EI/ECI)-MS	R: 71.9 - 124.2 % LOQ: 0.33 - 5.34	Li et al., 2015
Honey bees and pollen	Neonicotinoids, organophosphates, pyrethroids and others (n= 19)	QuEChERS + dSPE	LC-(ESI)-QqQ-MS/MS	R: 70 - 120 % LOQ : 0.1 - 50	Niell et al., 2015
Honey bees	Neonicotinoids, organophosphates, pyrethroids and others (n= 58)	QuEChERS + dSPE	LC-(ESI)-QqQ-MS/MS	R: 70 - 96 % LOD: 0.3 - 3 LOQ: 1 - 10	Calatayud-Vernich et al., 2016a
Honey	Neonicotinoids, organophosphates, pyrethroids and others (n= 52)	QuEChERS + dSPE	LC-(ESI)-QqQ-MS/MS	R: 63 - 99 % LOQ: 0.5 - 10	Calatayud-Vernich et al., 2016b
Beeswax	Neonicotinoids, organophosphates, pyrethroids and others (n= 120)	QuEChERS + dSPE	LC-(ESI)-QqQ-MS/MS	R: 70 - 120 % LOQ: 5	Herrera-López et al., 2016
Honey bees	Neonicotinoids, organophosphates, pyrethroids and others (n= 200)	QuEChERS + dSPE	LC-(ESI)-QqQ-MS/MS GC-(EI)-QqQ-MS/MS	R: 70 - 120 % LOQ: 1 - 100	Kiljanek et al., 2016
Pollen	Neonicotinoids (n=7)	QuEChERS + dSPE	LC-(ESI)-qTOF-MS	R: 91 - 105 % LOD: 0.6 - 1.3 LOQ: 2.1 - 4.0	Valverde et al., 2016
Honey bees, honey and beeswax	Neonicotinoids, organophosphates, pyrethroids and others (n= 38)	QuEChERS + dSPE	LC-(ESI)-QqQ-MS/MS GC-ECD	R: 39 - 366 % LOD: 0.003 - 0.59 LOQ: 0.01 - 2	Amulen et al., 2017

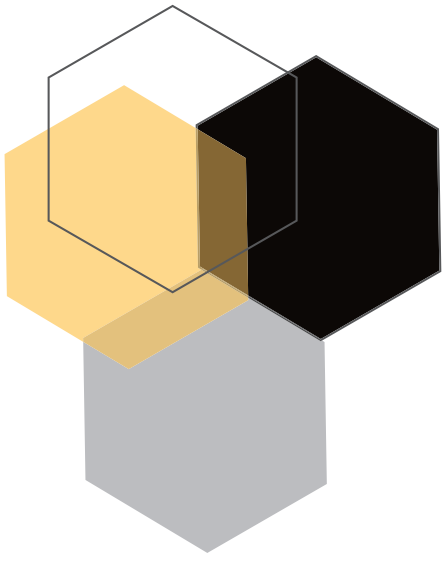
Table 1. Cont

Matrix	Pesticide residues	Extraction approach	Determination	Validation parameters*	Reference
Beeswax	Neonicotinoids, organophosphates, pyrethroids and others (n= 58)	QuEChERS + dSPE	LC-(ESI)-QqQ-MS/MS	R: 50 - 120 % LOD: 0.3 - 4.2 LOQ: 1 - 12.5	Calatayud-Vernich et al., 2017
Beeswax	Organophosphates, pyrethroids and others (n= 160)	QuEChERS + dSPE	GC-(EI)-QqQ-MS/MS	R: 60 - 120 % LOQ: 10 - 20	Gil-García et al., 2017
Honey bees	Neonicotinoids, organophosphates, pyrethroids and others (n= 150)	QuEChERS + dSPE	LC-(ESI)-MS GC-ECD	R: 60 - 140 % LOQ: 10	Martinello et al., 2017
Sugar food, Beebread, honey bees and beeswax	Neonicotinoids, organophosphates, pyrethroids and others (n= 80)	QuEChERS + dSPE SPE (Beeswax)	LC-(ESI)-QqQ-MS/MS GC- MS GC-ECD	R: 60 - 110 % LOD: 0.1 - 500 LOQ: 1 - 1000	Pohorecka et al., 2017
Pollen and Beebread	Neonicotinoids, organophosphates, pyrethroids and others (n= 112)	QuEChERS + dSPE	LC-(ESI)-QqQ-MS/MS GC-QqQ-MS/MS	R: 2 - 104 % LOQ: 0.23 - 13.38	Beyer et al., 2018
Pollen and beebread	Neonicotinoids, organophosphates, pyrethroids and others (n= 63)	QuEChERS + dSPE	LC-(ESI)-QqQ-MS/MS	R: 55 - 116 % LOD: 0.3 - 1.7 LOQ: 1 - 5	Calatayud-Vernich et al., 2018
Honey bees, beebread and beeswax	Neonicotinoids, Pyrethroids and others (n= 13)	QuEChERS + dSPE	LC-(ESI)-QqQ-MS/MS	R: 48 - 119 % LOD: 0.01 - 40 LOQ: 0.03 - 50	Daniele et al., 2018
Honey bees	Organophosphates, pyrethroids and others (n= 25)	LLE + SPE	GC-ECD GC-NPD	R: 57.6 - 120 % LOD: 0.3 - 3 LOQ: 1 - 10	Martel et al., 2018
Beeswax	Neonicotinoids, organophosphates, pyrethroids and others (n= 247)	QuEChERS + dSPE	LC-(ESI)-QqQ-MS/MS GC-QqQ-MS/MS	R: 78 - 110 % LOD: 5 LOQ: 10	Perugini et al., 2018
Pollen	Neonicotinoids, organophosphates, pyrethroids and others (n= 66)	SLE + MSPD	LC-QqQ-MS/MS	LOD: 0.25 - 3 LOQ: 2.5 - 10	Tosi et al., 2018
Honey, beebread and beeswax	Neonicotinoids, organophosphates, pyrethroids and others (n= 325)	QuEChERS + dSPE	LC-(ESI)-QqQ-MS/MS GC-QqQ-MS/MS	R: 70 - 120 % LOQ: 5 - 50	Lozano et al., 2019

Electron Capture detector (ECD); Electron ionization (EI); electrospray ionization (ESI); dispersive solid phase extraction (dSPE); Gas chromathography (GC); Liquid chromathography (LC); Liquid-Liquid Extraction (LLE); Limit of detection (LOD); Limit of Quantification (LOQ); Mass spectrometry (MS); Matrix solid phase dispersion (MSPD); Nitrogen-phosphorus detector (NPD); Quick, Easy, Cheap, Efficient, Rugged and Safe (QuEChERS); Solid-Liquid Extraction (SLE); Time of flight (TOF); Triple-quadrupole (QqQ)
R = Recovery
*LOD and LOQ are in $ng \cdot g^{-1}$

03

DISTRIBUTION OF PESTICIDES RESIDUES IN APIARIES



Honey bees impregnate their bodies with pesticides while foraging, and in-hive they are exposed to acaricides applied by beekeepers. Incoming pollen, often contaminated with pesticides used in crops, is stored inside the combs and maturated into beebread, where is also contaminated by compounds used in beekeeping. The activity of the numerous inhabitants of the hive spread pesticide residues within the colony, and a great proportion of that pesticide load is accumulated in beeswax, which acts as a pesticide sink for non-polar compounds. Tables 2-3-4 show the worldwide distribution of pesticides in honey bees and hive products.

Table 2. Pesticide residues detected (top 5) in different wax sources (combs, cappings and foundation).

Wax source	Pesticide	Nº of analyzed samples	Detection (%)	Range (ng·g^{-1})	Mean (ng·g^{-1})	Reference
Combs	Fluvalinate	67	52.2	n/a	220	Chauzat et al., 2009
	Coumaphos	92	46.7	n/a	647.5	
	Endosulfan	93	12.9	n/a	51	
	Azinphos methyl	54	5.6	n/a	228.2	
	Lindane	87	2.3	n/a	18.8	
Combs and foundation	Fluvalinate	259	98.1	2 - 204000	7473.8	Mullin et al., 2010
	Coumaphos	259	98.1	1 - 91900	3300.4	
	Chlorpyrifos	258	63.2	1 - 890	24.5	
	DMF (Amitraz)*	177	60.5	9.2 - 43000	2199.8	
	Chlorothalonil	258	49.2	1 - 53700	1066.6	
Combs	Coumaphos	109	100	44.9 - 20500	1755.7	Traynor et al., 2016
	Fluvalinate	109	100	148 - 28700	4895.3	
	DMF (Amitraz)	109	83.5	9.2 - 43000	2411.2	
	Chlorothalonil	109	68.8	1 - 53700	1635	
	Endosulfan	109	56.9	1.4 - 16.8	5.4	
Combs and foundation	Coumaphos	22	100	25 - 26858	10459	Calatayud-Vernich et al., 2017
	Chlorfenvinphos	22	100	219.1 - 5284.8	959.7	
	Fluvalinate	22	100	289.6 - 3593.3	779	
	Acrinathrin	22	85.9	30.7 - 2584.4	332.8	
	Flumethrin	22	85.9	24.5 - 170.1	88.9	
Combs	Fluvalinate	50	100	19 - 1870	180	Gil-Garcia et al., 2017
	Chlorfenvinphos	50	98	13 - 1764	224	
	Coumaphos	50	82	8 - 9308	2215	
	Acrinathrin	50	60	2 - 178	22	
	Orthophenylphenol	50	52	1 - 6	2	
Combs	Coumaphos	43	100	18 - 53400	5410	Calatayud-Vernich et al., 2018
	Chlorfenvinphos	43	95.3	35 - 16900	1320	
	Fluvalinate	43	88.4	55 - 6310	742	
	Acrinathrin	43	74.4	70 - 7500	1020	
	DMF (Amitraz)	43	46.5	30 - 3520	180	
Cappings and foundation	Coumaphos	178	60.7	10 - 990	100	Perugini et al., 2018
	Fluvalinate	178	50	10 - 1070	90	
	Chlorfenvinphos	178	35.4	10 - 630	60	
	Piperonil butoxide	178	20.8	10 - 230	160	
	Amitraz	178	15.2	10 - 20	10	
Combs	Coumaphos	68	98.1	2.35 - 15500	n/a	Fulton et al., 2019
	Fluvalinate	68	98.1	2.33 - 6970	n/a	
	Coralox	68	89.9	1.93 - 370	n/a	
	Chlorpyrifos	68	63.2	< LOQ	n/a	
	Chlorothalonil	68	49.2	< LOQ - 13.6	n/a	

*Amitraz is detected through its degradate DMF. n/a = Not available. LOQ = Limit of quantification.

Table 3. Pesticide residues detected (top 5) in beebread and pollen.

Matrix	Pesticide	Nº of analyzed samples	Detection (%)	Range (ng·g^{-1})	Mean (ng·g^{-1})	Reference
Pollen	Imidacloprid	185	57.3	n/a	1	Chauzat et al., 2009
	Carbaryl	126	13.5	n/a	142.4	
	Fipronil	185	12.4	n/a	1.3	
	Endosulfan	198	7.6	n/a	45.8	
	Coumaphos	198	5.1	n/a	423.5	
Beebread	Fluvalinate	350	88.3	1.6 - 2670	95.1	Mullin et al., 2010
	Coumaphos	350	75.1	1 - 5828	180.4	
	Chlorpyrifos	350	43.7	0.1 - 830	53.3	
	Chlorothalonil	280	52.9	1.1 - 98900	3014.8	
	Pendimethalin	247	45.7	1.1 - 1730	44.6	
Pollen	Carbendazim	128	34.4	< LOQ - 2595	24.31	Lambert et al., 2013
	Amitraz	128	14.8	< LOQ - 129.4	7.39	
	Triphenylphosphate	128	9.4	< LOQ	0.7	
	Carbaryl	128	7.8	< LOQ - 14.67	0.7	
	Phosmet	128	7.4	< LOQ - 78.10	9.38	
Pollen	Coumaphos	313	46.6	1 - 163	5.8	Stoner and Eitzer, 2013
	Carbaryl	313	40.6	2 - 227	27.7	
	Phosmet	313	32.9	1 - 16556	226.5	
	Carbendazim	313	29.4	1 – 1800	49.8	
	Atrazine	313	26.8	0.5 - 80	2.8	
Pollen	Clothianidin	14	42.9	6.1 - 1273	n/a	Kasiotis et al., 2014
	Imidacloprid	14	14.3	72 - 73.9	n/a	
	Dimethoate	14	7.1	144.5	n/a	
Beebread	Fluvalinate	147	100	3.6 - 469	77.3	Traynor et al., 2016
	Coumaphos	147	90.5	1 - 3260	174	
	Chlorothalonil	147	59.2	1.2 - 26600	2750	
	Pendimethalin	147	45.6	1.1 - 143	18.9	
	DMF (Amitraz)	147	40.1	9.1 - 1117	138.3	
Beebread	Carbendazim	123	30.1	n.a - 44.6	7.1	Pohorecka et al., 2017
	Thiacloprid	123	20.3	n.a - 88.6	5.1	
	Boscalid	123	18.7	n.a - 1030	124.9	
	Pendimethalin	123	17.1	n.a - 286	9.5	
	Acetamiprid	123	15.4	n.a - 32.8	10.3	
Beebread	Thiacloprid	85	52	0.46 - 149.4	n/a	Beyer et al., 2018
	Chlorfenvinphos	85	40	0.57 - 266	n/a	
	Tebuconazole	85	22	2.95 - 52	n/a	
	Methiocarb	85	10	0.74 - 5.26	n/a	
	Flufenacet	85	8	0.74 - 2	n/a	
Pollen	Thiacloprid	154	29.4	0.57 - 133	n/a	Beyer et al., 2018
	Permethrin-cis	154	11.8	2.2 - 39.7	n/a	
	Permethrin-trans	154	10.5	2.75 - 46.8	n/a	
	Azoxystrobin	154	9.2	0.44 - 22.8	n/a	
	Clothianidin	154	7.8	0.39 - 1.4	n/a	

Table 3. Cont

Pollen	Thiacloprid	281	51.6	n/a - 470.4	n/a	Boehme et al., 2018
	Prothioconazole	281	35.6	n/a - 78.6	n/a	
	Boscalid	281	27.4	n/a -1496	n/a	
	Tebuconazole	281	28.8	n/a -484.5	n/a	
	Fluazifop-butyl	281	16	n/a -6832	n/a	
Beebread	Coumaphos	45	88.9	4 - 374	56.2	Calatayud-Vernich., 2018
	Fluvalinate	45	46.7	2 - 72	10.9	
	DMF (Amitraz)	45	37.8	4 - 246	17.6	
	Chlorpyrifos	45	31.1	1 - 100	9.8	
	Chlorfenvinphos	45	26.7	2 - 194	10	
Pollen	Chlorpyrifos	554	30.3	n/a - 179	10	Tosi et al., 2018
	Mandipropamid	554	19.5	1 - 261	9	
	Metalaxyl	554	15.9	n/a -2463	60	
	Spiroxamine	554	15	n/a -18	2	
	Imidacloprid	554	12.5	1 - 19	2	
Beebread	DMF (Amitraz)	33	97	2 - 496	71.2	Calatayud-Vernich et al., 2019
	Coumaphos	33	94	4 - 174	31.6	
	Chlorpyrifos	33	45	2 - 167	16.2	
	Carbendazim	33	30	2 - 29	2.0	
	Acetamiprid	33	27	1 - 19	1.7	
Pollen	Fluvalinate	160	88.3	< LOQ - 25.3	n/a	Fulton et al., 2019
	Coumaphos	160	75.4	3.32 - 338	n/a	
	Chlorothalonil	160	52.9	< LOQ - 130	n/a	
	Chlorpyrifos	160	43.7	4.08 - 4.48	n/a	
	Cyhalothrin	160	10.9	25.2 - 32.4	n/a	

n/a = Not available. LOQ = Limit of quantification.

Table 4. Pesticide residues detected (top 5) in live and dead bees.

Honey bees	Pesticide	Nº of analyzed samples	Detection (%)	Range ($ng \cdot g^{-1}$)	Mean ($ng \cdot g^{-1}$)	Reference
Live	Imidacloprid	187	26.2	n/a	1.2	Chauzat et al., 2009
	Fipronil	187	9.1	n/a	0.7	
	Deltamethrin	307	5.9	n/a	16.9	
	Endosulfan	307	5.5	n/a	8.3	
	Coumaphos	307	4.6	n/a	1545.6	
Dead	Tebuconazole	25	48	10 - 1146	82.7	Walorczyk and Gnusowski, 2009
	Fipronil	25	40	10 - 64	9.9	
	Dimethoate	25	36	238 - 4864	603.5	
	Vinclozolin	25	32	185 - 657	112.6	
	Chlorpyrifos	25	20	10 - 56	4.7	
Live	Fluvalinate	140	83.6	1.1 - 5860	357.7	Mullin et al., 2010
	Coumaphos	140	60	1 - 762	50.4	
	Chlorpyrifos	140	8.6	1 - 10.7	3.4	
	Chlorothalonil	140	7.1	1.5 - 878	100.2	
	Cypermethrin	140	6.4	2 - 25.8	10.1	
Live	Carbendazim	141	41.1	< LOQ - 66.3	2.04	Lambert et al., 2013
	Triphenylphosphate	141	24.8	< LOQ - 61.6	1.95	
	Coumaphos	141	17.8	< LOQ - 47.3	1.04	
	Amitraz	141	16.3	< LOQ - 17	3.07	
	Fluvalinate	141	7.1	< LOQ - 52.9	3.41	
Dead	Cypermethrin	33	45.45	20 - 6300	598	Lozowicka, 2013
	Bifenthrin	33	21.21	20 - 130	13.6	
	Chlorpyrifos	33	18.18	10 - 576576	17705	
	Dimethoate	33	12.12	11 – 7280	247.8	
	Tebuconazole	33	12.12	60 - 1780	85.1	
Dead	Heptenophos	19	68.4	< LOQ - 18.5	n/a	Barganska et al., 2014
	Bifenthrin	19	52.6	< LOQ	n/a	
	Methidathion	19	47.4	< LOQ - 22.4	n/a	
	Diazinon	19	31.6	< LOQ - 13.3	n/a	
	Pyrazophos	19	31.6	< LOQ - 14.3	n/a	
Dead	Clothianidin	44	47.7	0.7 – 39.9	n/a	Kasiotis., et al 2014
	Chlorpyrifos	44	9.09	< LOQ - 46	n/a	
	Thiamethoxam	44	6.8	0.5 - 49.6	n/a	
	Coumaphos	44	4.5	< LOQ - 20	n/a	
	Imidacloprid	44	4.5	0.3 - 5.74	n/a	
Dead	Chlorpyrifos-ethyl	40	40	n/a	92	Porrini et al., 2014
	Fenitrothion	40	27.5	n/a	971	
	Pirimiphos-methyl	40	25	n/a	15	
	Dimethoate	40	15	n/a	16	
	Chlorpyrifos-methyl	40	7.5	n/a	173	
Dead	Coumaphos	34	94	7 - 150	28	Calatayud-Vernich et al., 2016
	Chlorpyrifos	34	79	3 - 751	100	
	Dimethoate	34	68	13 - 403	102	
	Omethoate	34	62	2 - 109	34	
	Imidacloprid	34	32	12 - 223	53	

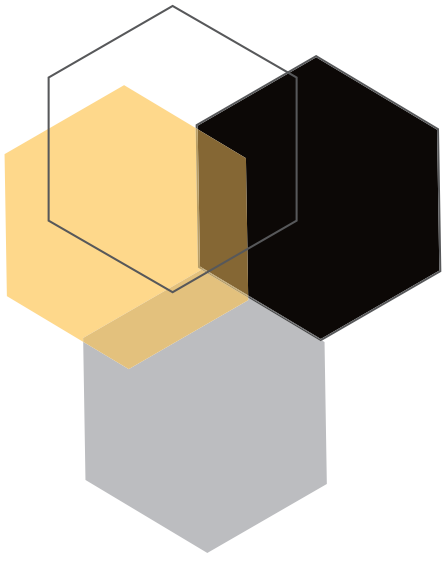
Table 4. Cont

Honey bees	Pesticide	Nº of analyzed samples	Detection (%)	Range (ng·g^{-1})	Mean (ng·g^{-1})	Reference
Dead	Chlorpyrifos	73	54.3	1.5 - 3290	272	Kiljanek et al., 2016
	Dimethoate	73	41.1	1.4 - 1596	399	
	Clothianidin	73	30.1	5.3 - 76.2	17.1	
	Tebuconazole	73	24.7	1.6 - 1245	93.1	
	DMF (Amitraz)	73	24.3	5.9 - 147	40.4	
Live	Fluvalinate	38	81.6	1.1 - 172.6	8.42	Traynor et al., 2016
	Coumaphos	38	23.7	1 - 11	1.1	
	DMF (Amitraz)	38	5.3	171 - 223	26	
	Pendimethalin	38	5.3	25.1 - 27.6	1.25	
	Fipronil	38	2.6	9.9	9.9	
Live	DMF (Amitraz)	343	14	5.9 - 429	37.1	Kiljanek et al., 2017
	Chlorpyrifos	343	12.2	1.2 - 10.7	3.5	
	Tebuconazole	343	5.8	1.3 - 226	36	
	Boscalid	343	5.5	1.3 - 40.1	6.6	
	Thiacloprid	343	4.7	1.3 - 14	3.3	
Dead	Imidacloprid	79	24	15 - 3164	n/a	Martinello et al., 2017
	Chlorpyrifos	79	22	54 - 5154	n/a	
	Fluvalinate	79	20	31 - 2846	n/a	
	Cyprodinil	79	15	28 - 508	n/a	
	Thiacloprid	79	10	15 - 571	n/a	
Dead*	Acetamiprid	155	5.8	n/a - 1.3	1.1	Pohorecka et al., 2017
	Dimethoate	155	3.2	n/a - 1.5	1.3	
	Fenpropimorph	155	3.2	n/a - 1.2	1.2	
	Carbendazim	155	1.9	n/a - 1.3	1.1	
	Imidacloprid	155	1.9	n/a - 5.3	4.1	
Live	Coumaphos	45	33.3	1 - 34	2.4	Calatayud-Vernich et al., 2018
	Fluvalinate	45	26.7	2 - 168	7.2	
	DMF (Amitraz)	45	15.6	1 - 104	3.5	
	Chlorpyrifos	45	8.9	1 - 24	0.6	
	Dichlofenthion	45	2.2	18	0.4	
Live	Coumaphos	38	55.3	2 - 34	5.2	Calatayud-Vernich et al., 2019
	DMF (Amitraz)	38	42.1	2 - 56	11.5	
	Dimethoate	38	5.3	12 - 36	1.3	
	Chlorpyrifos	38	2.6	22	0.6	
	Carbendazim	38	2.6	3	< 0.1	
Dead	Dimethoate	17	76.5	4 - 338	89.9	Calatayud-Vernich et al., 2019
	Omethoate	17	52.9	10 - 48	13.8	
	Chlorpyrifos	17	41.2	2 - 2702	232.9	
	Fluvalinate	17	35.3	6 - 108	19.4	
	Hexythiazox	17	17.6	4 - 266	16.2	
Live	Fluvalinate	288	83.6	< LOQ - 18.8	n/a	Fulton et al., 2019
	Coumaphos	288	60	3.25 - 770	n/a	
	Chlorpyrifos	288	8.6	0.95 - 1.64	n/a	
	Chlorothalonil	288	7.1	< LOQ - 5.82	n/a	
	Cyfluthrin	288	6.9	< LOQ	n/a	

n/a = Not available. LOQ = Limit of quantification. *Dead bee samples were collected from the bottom board of the hives without intoxication signs.

04

EFFECTS OF PESTICIDES RESIDUES ON HONEY BEE COLONIES



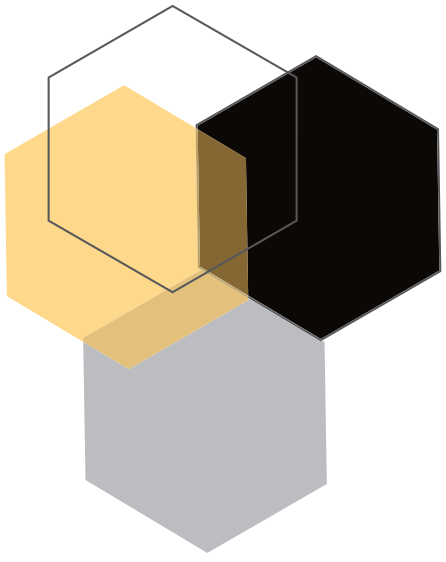
Pesticides in the apiaries are mixtures of compounds used in plant protection and acaricides applied by beekeepers against varroa. Such mixtures can impair immune responses, and combined with parasites, can result in an increased stress with severe consequences for bee health and colony fitness (**Goulson et al., 2015**; **Grassl et al., 2018**). By nature, herbicides are mostly innocuous to bees, while insecticides are the most toxic compounds to honey bees (**Table 1**). Insecticides can provoke acute mortality episodes in the apiaries when sprayed during bloom (**Kiljanek et al., 2016**; **Calatayud-Vernich., 2019**), and reduce foraging performance (**Colin et al.,**

2019), affect the larvae development (**Dai et al., 2019**) and the olfactory-mediated memory (**Urlacher et al., 2016**) when present in hives at sublethal doses. Fungicides are widely detected in hive products and its toxicity to bees is considered low, however, these compounds can reduce the population of beneficial fungi that are crucial in the maturation of pollen into beebread, and impair bee nutrition (**Yoder et al., 2012; Steffan et al., 2017**). Although acaricides are not very harmful, the toxicity of such compounds, when found simultaneously in hives, can alter honey bee immunity, development and nutrition (**Reeves et al., 2018**). Their toxicity is increased in part because of the competition for P450 detoxification enzymes, which is a serious problem because honey bees have fewer number of detoxifying genes compared to other insects (**Gong and Diao, 2017**).

The impact of pesticide residues in bee colonies is a serious and ongoing issue. The lack of up-to-date studies dealing with pesticide occurrence in Spanish apiaries and a global knowledge gap in assessing integrated honey bee exposure to pesticides (**Benuszak et al., 2017**) motivated the aim of the present thesis. Quantifying the threat that pesticides pose to bees is a crucial step in elucidating their potential repercussion on them. The hazard quotient (HQ) is a simple way to estimate such threat by considering all pesticide residues detected in each sample, and translating pesticide residue data into easily understood relationships to the LD_{50}. The hazard quotient (HQ) approach is an additive model that include all pesticide residue concentrations detected in each sample ($ng \cdot g^{-1}$) divided by their respective contact or oral LD_{50} ($\mu g \cdot bee^{-1}$). This model could help to standardize pesticide data results and contribute to clearer communication among scientists, beekeepers and general public about risks posed to honey bees by their exposure to pesticides (**Stoner and Eitzer, 2013**). The thesis here presented pretends to be a valuable contribution to current knowledge on honey bee pesticide exposure and serve as a basis for future research in this field.

05

References



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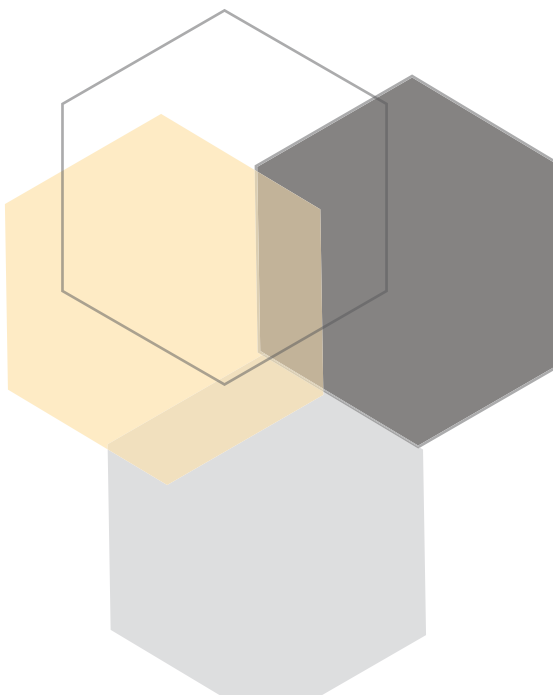
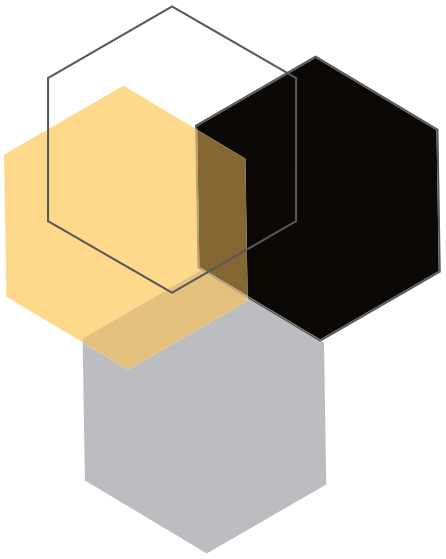


CHAPTER 2:

Show the validation study of the methodology used through a scientific publication. The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) procedure was used for the extraction of the pesticides from samples and HPLC-QqQ-MS/MS for the determination.

ARTICLE 01



EFFICIENCY OF QuEChERS APPROACH FOR DETERMINING 52 PESTICIDE RESIDUES IN HONEY AND HONEY BEES

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Efficiency of QuEChERS approach for determining 52 pesticide residues in honey and honey bees

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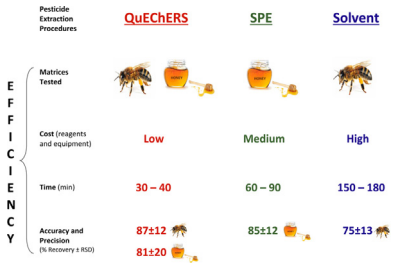
GRAPHICAL ABSTRACT



ABSTRACT

A comparison between QuEChERS and other pesticide extraction procedures for honey and honey bee matrices is discussed. Honey bee matrix was extracted by solvent based procedure whereas solid phase extraction was the protocol for the honey matrix. The citrate buffered QuEChERS method was used for both matrices. The methods were evaluated regarding cost (equipment and reagents), time, accuracy, precision, sensitivity and versatility. The results proved that the QuEChERS protocol was the most efficient method for the extraction of the selected pesticides in both matrices.

- QuEChERS is the most economical and less time-consuming procedure.
- SPE and solvent-based extraction procedures show equivalent recoveries to QuEChERS.
- QuEChERS can be used to extract pesticide residues from both matrices.

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Method names: QuEChERS (quick, easy, cheap, effective, rugged and Safe), Solvent extraction, SPE (solvent phase extraction)
Keywords: QuEChERS, solid phase extraction (SPE), solvent extraction, honey, honey bee, pesticide, LC–MS/MS
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Method details

QuEChERS approach for the extraction of pesticide residues in honey and honey bee matrices [1–3].

1) Weigh 5 g of honey or honey bees into 50 mL centrifuge tubes and add 7.5 mL of water, 10 mL of acetonitrile, 6 g of MgSO4 and 1 g of NaCl. Homogenize the mixture immediately and then, centrifuge for 5 min at 300 rpm.
2) Put 2 mL of the supernatant into another 15 mL centrifuge tube containing 50 mg C18, 50 mg PSA, and 150 mg MgSO4. Vortex the mix and centrifuge it for 5 min at 3000 rpm.
3) Finally, filter the supernatant using a PTFE 13 mm × 0.22 μm into the autosampler vials for LC–MS analysis.

Solvent approach for the extraction of pesticide residues in honey bee matrix [4].

1) Weigh 5 g of honey bees and pound thoroughly in a glass mortar. When homogenized place in a 250 mL flask and mix it vigorously for 10 min with 20 mL of acetone.
2) Filter the mixture in a Kitassato flask through a Buchner funnel of 13 cm with a paper filter packed with a layer of Celite 545 (5–10 mm) and wash the filter cake with 20 mL of acetone.
3) Prepare 100 mL, with 1% weight/volume (w/v) ammonium chloride and 2% volume/volume (v/v) ortophosphoric acid (85%) and add it to the filtrate. Allow it to stand for 30 min with occasional stirring and then filter with Celite 545.
4) After filtration, dilute the sample with 200 mL of 2% aqueous sodium chloride (w/v) and extract twice with 100 mL of dichloromethane.
5) Pass the resultant organic phase through a filter containing anhydrous sodium sulfate and evaporate it to dryness in a rotary evaporator at 35 °C.
6) Dissolve the extract obtained from the honey bee samples in acetone, up to 2 mL, for GC analysis. For LC–MS determination, evaporate to dryness a 1-mL aliquot of the previous extract using a gentle stream of nitrogen and then dissolve it in the same volume of methanol.

Solid phase extraction (SPE) approach for the extraction of pesticide residues in honey matrix [5].

1) Weigh honey (1.5 g) and mix it with 30 mL of hot water (<80 °C). Agitate by a stir bar for 10 min.
2) Pre-condition an Oasis HLB cartridge [poly (divinylbenzene-co-*N*-pyrrolidone)] with 5 mL of methanol and 5 mL of Milli-Q water.
3) Pass the mix through the cartridge at a flow rate of 10 mL min^{-1}.
4) Rinse the cartridge with 5 mL of Milli-Q water.
5) Dry the cartridge under vacuum for 15 min.
6) Elute the retained pesticides by passing 10 mL of methanol–dichloromethane (3:7).
7) Evaporate the eluate to 0.5 mL using a gentle steam of nitrogen.
8) Then, transfer it into 1-mL volumetric flask with methanol, obtaining a final extract in 100% methanol.

Liquid chromatography–mass spectrometry

Inject 5 μL of the extract in the LC–MS/MS according to the conditions already reported [1] and detailed below.

Ionization and fragmentation settings were optimized by direct injection of pesticide standard solutions. MS/MS was performed in the SRM mode using ESI in positive mode. For each compound, two characteristic product ions of the protonated molecule $[M+H]^+$ were monitored, the first and most abundant one was used for quantification, while the second one was used as a qualifier. Collision energy and cone voltage were optimized for each pesticide (Table 1). Nitrogen was used as collision, nebulising and desolvation gas. The ESI conditions were: capillary voltage 4000 V, nebulizer 15 psi, source temperature 300 °C and gas flow 10 L min^{-1}. In order to maximize sensitivity, dynamic MRM was used, with MS_1 and MS_2 at unit resolution and cell acceleration voltage of 7 eV for all the compounds.

Table 1
Dynamic MRM conditions used for LC–MS/MS determination of pesticide residues.

Target Pesticide	t_R[a] (min)	Δ t_R[b]	Precursor Ion	SRM_1[c]	Frag[d] (V)	CE[e] (V)	SMR_2[f]	Frag[d] (V)	CE[e] (V)	SMR_2/SRM_1 (%) (% RSD)[g]
Acetamiprid	2.67	3.21	223	126	111	22	56	111	14	37.4 (12)
Acetochlor	10.07	2	270	224	120	10	148	120	10	46.8 (22)
Alachlor	10.07	2	270	238	80	15	162	80	10	50.4 (13)
Atrazine	6.52	2.63	216	132	120	15	174	120	20	17.3 (14)
Atrazine-desethyl	2.54	2.5	188	146	120	15	104	121	24	29.1 (15)
Atrazine-desisopropyl	1.75	2.08	174	96	120	15	132	120	15	78.6 (13)
Azinphos-ethyl	10.16	1.71	346	97	80	20	137	80	32	83.5 (12)
Azinphos-methyl	8.17	1.24	318	125	80	8	132	80	12	85.4 (11)
Buprofezin	14.5	1.1	306	201	120	10	116	120	15	64.6 (13)
Carbendazim	4.54	4.74	192	160	95	17	132	95	25	11.4 (14)
Carbofuran	4.37	2.91	222	123	120	10	165	70	15	98.0 (9.3)
Carbofuran-3-hydroxy	1.85	2.48	255	163	70	5	220	70	15	90.8 (9)
Chlorfenvinphos	11.74	1.61	359	155	120	10	127	120	15	63.8 (11)
Chlorpyriphos	15.33	2.23	350	350	92	13	198	97	13	78.6 (14)
Coumpahos	14.05	2.15	363	335	134	10	307	134	10	24.8 (10)
Diazinon	11.77	1.89	305	169	128	17	153	128	21	66.3 (12)
Dichlofenthion	14.68	2	315	259	120	10	287	120	5	44 (11)
Dimethoate	2.06	2.59	230	199	80	10	171	80	5	45.3 (12)
Diuron	7.5	1.25	233	72	120	20	160	120	20	3.2 (13)
DMF	5.14	4.5	150	132	111	10	107	111	15	41.6 (16)
Ethion	14.88	1.23	385	199	80	5	171	80	15	35.3 (11)
Fenitrothion	10.03	1.18	278	125	140	15	109	121	12	95.5 (12)
Fenthion	11.51	1.83	279	247	114	5	169	114	13	76.6 (10)
Fipronil	13.33	2.85	437	368	150	15	290	150	25	21.8 (11)
Flumethrin	18.53	1.85	527	267	50	10	239	50	10	48.3 (18)
Fluvalinate	18.11	1.81	503	208	50	10	181	50	26	73.4 (10)
Hexythiazox	15.11	1.15	353	228	120	20	168	120	10	67.4 (9)
Imazalil	11.4	1.71	297	159	120	20	201	120	15	56 (14)
Imidacloprid	1.61	1.96	256	209	80	10	175	80	10	75 (11)
Isoproturon	6.83	2.37	207	72	120	20	165	120	10	16.8 (12)
Malathion	9.36	1.96	331	99	80	10	127	80	5	98.5 (4)
Methiocarb	8.64	1.93	226	121	80	5	169	80	10	66.6 (11)
Metholachlor	10.49	2.04	284	252	120	15	176	120	10	10 (14)
Molinate	9.41	1.98	188	126	80	20	55	80	10	61.7 (11)
Omethoate	1.06	2.67	214	125	80	5	183	80	20	72.3 (12)
Parathion-ethyl	11.11	1.91	292	236	88	4	264	88	8	45.5 (13)
Parathion-methyl	8.17	1.5	264	125	120	20	232	110	5	34.5 (13)
Prochloraz	12.08	1.91	376	308	80	10	266	80	10	14.3 (9)
Propanil	8.6	2.01	218	162	120	20	127	120	15	92.4 (11)

Table 1 (*Continued*)

Target Pesticide	t_R[a] (min)	Δ t_R[b]	Precursor Ion	SRM_1[c]	Frag[d] (V)	CE[e] (V)	SMR_2[f]	Frag[d] (V)	CE[e] (V)	SMR_2/SRM_1 (%) (% RSD)[g]
Propazine	8.74	2	230	146	120	15	188	120	20	93.3 (14)
Pyriproxyfen	14.78	1.33	322	227	120	10	185	120	10	36.1 (12)
Simazine	4.53	1.76	202	124	120	20	132	120	20	93.8 (12)
Tebuconazole	13.82	2.87	308	125	95	25	70	95	21	6.6 (11)
Terbumeton	10.98	2.89	226	170	95	17	114	95	25	13.8 (14)
Terbumeton-desethyl	6.69	3.76	198	142	90	13	86	90	25	31.7 (12)
Terbuthylazine	11.1	3.01	230	174	95	13	96	95	25	16.4 (13)
Terbuthylazine-2-hydroxy	6.92	3.28	212	156	95	13	86	95	25	28 (13)
Terbuthylazine-desethyl	6.98	2.81	202	146	95	13	79	95	25	13.2 (14)
Terbutryn	10.63	1.2	242	186	120	20	71	120	15	4.6 (14)
Thiabendazole	5.06	3.5	202	175	95	25	131	95	25	29.1 (18)
Thiamethoxam	2	2.58	292	211	78	10	132	78	10	21.3 (11)
Tolclofos-methyl	12.13	1.71	301	125	115	12	269	120	15	73.8 (19)

[a] t_R = retention time.
[b] Δ t_R = delta retention time, that is the centered retention time window.
[c] SRM_1 = selected product ion for quantification.
[d] Frag = Fragmentor.
[e] CE = Collision energy.
[f] SRM_2 = selected product ion for qualification.
[g] (%RSD) = relative standard deviation of the ratio SRM_2/SRM_1, calculated from mean values obtained from the matrix-matched calibration curves.

Quality assurance/quality control (QA/QC)

In order to compare QuEChERS to other routine procedures, methods were validated according to the European Union Guideliness [6]. Furthermore, the main elements of uncertainty as the amount of sample used for a determination, the recovery value of the analytical procedure and the repeatability of determinations for a true sample [7], were considered through the validation process (for detailed information of the validation parameters, see Supplementary material Table S1 and S2).

The sensitivity of the method was estimated by establishing the limits of quantification (LOQs) (Fig. 1). The LOQs were determined in pure solvent and in spiked honey and honey bees samples. LOQs were calculated as the lowest concentration or mass of the analyte that has been validated with acceptable accuracy by applying the complete analytical method. LOQs were from 0.2 to 10 ng g^{-1} and

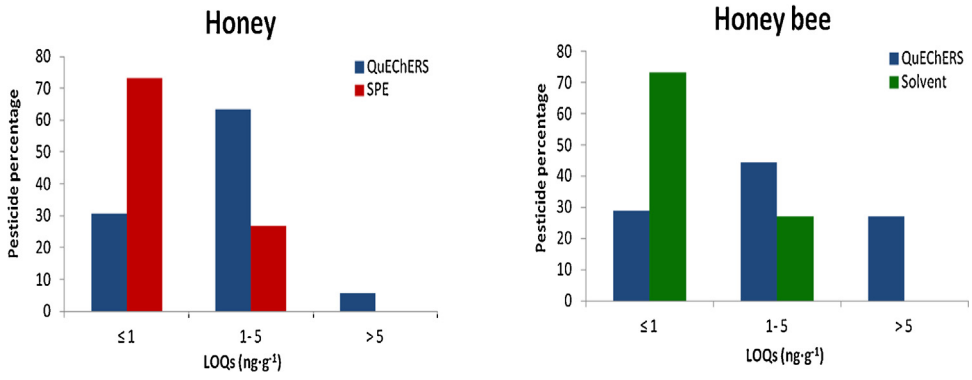

Fig. 1. Limits of quantitation (LOQs) of QuEChERS, SPE and solvent methods in honey and honey bee matrices.

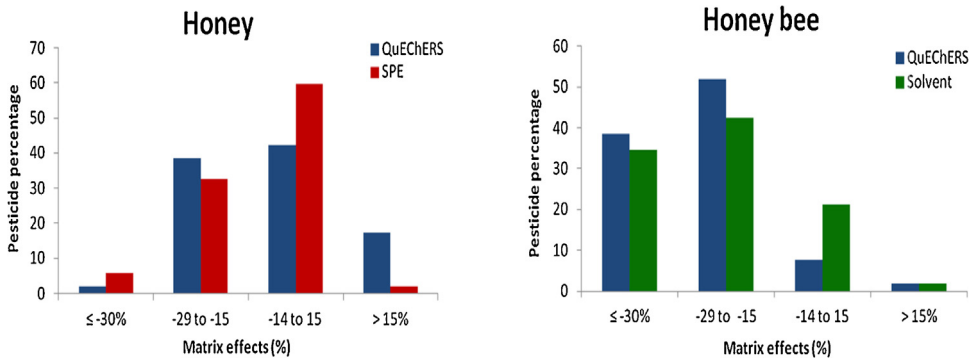

Fig. 2. Matrix effects of QuEChERS, SPE and solvent methods in honey and honey bee matrices.

from 0.03 to 10 ng g^{-1} for honey and honey bee matrices respectively. Solvent and SPE methods were slightly more sensitive than QuEChERS approach.

Matrix effects were evaluated by comparing the slope of the previous calibration curve and the slope of that prepared in the extract of honey or honey bee matrix with six concentration levels of

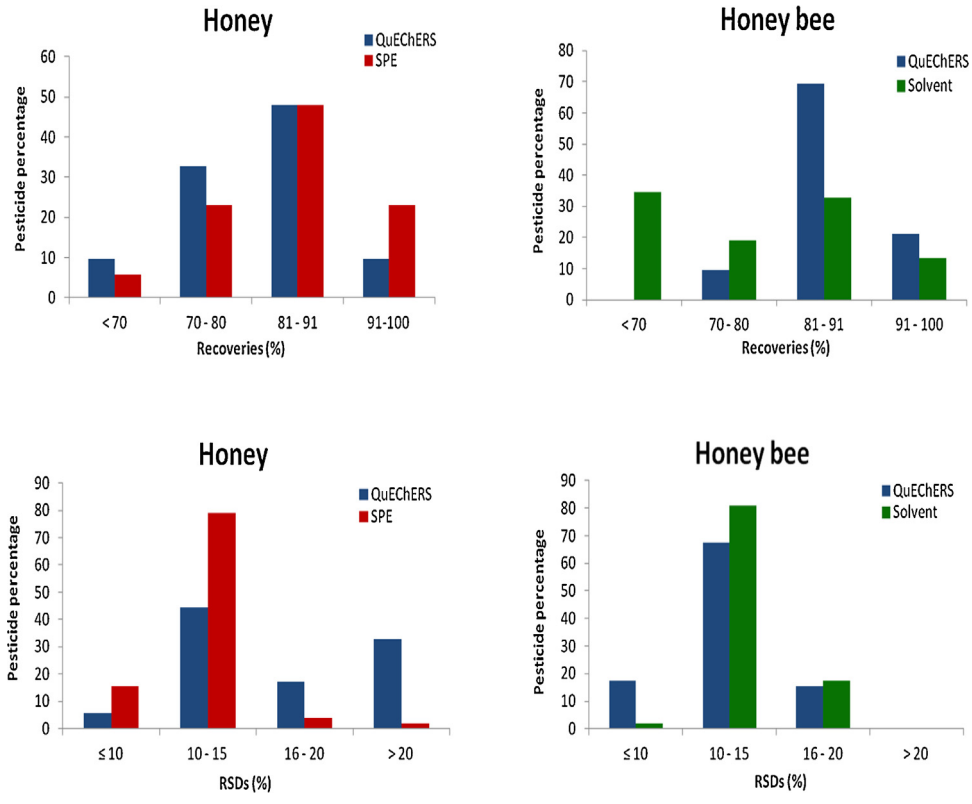

Fig. 3. Accuracy (Recoveries) and precision (RSDs) validation parameters of QuEChERS, SPE and solvent methods in honey and honey bee matrices.

standard solutions (Fig. 2). Matrix effects were mostly suppressive in both matrices and ranged from −60 to 50 and from −60 to 35% in honey and honey bee matrices, respectively.

Mean recovery (as accuracy) and relative standard deviation (as precision) were evaluated by spiking the samples at the LOQ and 10 x LOQ, with a minimum of 5 replicates (Fig. 3). Recovery values of honey bee matrix were from 34 to 96%, whereas RSDs were in all cases <20%. Honey matrix showed recoveries that ranged from 30 to 96% and RDS were <20% except for 17 compounds that were from 21 to 42%. QuEChERS approach showed better results than solvent method in the honey bee matrix while SPE was slightly better both in accuracy and precision than QuEChERS extraction procedure for honey.

Additional information

The use of pesticides in agricultural cropping systems is often discussed as a factor influencing honey bee health [1]. Furthermore, honey, which is considered a healthy natural product, can be contaminated during its production from both agricultural and beekeeping practices [8,5]. The development of extraction procedures able to process samples in an economic way is crucial.

This paper presents some of the currently applied sample preparation methods for the separation and pre-concentration of pesticides in honey and honey bee samples. The composition of honey and honey bees is very different but both are complex matrices. In order to achieve an accurate and reliable analytical result, an efficient pre-concentration/separation step is usually required prior to determination, even when such a sensitive detection method as LC–MS/MS is used.

From an analytical point of view, honey can be considered as a highly concentrated sugar solution (mostly fructose). Then, after water dilution it can be extracted using protocols similar to those applied to water as SPE. The protocol described here requires a medium cost in reagent and equipment because the SPE sorbents involve a high cost. The extraction of a sample requires between 60 and 90 min, being evaporation the step that takes more time. The performance of the method provides the best sensitivity and lower matrix effects.

On the contrary, honey bees are rich in lipids and proteins, requiring most sophisticated and extensive sample preparation methods. Traditional methods as the solvent approach are long, tedious and require high amounts of expensive organic solvents [4]. Considering the use of reagents and equipment this method has high cost, requires between 150 and 180 min to process a sample and provides recoveries slightly lower for more polar pesticides

The results pointed out that QuEChERS approach is used in many different matrices as hive products (beeswax, pollen, honey, honey bee) [9,3,10]. Honey and honey bee composition (Fig. 4) evidence the versatility of the QuEChERS method compared to other extraction procedures as those used in the present work. Appropriate results in terms of specificity, selectivity, accuracy and sensitivity, low cost and quickness make QuEChERS a suitable procedure for determining pesticides in less studied hive matrices as royal jelly and propolis. Furthermore, QuEChERS approach meets important components of green analytical chemistry [11] due to its small amounts of solvent needed compared to the traditional methods.

	Protein (%)	Fat (%)	Sugars (%)	Water (%)	Others (%)
Honey	0.3	0	79.7	17.2	0.7
Honey bee	14.5	7.9	6.3	68.4	2.9

Fig. 4. Honey and honey bee composition (%) [12,13,14].

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mex.2016.05.005.

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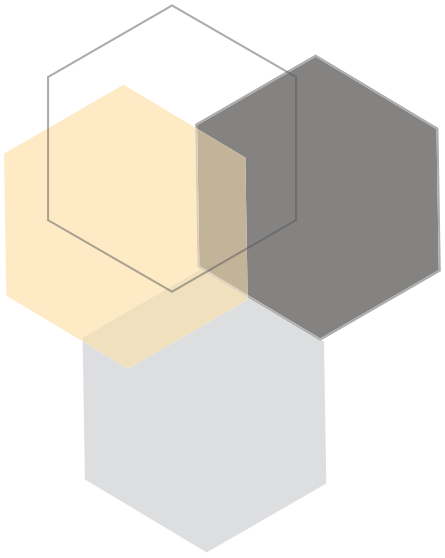
ARTICLE 01



SUPPLEMENTARY MATERIAL:

EFFICIENCY OF QuEChERS APPROACH FOR DETERMINING 52 PESTICIDE RESIDUES IN HONEY AND HONEY BEES

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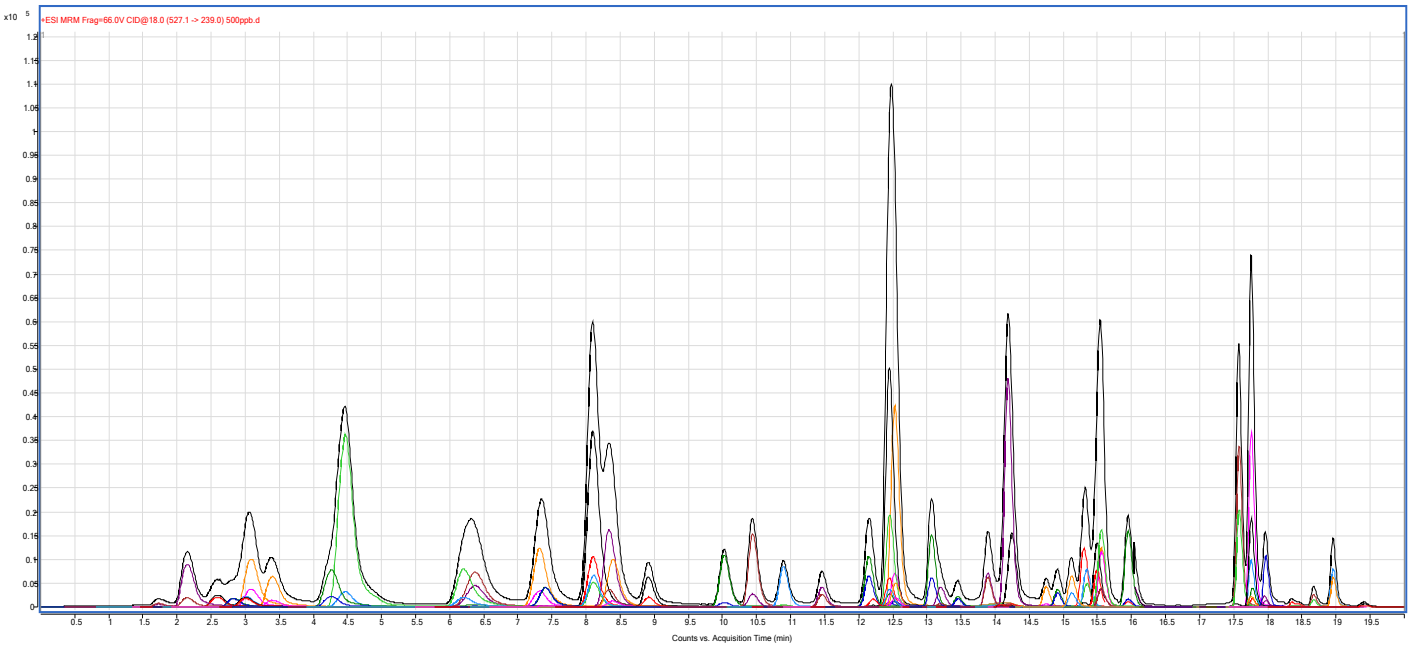


Figure S1. Chromatograms extracted from 500 $\mu g \cdot L^{-1}$ standard of all pesticides analyzed.

Table S1. Validation data of QuEChERS and solvent approaches for honey bee matrix. LOQ, recovery (R), precision (RSD) and matrix effects of the analyzed pesticides.

HONEY BEE						
	QuEChERS			Solvent		
Pesticide	LOQ $ng \cdot g^{-1}$	R ± RSD (%)	Matrix effects (%)	LOQ $ng \cdot g^{-1}$	R ± RSD (%)	Matrix effects (%)
Acetamiprid	3.9	92 ± 12	-25	0.8	56 ± 16	-30
Acetochlor	3.9	95 ± 15	-30	0.8	34 ± 18	-45
Alachlor	3.9	89 ± 13	-35	0.8	34 ± 18	-25
Atrazine	3.9	91 ± 16	-20	1.5	59 ± 17	-30
Atrazine-desethyl	7.5	85 ± 17	-28	1.5	57 ± 14	-55
Atrazine-desisopropyl	7.5	89 ± 15	-32	1.5	52 ± 14	-52
Azinphos-ethyl	3.9	93 ± 12	-15	0.8	96 ± 14	-15
Azinphos-methyl	3.9	78 ± 14	-15	0.8	91 ± 13	2
Buprofezin	1	94 ± 10	-18	0.03	69 ± 12	-13
Carbendazim	10	92 ± 10	-35	2	58 ± 14	-15
Carbofuran	1	73 ± 18	35	0.5	72 ± 12	-25
Carbofuran-3-hydroxy	10	90 ± 15	10	2	64 ± 12	-45
Chlorfenvinphos	10	94 ± 10	-40	2	74 ± 14	-30
Chlorpyrifos	1	95 ± 11	-15	0.04	81 ± 18	1
Coumaphos	3.9	87 ± 12	-10	0.9	88 ± 14	-15
Diazinon	1	83 ± 15	-30	0.06	91 ± 11	-12
Dichlofenthion	3.9	87 ± 12	-22	1.0	87 ± 11	-35
Dimethoate	3.9	88 ± 12	-27	0.8	92 ± 12	-16
Diuron	10	85 ± 11	-38	2	60 ± 16	-23
DMF	1	84 ± 6	-28	0.2	85 ± 12	-32
Ethion	1	88 ± 10	-42	0.2	91 ± 11	-4
Fenitrothion	3.9	83 ± 18	-30	0.8	83 ± 12	-15
Fenthion	10	90 ± 15	-5	2	82 ± 7	-20
Fipronil	1	82 ± 8	-19	0.2	70 ± 16	-15
Flumethrin	3.9	86 ± 8	-25	0.8	83 ± 14	-23
Fluvalinate	1	93 ± 10	-28	0.2	86 ± 15	-15
Hexythiazox	1	85 ± 12	-15	0.2	93 ± 13	-8

Imazalil	3.9	81 ± 10	-30	1	77 ± 12	-24
Imidacloprid	1	91 ± 15	-28	0.5	76 ± 11	-33
Isoproturon	3.9	86 ± 10	-35	0.8	70 ± 11	-23
Malathion	3.9	88 ± 9	-15	0.2	83 ± 12	-5
Methiocarb	10	95 ± 7	-33	1	68 ± 10	-35
Methoalachlor	1	80 ± 15	-22	0.12	76 ± 11	-34
Molinate	10	86 ± 15	-21	2	61 ± 12	-15
Omethoate	1	82 ± 19	-12	0.2	87 ± 13	23
Parathion-ethyl	10	81 ± 18	-16	2	94 ± 12	-7
Parathion-methyl	10	77 ± 15	-18	2	91 ± 13	-10
Prochloraz	3.9	96 ± 8	-24	0.8	92 ± 12	-14
Propanil	1	82 ± 8	-38	0.05	79 ± 15	-18
Propazine	1	78 ± 19	-22	0.1	60 ± 15	-23
Pyriproxifen	10	89 ± 16	-50	2	92 ± 11	-11
Simazine	10	83 ± 10	-60	2	42 ± 18	-56
Tebuconazole	3.9	91 ± 8	-24	0.8	79 ± 14	-25
Terbumeton	3.9	82 ± 10	-33	0.8	62 ± 13	-23
Terbumeton-desethyl	1	85 ± 10	-28	0.1	51 ± 14	-32
Terbuthylazine	3.9	89 ± 15	-38	0.8	60 ± 13	-27
Terbuthylazine-2-hydroxy	3.9	97 ± 10	-40	1	92 ± 13	-31
Terbuthylazine-desethyl	3.9	82 ± 10	-38	1	90 ± 10	-34
Terbutryn	3.9	87 ± 10	-22	0.8	59 ± 14	-43
Thiabendazole	10	82 ± 11	-25	2	80 ± 10	-22
Thiamethoxam	3.9	84 ± 9	-30	0.8	81 ± 15	-31
Tolclofos-methyl	3.9	90 ± 10	-20	0.8	85 ± 17	-15

Table S2. Validation data of QuEChERS and SPE approaches for honey matrix. LOQ, recovery (R), precision (RSD) and matrix effects of the analyzed pesticides.

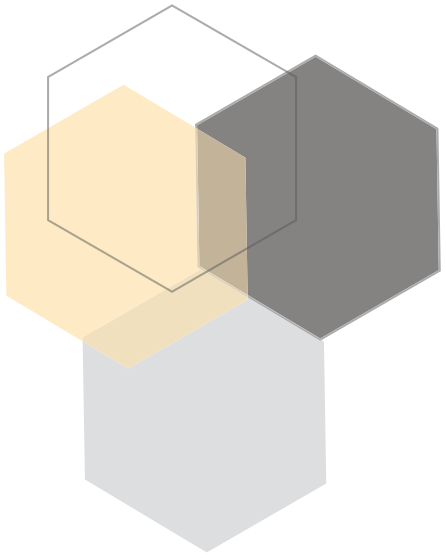
HONEY						
	QuEChERS			SPE		
Pesticide	LOQ $ng \cdot g^{-1}$	R ± RSD (%)	Matrix effects (%)	LOQ $ng \cdot g^{-1}$	R ± RSD (%)	Matrix effects (%)
Acetamiprid	3.5	90 ± 11	-18	1	94 ± 11	-5
Acetochlor	2.5	90 ± 18	23	1	92 ± 9	-8
Alachlor	2.5	78 ± 33	20	1	94 ± 11	-15
Atrazine	3	80 ± 18	-12	1	94 ± 10	-10
Atrazine-desethyl	5	76 ± 19	-15	2	78 ± 13	-30
Atrazine-desisopropyl	6	89 ± 41	-25	2	63 ± 13	-25
Azinphos-ethyl	3	94 ± 11	-8	1	90 ± 10	1
Azinphos-methyl	3	70 ± 13	-4	1	74 ± 13	3
Buprofezin	1	94 ± 11	-14	1	93 ± 10	-2
Carbendazim	5	79 ± 18	-35	5	82 ± 7	-8
Carbofuran	1	56 ± 15	26	0.5	90 ± 11	-3
Carbofuran-3-hydroxy	10	96 ± 28	15	2	80 ± 15	17
Chlorfenvinphos	5	91 ± 16	-8	2	81 ± 12	-15
Chlorpyrifos	0.5	87 ± 15	3	0.5	87 ± 12	-1
Coumaphos	2	88 ± 13	-12	1	91 ± 16	-4
Diazinon	0.5	73 ± 22	-10	0.5	89 ± 11	-17
Dichlofenthion	2	80 ± 14	-8	1	87 ± 13	-21
Dimethoate	1.5	82 ± 21	15	1	42 ± 12	-8
Diuron	5	80 ± 18	-15	2	85 ± 12	-10
DMF	1	85 ± 10	-22	0.5	85 ± 21	-24
Ethion	0.5	77 ± 23	-14	0.5	86 ± 13	-34
Fenitrothion	2	30 ± 16	4	2	88 ± 11	-15
Fenthion	5	79 ± 22	12	3	97 ± 16	-8
Fipronil	1	81 ± 8	-16	0.5	80 ± 10	-14
Flumethrin	3	88 ± 12	-21	0.5	78 ± 13	-15
Fluvalinate	1	95 ± 10	-25	0.1	78 ± 12	-10
Hexythiazox	0.5	78 ± 23	-2	0.5	89 ± 11	-15

Imazalil	1	82 ± 14	25	2	92 ± 12	-5
Imidacloprid	0.5	84 ± 25	24	0.5	82 ± 9	-7
Isoproturon	2	82 ± 14	-25	1	97 ± 10	-15
Malathion	3	70 ± 29	-4	1	92 ± 14	-1
Methiocarb	5	91 ± 7	-12	2	80 ± 11	-4
Methoalachlor	0.5	69 ± 23	15	0.5	75 ± 11	-15
Molinate	5	68 ± 17	-12	1	91 ± 10	-8
Omethoate	0.5	63 ± 24	33	0.2	83 ± 12	-12
Parathion-ethyl	5	84 ± 18	-14	2	89 ± 8	-8
Parathion-methyl	5	82 ± 14	-17	2	93 ± 8	-5
Prochloraz	2	81 ± 15	-12	1	91 ± 10	-6
Propanil	1	82 ± 26	-22	0.2	77 ± 13	-15
Propazine	1	78 ± 13	-15	0.3	90 ± 9	-5
Pyriproxifen	5	89 ± 12	-12	1	77 ± 11	-14
Simazine	10	79 ± 14	-60	2	75 ± 12	-17
Tebuconazole	2	85 ± 18	50	1	92 ± 8	-5
Terbumeton	2	83 ± 9	-21	1	95 ± 9	-8
Terbumeton-desethyl	0.5	82 ± 11	-25	0.4	90 ± 10	-26
Terbuthylazine	2	74 ± 25	-27	1	89 ± 12	-12
Terbuthylazine-2-hydroxy	3	99 ± 30	-24	1	61 ± 15	-8
Terbuthylazine-desethyl	2	82 ± 13	-15	1	80 ± 13	-32
Terbutryn	3	74 ± 26	-15	0.4	83 ± 11	-15
Thiabendazole	5	74 ± 15	22	2	86 ± 11	-12
Thiamethoxam	3	84 ± 11	-25	1	86 ± 12	-24
Tolclofos-methyl	3.9	89 ± 13	-15	1	86 ± 11	-15

CHAPTER 3:

Study the pesticide residues in beeswax, pollen and honey bees from 45 different apiaries. The results give a detailed profile about pesticide content in Spanish beehives and allow to evaluate the hazard that pose to honey bee through HQ.

ARTICLE 02



PESTICIDE RESIDUES IN HONEY BEES, POLLEN AND BEESWAX: ASSESSING BEEHIVE EXPOSURE.

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Pesticide residues in honey bees, pollen and beeswax: Assessing beehive exposure

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ABSTRACT

In order to study the distribution of pesticide residues in beekeeping matrices, samples of live in-hive worker honey bees (*Apis mellifera*), fresh stored pollen and beeswax were collected during 2016–2017 from 45 apiaries located in different landscape contexts in Spain. A total of 133 samples were screened for 63 pesticides or their degradation products to estimate the pesticide exposure to honey bee health through the calculation of the hazard quotient (HQ). The influence of the surrounding environment on the content of pesticides in pollen was assessed by comparing the concentrations of pesticide residues found in apiaries from intensive farming landscapes to those found in apiaries located in mountainous, grassland and urban contexts. Beeswax revealed high levels of miticides used in beekeeping such as coumaphos, chlorfenvinphos, fluvalinate and acrinathrin, which were detected in more than 75% of samples. Pollen was predominantly contaminated by miticides but also by insecticides used in agriculture such as chlorpyrifos and acetamiprid, which showed concentrations significantly higher in apiaries located in intensive farming contexts. Pesticides residues were less frequent and at lower concentrations in live honey bees. Beeswax showed the highest average hazard scores (HQ > 5000) to honey bees. Pollen samples contained the largest number of pesticide residues and relevant hazard (HQ > 50) to bees. Acrinathrin was the most important contributor to the hazard quotient scores in wax and pollen samples. The contributions of the pesticides dimethoate and chlorpyrifos to HQ were considered relevant in samples.

1. Introduction

Pollination is a crucial process in terrestrial ecosystems. Most of the flowering plants species need pollinators to survive, and insect pollination is necessary for 35% of crops destined for human food (Ollerton et al., 2011; Klein et al., 2007). In Europe, the 84% of the crop species need pollinators —especially honey bees— to guarantee an optimum productivity (Gallai et al., 2009). Beekeeping, as source of managed pollinators, is an essential sector for agriculture and rural environments where wild pollinators are too sparse. However, during the last decades honey bee colonies have suffered a worldwide decline in their populations (van Engelsdorp et al., 2008; Porrini et al., 2016; Seitz et al., 2016). Global effects of varroa parasite (*Varroa destructor*) and associated viruses (Le Conte et al., 2010; Wells et al., 2016; Benaets et al., 2017), nutritional deficiencies (Tritschler et al., 2017; Annoscia et al., 2017), and pesticides appears to be the main causes of honey bee morbidity and mortality (Kasiotis et al., 2014; Calatayud-Vernich et al., 2016; Porrini et al., 2016; Sánchez-Bayo et al., 2016; Traynor et al., 2016).

Honey bees can patrol extensive areas during their foraging flights in search of nectar and pollen. Sprayed crops are visited by honey bees and pesticides are transported inside the hive, where both, agrochemicals from plant protection and those used in-hive against varroosis by beekeepers are deposited in pollen, honey, beeswax and honey bees. For that reason, honey bees are good sentinels of environmental contamination (Niell et al., 2015; Gomez-Ramos et al., 2016). Pesticide presence in hive matrices have been reported worldwide (Ghini et al., 2004; Chauzat et al., 2011; Mitchell et al., 2017). In the USA, fluvalinate and coumaphos miticides and the insecticide chlorpyrifos were the most

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frequently detected pesticides, which had the highest concentrations in honey bees, beeswax and pollen (Mullin et al., 2010). France surveys detected the widely used fungicide carbendazim and the acaricides amitraz and coumaphos in honey bees and pollen (Lambert et al., 2013). Italian beekeeping matrices were contaminated with the miticides chlorfenvinphos, coumpahos and amitraz, and the insecticide chlorpyrifos was detected in pollen samples (Boi et al., 2016; Tosi et al., 2017b). Fluvalinate and coumaphos were the miticides most frequently found in Belgian beeswax (Ravoet et al., 2015). In Slovenia, coumaphos appeared in honey bee brood and beeswax (Bajuk et al., 2017). In Uganda, beekeeping matrices contained the fungicides carbendazim and cyprodinil, together with the organophosphate fenitrothion (Amulen et al., 2017). In Spain, the EU country with the highest hive census (Agriculture and rural development - EC, 2017), beekeeping matrices showed a similar pattern of contamination. Spanish beeswax was contaminated by coumaphos, fluvalinate and chlorfenvinphos miticides (Serra-Bonvehi and Orantes-Bermejo, 2010; Calatayud-Vernich et al., 2017). Acrinathrin and amitraz levels in beeswax have been increasing during the last years (Herrera-López et al., 2016; Calatayud-Vernich et al., 2017). Furthermore, high levels of the organophosphates dimethoate and chlorpyrifos, together with imidacloprid in honey bee dead bodies were reported during intoxication episodes (Calatayud-Vernich et al., 2015).

Given that honey bees appear to be deficient in detoxifying enzymes they can be regarded as susceptible to pesticides (Atkins, 1992; Claudianos et al., 2006). Many studies have demonstrated adverse effects of pesticides ranging from acute poisoning episodes that produce high mortality rates to chronic exposure to pesticides that can impair honey bee flight ability (Tosi et al., 2017a), sperm viability (Chaimanee et al., 2016) and larvae survival (Tavares et al., 2017). Pesticides can also alter gene expression (Wu et al., 2017) and affect honey bee immunocompetence (Di Prisco et al., 2013).

In view of these concerns, the present work aimed at evaluating the pesticide occurrence in three different beekeeping matrices (live in-hive worker bees, fresh stored pollen and beeswax), to study possible influences of the surrounding environment in the pollen pesticide content, and to discuss the potential risks of pesticide exposure to honey bee health. Hive matrix little reported in the literature such as beeswax was studied because its capacities for long-term pesticide storage (Benuszak et al., 2017). Furthermore, pollen was analyzed because is the only source of protein, and essential for the immunocompetence of the honey bees (Di Pasquale et al., 2013). Live worker bees were included in this study due to the lack of literature analyzing residues in living honey bees from the inside of the hive. Methodology used in this study (LC-MS) has been widely used to detect pesticides in beekeeping matrices (Kasiotis et al., 2014; Calatayud-Vernich et al., 2016; Herrera-López et al., 2016; Daniele et al., 2017). Further, LC-MS offers a wider scope and better sensitivity than GC-MS when analyzing most of the selected pesticides (Alder et al., 2006). Compounds included in the analysis were the most relevant miticides used by beekeepers against varroa parasite as well as many insecticides, fungicides, herbicides and nematicides extensively used in crop protection.

2. Material and methods

2.1. Study area and sampling

Beeswax, fresh stored pollen and live in-hive worker bees were collected in June and July during 2016–17 from 45 apiaries in 39 locations in Spain that covered a wide range of landscapes, from intensive farming areas to grasslands, holm oak woodlands, mountainous and urban surroundings (Fig. 1). At each apiary, five hives were selected for the sampling. These hives were free of any veterinary treatment during the collecting period and bee colonies were apparently healthy. Beeswax samples (n = 43) were obtained by cutting a comb portion from each of five selected hives at a given location and pooled together to obtain a single sample

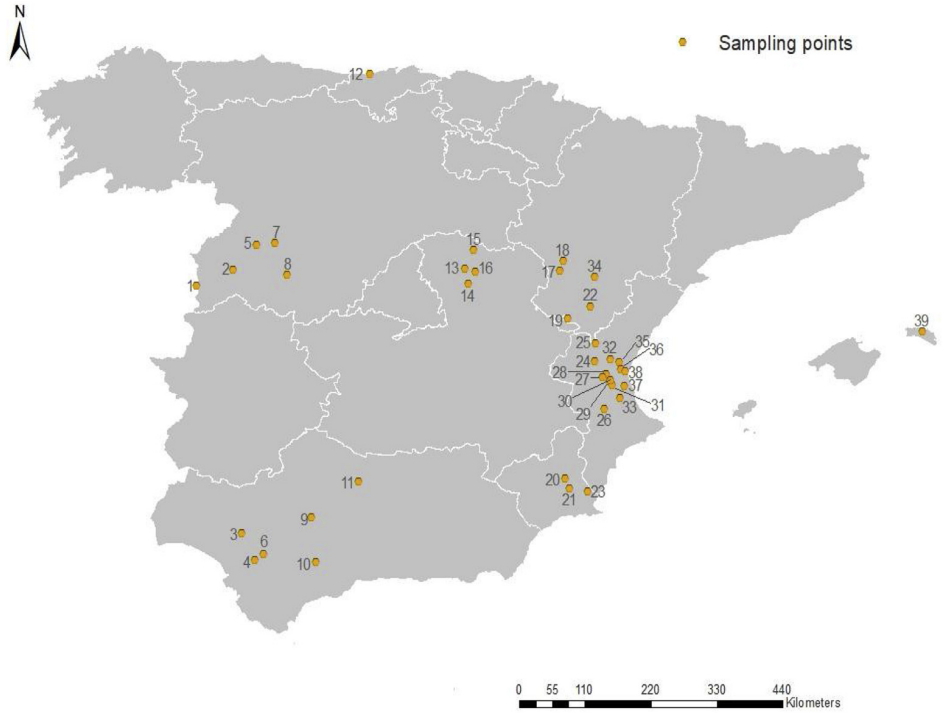

Fig. 1. Situation of the apiaries distributed in 39 Spanish locations.

representative of each apiary. Recently-stored pollen samples (n = 45) were taken from combs using disposable wooden sticks (1 stick per hive, and with five samples from the same apiary pooled in the same way). Since honey bees preferentially consume freshly-stored pollen (Carroll et al., 2017), then, the hazard score calculations based on pesticide loads from this type of pollen are more relevant than those obtained from residues found in old beebread. In order to assess the influence of the surrounding environment, the pollen samples were classified in 2 groups according to their origin, surroundings dominated or not by agricultural landscapes. A proportion of agricultural area was used to define the two groups. Surroundings with more than 50% of agricultural areas were considered as high, and surroundings with less than 50% as low (a detailed characterization of the samples environment is provided in Table 1). Worker bee samples (n = 45) taken from lateral combs were also a pool of bees from 5 hives. Bees were not collected nearby brood nest to avoid new emerging individuals. The samples were transported to the laboratory in an insulated cooler and stored at −20 °C until analysis.

2.2. Chemicals and reagents

High purity (98–99.9%) standards of the 60 selected pesticides together with the transformation products of amitraz; 2,4-dimethylaniline (DMA), 2,4-dimethylphenylformamide (DMF) and N-(2,4-dimethylphenyl)-N′-methylformamidine (DMPF) were from Sigma-Aldrich (Steinheim, Germany) (listed in supplementary material Table S1). Individual standard solutions were prepared in methanol at a concentration of 1000 mg L^{-1}. The working standard solutions were prepared by mixing the appropriate amounts of individual standard solutions and diluting them with methanol to a final concentration of 1 and 10 mg L^{-1}. Solutions were stored in 15 mL vials at 4 °C in the dark. Magnesium sulfate was obtained from Alfa Aesar (Karlsruhe, Germany), ammonium formate, sodium hydroxide, sodium chloride, acetonitrile and formic acid were purchased from Sigma-Aldrich (Steinheim, Germany). PSA and C18 sorbents, and PTFE (13 mm × 0.22 μm) filters were purchased from Análisis Vínicos S.L. (Tomelloso, Spain). Methanol was obtained from VWR chemicals (Radnor, Pennsylvania). Deionized water was from a MilliQ SP Reagent Water System (Millipore, Bedford, MA, USA).

2.3. Analysis

The samples were extracted by a slightly modified QuEChERS procedure and screened for 63 pesticides and its degradation products by liquid chromatography mass spectrometry (LC-MS/MS). The QuEChERS protocol using acetonitrile as extraction solvent and primary-secondary amine (PSA) and C18 as cleaner sorbents was applied to honey bees, pollen and beeswax samples (see Supplementary material) (Garrido Frenich et al., 2008). Beeswax extraction procedure adapted from Niell et al. (2014), and honey bee extraction protocol used were validated in previous works available online (Calatayud-Vernich et al., 2015; Calatayud-Vernich et al., 2017). Pollen was extracted using the same method as for honey bees and the validation parameters are described in Quality Assurance and Quality Control (QA/QC) section.

The chromatographic instrument was an HP1200 series LC equipped with an automatic injector, a degasser, a quaternary pump and a column oven-combined with an Agilent 6410 triple quadrupole (QQQ) mass spectrometer with an electrospray ionization (ESI) interface (Agilent Technologies, Waldbronn, Germany). Data were processed using a MassHunter Workstation Software for qualitative and quantitative analysis (A GL Sciences, Tokyo, Japan).

2.4. Quality Assurance/quality control (QA/QC)

The pollen multiresidue method was evaluated regarding sensitivity, accuracy, precision and robustness according to SANTE

Table 1
Detailed information of the apiaries environment.

Apiaries	Nº samples			Apiaries environment	Agricultural surroundings proportion[a]
	Wax	Pollen	Bees		
1, 2,5,7,8	5	5	5	Rural-grassland landscapes - Holm oak grasslands - Sunflower and cereals crops (oat, wheat and barley)	Low
3,4a-b-c,6,9,10,11	8	8	8	Intensive farming landscape - Drylands crops: sunflower and cereals (wheat, canola cotton) - Mediterranean vegetation	High
12	–	1	1	Mountainous landscape - Ash, laurel, hazel, maple,poplar, birch, eucalyptus and oak mixed forests - Grasslands	Low
13,14,15,16,17,18,19,22,34	9	9	9	Mountainous landscape - Pines, holms oaks, and mediterranean-continental vegetation - Some scattered cereal crops (oat, wheat)	Low
20,21a-b-c, 23,33,35,37	8	8	8	Intensive farming landscape - Irrigation crops: citrus and persimmon - Mediterranean vegetation	High
24,25	2	2	2	Mountainous landscape -Pine forests and mediterranean vegetation -Some scattered dryland crops: vineyard,olive, carobs and almonds	Low
26a-b,27,28,29a-b,30,31,32,36	10	10	10	Intensive farming landscape - Irrigation crops: citrus and nectarines - Drylands crops: vineyard,olive, carobs and almonds - Mediterranean vegetation	High
38	1	1	1	Urban-horticultural landscape - Horticulture (tomatoe, zucchini, cucumber) - Rice crops - Urban ornamental gardens	Low
39	–	1	1	Rural-grassland landscapes - Mediterranean vegetation - Cereal crops (oat, wheat, barley)	Low

[a] Surroundings with more than 50% of agricultural areas were considered as high, and surroundings with less than 50% as low.

guidance document on analytical quality control and validation procedures for pesticides (SANTE/11945/2015) (Table S1).

The linearity of the MS/MS method was established with seven calibration points, using external standards over a concentration range of 10–500 ng·mL^{-1}. The peak area of target analytes was calculated using Mass Hunter software (Agilent). Each point was obtained as the mean of three injections. The data were fit to a linear least-squares regression line with a 1/x weighting, and not forced through the origin. The R-squared was >0.99 with residuals <30%. Matrix effects were evaluated by comparing the slope of the previous calibration curve and the slope of that prepared in the extract of the matrix validated with seven concentration levels of standard solutions. To validate the method and to quantify the samples, matrix matched standards prepared in pollen extracts were used.

The sensitivity of the method was estimated by establishing the limits of detection (LODs) and quantification (LOQs) (Table S1). LODs were calculated using standard solutions prepared in spiked samples that were free of pesticides. As it was difficult to find a sample without any of the selected pesticides, if one compound was initially in the samples (e.g. coumaphos), another pollen sample free of the compound was used to establish LODs and LOQs for it. The LODs were determined as the lowest pesticide concentration whose qualified transition (SRM2) presented a signal-to-noise ratio (S/N) $\geq$ 3. The LOQs were determined also in spiked samples as the minimum detectable amount of analyte with S/N $\geq$ 10 for the quantifier (SRM1) transition. All the LOQs were verified spiking the samples and analyzing them. Recovery, as accuracy, and precision, expressed as relative standard deviation (RSD), were determined by analyzing quintuplicate samples spiked at 10, 50 and 100 ng g^{-1}. The average recoveries values at 10, 50 and 100 ng g^{-1} spiked levels were 90, 86 and 91%, respectively. Recovery values ranged from 70 to 116%, and only 7% of the compounds produced recoveries between 55 and 69%. Precision, expressed as relative standard deviation (RSD), was <20% in most pesticides analyzed. Limits of detection (LOD) were lower than 2 ng g^{-1} and limits of quantification (LOQ) were below 5 ng g^{-1} for all pesticides. Matrix effects were mostly suppressive and ranged from-54 to 50 (Table S1).

2.5. Calculating hazard

In order to evaluate the pesticide exposure in the studied beekeeping matrices, the hazard quotient scores (HQ = pesticide concentration in ng·g^{-1} ÷ pesticide topical/oral LD_{50} as μg/bee) proposed by Stoner and Eitzer (2013), were calculated (Tables S5–S6). This is, the sum of all pesticide residue concentrations detected (ng·g^{-1}) divided by their respective contact or oral LD_{50} in μg/bee for each residue in a given sample. The HQ score provides an estimate based on percentages of LD_{50} equivalents present in pollen or wax samples. As pollen is an essential nutrient for honey bee colony members, the pollen hazard quotients were calculated with oral acute LD_{50} values (if an oral LD_{50} value was not available, then a contact LD_{50} value was used instead). If we consider an individual pollen consumption of 100 mg by a nurse bee during the first 8–10 days of life (Rortais et al., 2005), then a nurse bee that consumed a pollen with a HQ of 1000 would have consumed approximately 10% of the LD_{50} for the pesticide during development stage. The HQ provides an easy tool to understand the potential risk to honey bees of measured pesticide load, establishing a simple relationship to the LD50. HQ scores in pollen could underestimate the pesticide exposure because in the HQ approach the toxic effects are considered additive, and the toxic synergistic effects between compounds are not contemplated. In beeswax matrix, pesticides residues are embedded in a polymer matrix and only a fraction of the molecules (e.g. those on the surface) may become in contact with the bees, therefore the HQs calculated for this matrix overestimate the real exposure to pesticides. The hazard quotients for beeswax were calculated using the contact acute LD_{50}. Pollen samples had a relevant HQ score when it was greater than 50, and the HQ score was considered as elevated when it was greater than 1000. HQ in beeswax samples was considered as relevant when it was greater than 250 because pesticide contact through this matrix is poorly understood. Samples with $HQ_{wax} > 5000$ were considered to have an elevate pesticide load (Traynor et al., 2016). Pesticides LD_{50} used for the hazard quotient were taken from Sanchez-Bayo and Goka (2014), and University of Hertfordshire Pesticide Properties Database (Hertfordshire, 2017). Amitraz concentrations in the samples were calculated through its main breakdown products DMF and DMPF (Korta et al., 2001). Amitraz parent compound ecotoxicological data was used to HQ calculations when detected. Dichlofenthion pesticide was excluded from the hazard quotient because no honey bee ecotoxicological data was available (see Supplementary material).

2.6. Statistical analysis

In order to evaluate the influence of the surrounding environment in pollen pesticide content, samples of pollen were classified according to their origin in 2 groups, surroundings dominated or not by agricultural landscapes. A proportion of agricultural area in the surroundings with more than 50% was considered as high, and surroundings with less than 50% as low. Pesticide residues were compared between both groups. The IBM SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The Shapiro-Wilk test was used to determine whether the data fit the normal distribution. For the different groups comparison, the U Mann–Whitney non parametric test ($P \leq 0.05$) was applied because normality of the data could not be assumed.

3. Results and discussion

3.1. Pesticide residues in beekeeping matrices

Pesticide residues detected in beeswax, fresh stored pollen and live in-hive worker bees samples showed that miticides used by beekeepers against the ectoparasite varroa were the main source of hive matrices contamination. Authorized active subtances used in beekeeping against varroa, such as coumaphos, fluvalinate, flumethrin and amitraz degradates (DMF and DMPF) were detected. Two non-authorized products against varroosis such as acrinathrin and chlorfenvinphos were also detected in the three matrices. Although acrinathrin is also used in agriculture, high levels found in beeswax and pollen could indicate an irregular use of this pyrethroid by beekeepers together with the organophosphate chlorfenvinphos in some apiaries (Tables S2–S4). Pesticides used in agriculture and transported to the colony by forager honey bees were less frequent, and represented the other source of hive contamination. Our sampling period could be in part responsible of this fact, considering that the use of pesticides in plant protection is less frequent during summer season in Spain. Chlorpyrifos, dimethoate, acetamiprid, hexythiazox and pyriproxyfen agricultural pesticides were present in the samples. Pesticides dichlofenthion, carbendazim and fenitrothion, not approved in the EU through Regulation (EC) 1107/2009 (2013), were detected in few samples of pollen and honey bees. Frequency and concentrations found in the study were low, however the spraying with these illegal pesticides in the surrounding environment of the apiaries could not be discarded.

3.2. Honey bees

The 45 honey bee samples analyzed in this study were contaminated with 7 different pesticides, and the highest number of pesticides found per bee was 4, detected in 3 samples (Table 2). The acaricides used in beekeeping coumaphos, fluvalinate and amitraz (DMF) were the most frequently detected at mean concentrations of 2.4, 7.2 and 3.5 ng g^{-1}, respectively. Honey bees are more in contact with acaricides used in the hives and are therefore more exposed to these than to pesticides applied on crops. These miticides were also found in honey bee samples around Europe (Lambert et al., 2013; Porrini et al., 2016). The organophosphate chlorpyrifos was the agricultural insecticide most frequently detected (8.9%), as occurred in North American (Mullin et al., 2010) and Spanish apiaries (Calatayud-Vernich et al., 2015). Previous studies in Spain and Poland related the use of this organophosphate in the surroundings of apiaries to honey bee poisoning episodes (Calatayud-Vernich et al., 2015; Pohorecka et al., 2017). Dichlofenthion, chlorfenvinphos and acrinathrin, were detected once at concentrations below 20 ng g^{-1} (average in the samples was <1 ng g^{-1}). With a common frequency of 1 pesticide per sample and 22 samples pesticide-free (Table S2 Supplementary Material), honey bees are the less contaminated matrix. These results are in agreement with previous studies (Mullin et al., 2010; Lambert et al., 2013).

The analysis of healthy and alive honey bees collected from the inside of the hives underestimate their real contact with pesticides and gives a biased vision of pesticides exposure in honey bees. Residues in bees are an indication that they are really exposed at least to the pesticides found in their bodies, but probably to many more. Biotransformation and rapid excretion could reduce pesticide load in their bodies. Moreover, when exposed to sublethal doses of pesticides, forager honey bees often disorient and are unable to realize the homing-flight (Vandame et al., 1995; Tosi et al., 2017a). So, honey bees with considerable pesticide loads are lost in the fields and excluded from the analysis.

3.3. Pollen

Fresh stored pollen analysis showed 14 different pesticide residues, with 8 pesticides derived from agricultural use and 6 used in beekeeping (Table 3). One sample contained 10 different pesticide residues and 4 samples were pesticide-free, 16 samples had more than 3 pesticides and an average of 3 pesticides per sample was detected (Table S3 Supplementary Material). As in honey bees, the most frequently detected pesticides were coumaphos, fluvalinate and amitraz degradate DMF, found in 88.9, 46.7 and 37.8% of samples, and which mean concentrations were 56.2, 10.9 and 17.6 ng g^{-1}, respectively. Chlorfenvinphos, acrinathrin and amitraz degradate DMPF mean concentrations were 10, 16.8 and 1.2 ng g^{-1}, respectively. Chlorpyrifos was the most frequent insecticide found in hive matrices, and in pollen was detected in 31% of samples at a mean concentration of 9.8 ng g^{-1}. The agricultural pesticides acetamiprid, dimethoate, hexythiazox, dichlofenthion, carbendazim, fenitrothion and pyriproxyfen were detected in frequencies ranging from 2 to 11% of samples and at concentrations up to 190 ng g^{-1}. Recent stored pollen was the most contaminated hive product regarding the number of pesticides detected. This observation agrees with those also reported by French and Italian studies (Porrini et al., 2016; Daniele et al., 2017). The European ban of common neonicotinoids like imidacloprid, clothianidin and thiamethoxam, implemented before the sampling reported here took place, explain the absence, in part, of these three products in the samples.

Pollen, collected and transported from field to hive by forager honey bees, is known to contain pesticides used in agriculture as several studies have demonstrated (Krupke et al., 2012; David et al., 2016; Hakme et al., 2017). Once the pollen is stored in honeycombs, can also be contaminated with other pesticides present in wax. The stored pollen analyzed in the present study revealed the presence of compounds used in-hive before sampling (amitraz) and not applied in apiaries for months (coumaphos), thus indicating that beeswax can act as a source of contamination of incoming pollen. Results from Tosi et al. (2017b) showed that pollen, collected outside the hives from returning foragers honey bees, was only contaminated by pesticides applied in agriculture. Apiaries environment is an additional factor to consider when evaluating the effect of pesticides on bees and their products (Calatayud-Vernich et al., 2015; Amulen et al., 2017). So, apiaries used for sampling in this study were classified according to its environment, whether were located in areas with a high or low agricultural environment (Table 1). No differences were observed between groups when comparing the number of detected pesticides (11) or the average of pesticides detected per sample (3). Compounds used in beekeeping against varroa showed similar concentrations and frequencies and no statistical differences were observed between both groups. The insecticides chlorpyrifos and acetamiprid concentrations were significantly more elevated in intensive farming landscapes where both pesticides are widely used, compared with mountainous and grasslands areas (Table 4). As miticide chlorfenvinphos is not used in agriculture, statistical differences between both groups could be explained attending to differences in beekeepers treatments applied in the apiaries.

3.4. Beeswax

Beeswax from honeycombs was contaminated with 8 pesticides residues (Table 5). An average of 4.5 pesticides per sample was detected and 7 pesticide residues were found simultaneously in 6 samples. Coumaphos, chlorfenvinphos, fluvalinate and acrinathrin

Table 2
Summary of pesticide residues detected in honey bee workers.

Worker honey bees samples (n = 45)					
Pesticide	Class	Use	Positive cases (%)	Range (ng·g^{-1})	Mean[a] (ng·g^{-1})
Coumaphos	Organophosphate	Miticide	15 (33.3%)	1–34	2.4
Fluvalinate	Pyrethroid	Miticide	12 (26.7%)	2–168	7.2
[b]DMF (amitraz)	Formamidine	Miticide	7 (15.6%)	1–104	3.5
Chlorpyrifos	Organophosphate	Insecticide	4 (8.9%)	1–24	0.6
Dichlofenthion	Organophosphate	Insecticide	1 (2.2%)	18	0.4
Chlorfenvinphos	Organophosphate	Miticide/Insecticide	1 (2.2%)	6	0.1
Acrinathrin	Pyrethroid	Miticide/Insecticide	1 (2.2%)	6	0.1

[a] If a compound was not detected in a sample, concentration value was considered as 0.
[b] DMF is a degradation product of the amitraz pesticide.

Table 3
Summary of pesticide residues detected in pollen samples.

Pollen samples (n = 45)					
Pesticide	Class	Use	Positive cases (%)	Range ($ng \cdot g^{-1}$)	Mean[a] ($ng \cdot g^{-1}$)
Coumaphos	Organophosphate	Miticide	40 (88.9%)	4–374	56.2
Fluvalinate	Pyrethroid	Miticide	21 (46.7%)	2–72	10.9
[b]DMF (amitraz)	Formamidine	Miticide	17 (37.8%)	4–246	17.6
Chlorpyrifos	Organophosphate	Insecticide	14 (31.1%)	1–100	9.8
Chlorfenvinphos	Organophosphate	Miticide/Insecticide	12 (26.7%)	2–194	10.0
Acrinathrin	Pyrethroid	Miticide/Insecticide	9 (20.0%)	1–458	16.8
Acetamiprid	Neonicotinoid	Insecticide	5 (11.1%)	7–104	5.4
Dimethoate	Organophosphate	Insecticide	4 (8.9%)	14–22	1.5
[b]DMPF (amitraz)	Formamidine	Miticide	4 (8.9%)	8–22	1.2
Hexythiazox	Carboxamide	Miticide	3 (6.7%)	14–190	5.1
Dichlofenthion	Organophosphate	Insecticide	2 (4.4%)	18–42	1.3
Carbendazim	Benzimidazole	Fungicide	2 (4.4%)	22–29	1.1
Fenitrothion	Organophosphate	Insecticide	1 (2.2%)	14	0.3
Pyriproxyfen	Insect growth regulator	Insecticide	1 (2.2%)	6	0.1

[a] If a compound was not detected in a sample, concentration value was considered as 0.
[b] DMF and DMPF are the degradation products of the amitraz pesticide.

Table 4
Summary of pesticide residues detected in pollen from high or low agricultural surroundings proportion.

Pesticide	Pollen samples (n = 45)					
	Agricultural surroundings proportion					
	High (n = 26)			Low (n = 19)		
	Positive cases (%)	Range ($ng \cdot g^{-1}$)	Mean[a] ($ng \cdot g^{-1}$)	Positive cases (%)	Range ($ng \cdot g^{-1}$)	Mean[a] ($ng \cdot g^{-1}$)
Coumaphos	24 (92.3%)	4–228	59.3	16 (84.2%)	4–374	52.0
Fluvalinate	11 (42.3%)	10–72	16.5	10 (52.6%)	2–18	3.3
[b]DMF (amitraz)	8 (30.8%)	18–102	13.8	9 (47.4%)	4–246	22.6
[a,b]Chlorpyrifos	13 (50.0%)	10–100	17.0	1 (5.3%)	1	0.1
[a,b]Chlorfenvinphos	3 (11.5%)	14–194	13.4	9 (47.4%)	2–60	5.5
Acrinathrin	4 (15.4%)	32–458	24.2	5 (26.3%)	1–80	6.6
[a,b]Acetamiprid	5 (19.2%)	7–104	9.4	–	–	0.0
Dimethoate	3 (11.5%)	16–22	2.1	1 (5.3%)	14	0.7
[b]DMPF (amitraz)	3 (11.5%)	8–16	1.2	1 (5.3%)	22	1.2
Hexythiazox	3 (11.5%)	14–190	8.8	–	–	0.0
Dichlofenthion	–	–	0.0	2 (10.5%)	18–42	3.2
Carbendazim	2 (7.7%)	22–29	2.0	–	–	0.0
Fenitrothion	–	–	0.0	1 (5.3%)	14	0.7
Pyriproxyfen	–	–	0.0	1 (5.3%)	6	0.3

[a,b] Different letters indicate statistical differences between the pesticides among both groups.
[a] If a compound was not detected in a sample, concentration value was considered as 0.
[b] DMF and DMPF are the degradation products of the amitraz pesticide.

miticides were detected in >70% of wax samples. Compared to residues in honey bees, levels found in wax were 103, 2252, 10168 and 13204 times higher for fluvalinate, coumaphos, acrinathrin and chlorfenvinphos, respectively. Wax miticides levels were also higher than concentrations detected in pollen samples, and ranged from 60 (acrinathrin) to 132 (chlorfenvinphos) times higher. Amitraz degradate DMF and flumethrin acaricides were detected <50% of samples, and mean concentrations found were lower, 180 and 10 ng g^{-1}, respectively. Agricultural pesticides, such as chlorpyrifos and hexythiazox were detected in 20.9 and 2.3% of wax samples, and reached a mean concentration of 4.9 and 1.3 ng g^{-1}, respectively. Chlorpyrifos and hexythiazox insecticides residues provide evidence that beeswax receives pesticides applied in crops through forager honey bees activity. Incoming pollen contaminated by pesticides

Table 5
Summary of pesticide residues detected in beeswax.

Beeswax samples (n = 43)					
Pesticide	Class	Use	Positive cases (%)	Range ($ng \cdot g^{-1}$)	Mean[a] ($ng \cdot g^{-1}$)
Coumaphos	Organophosphate	Miticide	43 (100%)	$18–5.34 \cdot 10^4$	$5.41 \cdot 10^3$
Chlorfenvinphos	Organophosphate	Miticide/Insecticide	41 (95.3%)	$35–1.69 \cdot 10^4$	$1.32 \cdot 10^3$
Fluvalinate	Pyrethroid	Miticide	38 (88.4%)	$55–6.31 \cdot 10^3$	742
Acrinathrin	Pyrethroid	Miticide/Insecticide	32 (74.4%)	$70–7.5 \cdot 10^3$	$1.02 \cdot 10^3$
[b]DMF (amitraz)	Formamidine	Miticide	20 (46.5%)	$30–3.52 \cdot 10^3$	180
Flumethrin	Pyrethroid	Miticide/Insecticide	11 (25.6%)	10–100	11.0
Chlorpyrifos	Organophosphate	Insecticide	9 (20.9%)	1–60	5.0
Hexythiazox	Carboxamide	Miticide	1 (2.3%)	60	1.0

[a] If a compound was not detected in a sample, concentration value was considered as 0.
[b] DMF is a degradation product of the amitraz pesticide.

used in agriculture could also act as a source of beeswax insecticide contamination. As occurs with fat soluble carotenoids pigments that migrates from pollen to beeswax and produce beeswax progressive coloring, hydrophobic pesticides could be transferred through honey bee interactions to beeswax matrix. It has been suggested that residues in wax represent an excretion product of the bees, a way to eliminate these xenobiotic substances from their bodies (Niell et al., 2017). Beeswax analyzed was highly contaminated by miticides and previous surveys in Spain (García et al., 2017; Calatayud-Vernich et al., 2017), Italy (Perugini et al., 2018) and North America (Mullin et al., 2010) support this finding. Beeswax lipophilic nature and a low replacement rate in hive, together with pesticides high hydrophobicity (Log $K_{ow} > 4$) and stability, are the main factors involved in beeswax pesticide storage. Coumaphos miticide was found up to 53400 ng g^{-1}, is stable in wax ($t_{1/2} = 115–346$ days) and its content in this matrix do not decrease after being exposed to high temperatures (140 °C) (Bogdanov et al., 1998; Martel et al., 2007). Despite Amitraz (Apivar®, Apitraz® and Amicel®) being used in the apiaries of this study as the principal miticide, the mean content of amitraz degradates in beeswax were significantly lower compared with other miticides detected. The high polarity of DMF (Kow = −1.1) implies that this metabolite would be washed off during commercial recycling processes of wax.

3.5. Pesticide hazard assessment

Pesticides applied in crops are carried in honey bee bodies and through collected nectar and pollen, and then, transported to the hive where they are mixed with pesticides applied by beekeepers. Once the pesticides are inside the hive, the distribution of the pesticides across beekeeping matrices is a complex process driven principally by food transfer interactions between members and the pesticides physicochemical properties (Tremolada et al., 2004; Sponsler and Johnson, 2017). So, a part from the wide contamination of honey bees, pollen and wax samples analyzed here, it is expected that such interactions between honey bees individuals also impregnate with pesticides honey and propolis, as reported by previous surveys (Bogdanov et al., 1998; Mitchell et al., 2017). As a result, colony members are reared and inhabit a toxic hive, exposed to different pesticides cocktails that have been proved to produce toxic synergistic effects on honey bees (Johnson et al., 2009; Johnson et al., 2013). Further, pesticides simultaneously detected in the beekeeping matrices analyzed in this study can impair mating and health of honey bee queens (Rangel and Tarpy, 2015), alter honey bee gut microbiome (Kakumanu et al., 2016) and foster varroa resistance to acaricides (Kamler et al., 2016). In order to estimate the pesticide hazard, the HQs for beeswax and pollen was calculated (Fig. 2, Tables S5–S6).

Pollen: Samples with relevant and low HQ were detected in the same frequency (49%), and one sample (2%) was considered to have an elevated pesticide risk to honey bees. The average HQ_{pollen} was 222, 4 times higher than the lower threshold stablished for relevant HQs. Acrinathrin was the main contributor to the highest HQ scores, whereas the contributions of dimethoate and chlorpyrifos were moderate in two of the 5 highest HQ scores. The contribution of the chlorfenvinphos acaricide was significant in the fifth sample with the highest HQ score (Fig. 3). Despite most of HQ highest scores were calculated in samples from intensive agriculture environment, the main contribution to HQ was due to acrinathrin pesticide, likely used against varroosis in some apiaries. The samples where insecticides dimethoate and chlorpyrifos showed a relevant HQ contribution (>100 points) came from apiaries located in an intensive agriculture environment.

Beeswax: Samples with a relevant (49%) or elevated (39%) HQ were majority. Only 12% of beeswax showed a low pesticide risk to honey bees. The average HQ_{wax} (6948) was 30 times higher than the average HQ_{pollen}. Although miticides coumaphos, fluvalinate and amitraz degradate DMF were the most frequently detected and its mean concentrations were high, these acaracidies did not contributed substantially to HQ_{wax} scores. The reason was the low toxicities of coumaphos ($LD50_{oral} = 4.6\ \mu g \cdot bee^{-1}$), fluvalinate ($LD50_{oral} = 45\ \mu g \cdot bee^{-1}$) and amitraz ($LD50_{oral} = 50\ \mu g \cdot bee^{-1}$) to honey bees. As occurred in pollen samples, the acrinathrin miticide was the main contributor to HQ_{wax} scores (Fig. 3). In the highest HQ_{wax} score (44544), acrinathrin pesticide contributed 44118 points. Flumethrin and chlorfenvinphos miticides showed relevant contributions to hazard scores in several samples (>1000 points). Insecticide chlorpyrifos contributed >400 points to the HQ_{wax} scores in 9% of the analyzed samples.

Based on HQ model assumptions, a nurse bee that fed on pollen from the apiary with the highest HQ_{pollen} (sample 21c), would be consuming 38% of acrinathrin DL_{50}, 0.12% of coumaphos DL_{50} and 0.005% of fluvalinate DL_{50} (during its first 10 days of life). If we also consider the toxicity load ($HQ_{wax} = 44543$) of the wax from this colonies, the honey bee health could be seriously compromised. Given the toxicity of some pesticides detected in the samples, their stability in-hive, and the potential distribution through the beekeeping matrices, it is necessary to adopt measures to reduce pesticide load in beekeeping matrices. The use of wax sources less contaminated as capping wax (Calatayud-Vernich et al., 2017), the use of less persistent compounds, and the right application of authorized veterinary treatments as well as the implementation of new and sustainable management practices are encouraged.

4. Conclusions

Live in-hive worker honey bees, recent stored pollen and beeswax analyzed in the present study were contaminated principally by miticides used in beekeeping, and to a lesser extent with insecticides and fungicides from the surrounding sprayed crops.

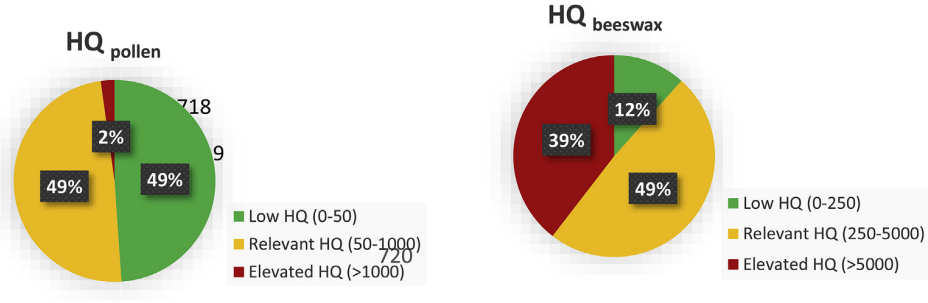

Fig. 2. Percentage of HQ scores classified as low, relevant or elevated, for pollen and beeswax samples.

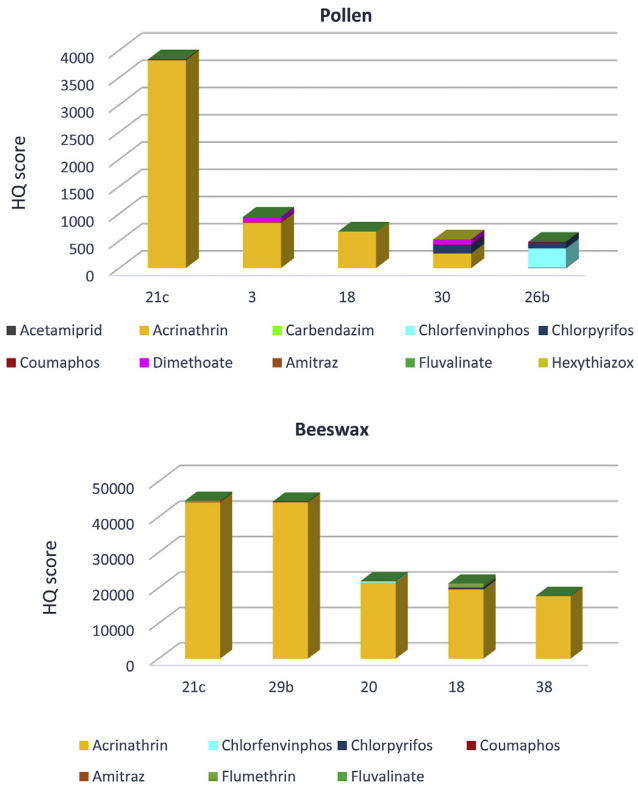

Fig. 3. Contribution of the detected pesticides to the highest HQ scores in pollen and wax samples. The top 5 most elevated HQ scores of each matrix are illustrated.

Beeswax is the most contaminated hive compartment regarding quantities of pesticides detected, whereas pollen samples revealed the highest number of different pesticide residues detected in the samples. Pollen from apiaries located in intensive farming landscapes showed concentrations of chlorpyrifos and acetamiprid significantly higher than those pollen samples collected in rural, grassland or horticultural landscapes. Honey bees were less contaminated, both in quantities and number of pesticides detected. However, it should be taken into account that the study was based in the sampling of apparently healthy bees. So residues found in honey bees analyzed in the present work are not reliable nor representative of the full exposure of bees to pesticides. Beeswax was the beekeeping matrix with the highest highest hazard to honey bees. The hazard of pollen residues was considered relevant for honey bees. Acrinathrin was the most important contributor to the HQ scores in wax and pollen samples. The contributions of the insecticides dimethoate and chlorpyrifos, and miticides chlorfenvinphos and flumethrin to the HQ were considered relevant in the samples. It is strongly recommended to reduce pesticide load in beekeeping matrices that could be adversely affecting honey bee colonies fitness.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.05.062.

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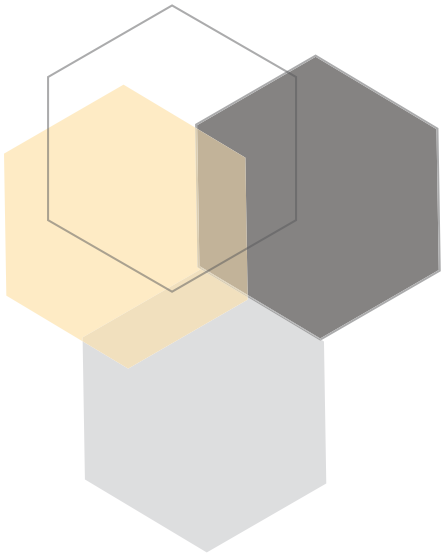
ARTICLE 02

SUPPLEMENTARY MATERIAL:

PESTICIDE RESIDUES IN HONEY BEES, POLLEN AND BEESWAX: ASSESSING BEEHIVE EXPOSURE.



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MATERIAL AND METHODS

LC-MS/MS conditions

The chromatographic column was a Luna C18 (15.0 cm × 0.21 cm) with a 3 µm particle size (Phenomenex, Torrance, USA). The column temperature was kept at 30 °C and the volume injected was 5 µL. A binary mobile phase at flow rate of 0.3 mL·min^{-1} with a gradient elution was used. Solvent A was Milli-Q water with 10 mM ammonium formate, and solvent B was methanol with 10 mM ammonium formate. The linear gradient was as follows: 0 min (50 % B), 10 min (83 % B), 12 min (83 % B), 12.5 min (98 % B) and 15.5 min (98 % B). Then, the mobile phase returns to the initial conditions with an equilibration time of 12 min.

Ionization and fragmentation settings were optimized by direct injection of pesticide standard solutions. MS/MS was performed in the SRM mode using ESI in positive mode. For each compound, two characteristic product ions of the protonated molecule [M+H] + were monitored, the first and most abundant one was used for quantification, while the second one was used as a qualifier. Collision energy and cone voltage were optimized for each pesticide. Nitrogen was used as collision, nebulising and desolvation gas. The ESI conditions were: capillary voltage 4000 V, nebulizer 15 psi, source temperature 300 °C and gas flow 10 L· min^{-1}. In order to maximize sensitivity, dynamic MRM was used, with MS1 and MS2 at unit resolution and cell acceleration voltage of 7 eV for all the compounds.

Analysis of honey bees, pollen and beeswax

Honeybee and stored pollen samples (5g) were weighed into 50 mL centrifuge tubes and a volume of 7.5 mL water and 10 mL of acetonitrile were added to the tubes containing the bees. After that, 6 g MgSO4 and 1 g NaCl were added and the samples were vortexed immediately for 1 min. The extracts were then centrifuged for 5 min at 3000 rpm. A volume of 1 mL from the supernatant was sampled into another 15 mL centrifuge tube containing 50 mg C18, 50 mg PSA and 150 mg MgSO4 and the samples were again vortexed for 1 min and centrifuged for 5 min at 3000 rpm. Finally, the supernatant was filtered using a PTFE 13mm x 0.22 µm into the autosampler vials for LC-MS analysis.

Beeswax (2 g) was weighed into 50 mL centrifuge tubes and 10 mL of acetonitrile were added. The tubes were closed and placed in a water bath at – 80 °C. Once the beeswax had melted, the tubes were vortexed vigorously for 30 s and placed again in the water bath to melt. This step was repeated four times to ensure adequate pesticide extraction. For beeswax precipitation, centrifugation tubes were left to cool to room temperature and put into the freezer (-18 °C) overnight. For the extract cleaning, a volume of 2 mL was sampled into a 15 mL centrifuge tube containing 50 mg C18 and 50 mg primary-secondary amine (PSA). The mixture was shaken for 15 s and centrifuged at 3000 rpm for 5 minutes. Finally, the supernatant was filtered using a PTFE 13 mm x 0.22 µm into the autosampler vials for LC-MS analysis and pH was adjusted to ca. 5 by adding a 5% formic acid solution in acetonitrile (v/v) (10 µL/mL extract).

Quality Assurance/Quality Control (QA/QC)

Table S1. LOD and LOQ, recovery, precision (RSD) and matrix effects of the analyzed pesticides in pollen matrix. Recoveries values are the mean of five independent determinations at 10, 50 and 100 ng·g $^{-1}$.

Pesticides	LOD (ng·g^{-1})	LOQ (ng·g^{-1})	Recoveries [average (R) and RSD]						Matrix effects (%)
			10 ng·g^{-1}		50 ng·g^{-1}		100 ng·g^{-1}		
			R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	
Acetamiprid	0.3	1	90	3	83	3	97	13	-14
Acetochlor	1.7	5	96	23	85	16	94	19	46
Acrinathrin	1.0	3	78	13	74	21	73	2	4
Alachlor	1.0	3	78	11	94	4	94	11	-3
Atrazine	0.3	1	84	6	86	4	92	4	2
Atrazine-desethyl	0.3	1	112	6	99	8	110	2	-32
Atrazine-desisopropyl	1.0	3	116	4	105	5	93	17	-39
Azinphos-ethyl	0.3	1	86	8	82	12	89	10	-7
Azinphos-methyl	0.3	1	93	6	91	5	94	14	7
Bifenthrin	0.3	1	75	10	83	8	76	13	-25
Buprofezin	0.3	1	114	7	93	15	95	7	-10
Carbendazim	0.3	1	107	1	83	9	80	2	-6
Carbofuran	0.3	1	95	3	95	2	100	13	1
Carbofuran-3-hydroxy	0.3	1	96	5	87	2	93	10	4
Chlorfenvinphos	0.3	1	91	9	86	4	94	10	-8
Chlorpyrifos	0.3	1	81	5	82	13	82	8	-5
Chlothianidin	1.0	3	105	5	104	3	108	10	2
Coumaphos	0.3	1	93	10	87	8	94	10	-10
Diazinon	0.3	1	77	14	79	12	95	9	-10
Dichlofenthion	0.3	1	73	3	83	14	85	12	-5
Dimethoate	0.3	1	76	1	78	1	89	13	-8

Diuron	0.3	1	70	6	79	2	92	13	-11
DMA (amitraz)	1.7	5	74	19	96	8	94	7	-12
DMF (amitraz)	0.3	1	87	9	87	1	97	1	-14
DMPF (amitraz)	0.3	1	107	5	82	8	92	8	-45
Ethion	0.3	1	75	6	83	11	90	11	-8
Etofenprox	0.3	1	108	8	74	8	75	8	-23
Fenitrothion	1.0	3	69	5	90	13	85	13	-17
Fenthion	0.3	1	82	7	80	14	96	14	-21
Fenthion-sulfone	0.3	1	100	5	95	13	106	13	-43
Fenthion-sulfoxide	0.3	1	95	10	98	13	113	13	-35
Fipronil	0.3	1	112	7	94	9	97	9	-12
Flumethrin	1.0	3	76	6	85	10	74	10	-4
Fluvalinate	0.3	1	116	13	96	3	77	3	5
Hexythiazox	0.3	1	106	18	90	8	91	8	-2
Imazalil	1.0	3	84	9	81	5	73	5	-9
Imidacloprid	0.3	1	81	4	84	15	95	15	-20
Isoproturon	0.3	1	86	7	90	15	101	15	-26
Lambda-cyhalothrin	1.7	5	67	3	78	6	73	6	-12
Malathion	0.3	1	76	7	81	11	92	11	-7
Methiocarb	0.3	1	82	4	88	14	101	14	50
Metolachlor	0.3	1	81	15	80	13	102	13	-15
Molinate	1.7	5	104	5	97	1	95	1	0
Omethoate	1.7	5	65	10	64	11	67	7	-23
Parathion-ethyl	0.3	1	83	2	84	3	93	12	-9
Parathion-methyl	0.3	1	114	8	91	3	100	11	-18
Prochloraz	0.3	1	70	10	68	13	77	9	-8
Propanil	0.3	1	100	5	85	5	87	11	-14
Propazine	1.0	3	112	4	85	1	97	12	-15
Pyriproxyfen	0.3	1	78	23	87	14	85	9	-10
Simazine	1.0	3	105	11	106	4	111	15	-39

Spinosyn A	0.3	1	71	8	85	9	87	5	-16
Spinosyn D	0.3	1	72	5	91	6	93	4	-2
Tebuconazole	0.3	1	108	5	89	20	103	12	-15
Terbumeton	0.3	1	68	7	89	10	107	13	-6
Terbumeton-desethyl	0.3	1	109	3	88	15	100	1	-22
Terbuthylazine	0.3	1	105	1	86	5	101	14	-17
Terbuthylazine-desethyl	0.3	1	105	3	93	4	105	14	-54
Terbuthylazine-2-hydroxy	1.7	5	58	10	56	6	55	9	-28
Terbutryn	0.3	1	112	13	86	13	104	14	-9
Thiabendazole	1.7	5	58	11	55	2	63	5	-18
Thiamethoxam	0.3	1	103	13	93	5	104	15	-31
Tolclofos-methyl	0.3	1	107	4	90	7	86	12	-17

Calculating Hazard

(HQ = pesticide concentration in ppb ÷ pesticide topical LD_{50} as µg/bee).

Contact-LD_{50} (µg/bee)[2]						
Hexythiazox	Imazalil	Pyriproxifen	Chlorfenvinphos	Fluvalinate	Flumethrin	Carbendazim
200	39	100	4.1	8.7	0.05	50
Acrinathrin	Chlorpyrifos	Coumaphos	Amitraz			
0.17	0.072	20	50			
Oral-LD_{50} (µg/bee)[2]						
Hexythiazox	Fenitrothion	Pyriproxifen	Dimethoate	Amitraz	Acetamiprid	Carbendazim
200	0.52	100	0.17	50	14	50
Acrinathrin	Chlorpyrifos	Coumaphos	Chlorfenvinphos	Fluvalinate		
0.12	0.24	4.6	0.55	45		

[2]LD_{50} values were from Sanchez-Bayo and Goka, (2014) and Hertfordshire, U. (2017). PPDB - Pesticides Properties DataBase.

RESULTS

Table S2. Determination of pesticides residues in the 45 honey bee samples analyzed are showed. Table units are expressed in $ng \cdot g^{-1}$.

	acrinathrin	chlorfenvinphos	Chlorpyrifos	coumaphos	dichlofenthion	DMF (amitraz)	DMPF (amitraz)	fluvalinate
29a	0	0	0	0	0	0	0	0
33	0	0	1	1	0	1	1	0
24	0	0	0	1	0	1	1	0
28	0	0	1	1	0	1	1	0
12	0	0	0	2	0	104	0	0
26a	0	0	0	34	0	0	0	20
35	0	0	0	0	0	0	0	0
31	0	0	0	10	0	0	0	24
30	0	0	1	1	0	1	1	0
29b	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0
32	0	0	24	0	0	0	0	0
21a	0	0	0	0	0	0	0	0
21b	0	0	0	0	0	0	0	26
21c	0	0	0	0	0	0	0	20
20	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0
38	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
37	0	0	0	0	0	0	0	0
26b	0	0	0	0	0	0	0	22
39	0	0	0	26	18	0	0	2
14	0	0	0	4	0	0	0	0
16	0	0	0	10	0	0	0	0
13	0	0	0	0	0	0	0	4
15	0	0	0	2	0	0	0	2
22	0	0	0	2	0	0	0	0
19	0	6	0	8	0	0	0	0
18	6	0	0	2	0	0	0	2
17	0	0	0	2	0	0	0	2
34	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
4a	0	0	0	0	0	0	0	0
4b	0	0	0	0	0	0	0	0
4c	0	0	0	0	0	0	0	0
9	0	0	0	0	0	16	0	168
11	0	0	0	0	0	0	0	32
10	0	0	0	0	0	34	0	0

Numbers and letters in the left column are the samples ID

Table S3. Determination of pesticides residues in the 43 beeswax samples analyzed are showed.

	acrinathrin	chlorfenvinphos	Chlorpyrifos	coumaphos	DMF	flumethrin	fluvalinate	hexythiazox
29a	1370	310	0	11375	0	0	420	0
33	0	295	0	5085	0	0	0	0
24	0	320	0	1935	190	0	0	0
28	0	35	1	880	0	0	0	0
26a	455	635	0	53395	505	0	910	0
35	0	850	60	13755	0	0	160	0
31	2500	715	0	6645	0	0	1490	0
30	0	320	0	1700	0	0	0	0
29b	7500	150	0	2565	0	0	145	0
36	810	1075	0	6405	685	0	355	0
32	0	260	0	2645	0	0	100	0
21a	1200	780	0	5915	290	0	100	0
21b	0	635	60	7770	3520	0	2610	0
21c	7500	0	0	1090	0	0	3235	0
20	3650	1435	0	950	0	0	155	0
23	2250	1575	0	4920	0	0	170	0
25	0	195	0	5065	0	0	0	0
27	300	8250	0	4850	0	0	140	0
38	3000	50	0	1135	0	0	105	0
7	335	185	0	11250	0	0	130	0
8	150	540	0	8085	0	0	55	0
5	400	175	0	3340	0	0	165	0
2	1000	635	0	2305	0	0	180	0
1	120	180	0	4785	0	0	95	0
37	0	110	0	5695	225	0	110	0
26b	0	16925	0	2760	0	0	535	0
14	345	280	0	6925	45	20	470	0
16	460	365	0	4085	185	100	830	60
13	465	2310	0	1170	410	10	1520	0
15	265	765	0	9560	130	25	6310	0
22	640	840	30	8860	80	50	1180	0
19	935	11200	5	6950	85	10	1710	0
18	3310	210	30	1415	245	55	715	0
17	1155	1555	5	7170	120	60	3800	0
34	1475	1230	0	1775	400	15	435	0
3	190	192	0	884	0	38	254	0
6	76	0	12	18	0	0	196	0
4a	458	132	8	1650	30	78	616	0
4b	0	56	0	390	0	0	102	0
4c	542	236	0	1594	110	0	660	0
9	412	164	0	1394	286	0	388	0
10	70	454	0	1482	44	0	1162	0
11	384	152	0	788	158	0	190	0

Numbers and letters in the left column are samples ID

Table S4. Determination of pesticides residues in the 45 fresh pollen samples analyzed are showed. Table units are expressed in $ng \cdot g^{-1}$.

	Acetamiprid	acrinathrin	carbendazim	chlorfenvinphos	Chlorpyrifos	coumaphos	dichlofenthion	dimethoate	DMF	DMPF	fenitrothion	fluvalinate	hexythiazox	pyriproxyfen
29a	0	0	0	0	0	108	0	0	0	0	0	0	0	0
33	7	0	0	0	32	142	0	0	42	16	0	10	0	0
24	0	0	0	0	1	80	0	14	66	22	0	8	0	0
28	19	0	29	0	50	70	0	22	36	8	0	20	14	0
12	0	1	0	0	0	28	0	0	84	0	14	0	0	0
26a	104	0	0	0	0	228	0	0	0	0	0	26	0	0
35	0	0	0	0	24	64	0	0	0	0	0	0	0	0
31	0	0	0	0	0	102	0	0	0	0	0	60	0	0
30	10	32	22	0	36	48	0	16	32	8	0	52	26	0
29b	0	0	0	0	24	0	0	0	0	0	0	0	0	0
36	0	0	0	0	26	14	0	0	0	0	0	0	0	0
32	0	0	0	0	28	28	0	0	0	0	0	0	0	0
21a	0	0	0	0	0	10	0	0	0	0	0	0	0	0
21b	0	0	0	0	30	18	0	0	0	0	0	24	0	0
21c	0	458	0	0	0	54	0	0	0	0	0	22	0	0
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	4	0	0	0	0	0	0	190	0
25	0	0	0	0	0	110	0	0	0	0	0	0	0	0
27	0	0	0	140	32	170	0	0	0	0	0	0	0	0
38	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	152	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	18	0	0	0	0	0	0	0	0
1	0	0	0	0	0	18	0	0	0	0	0	0	0	0
37	0	0	0	0	24	182	0	0	46	0	0	0	0	0
26b	104	0	0	194	26	48	0	0	0	0	0	48	0	0
39	0	0	0	0	0	374	42	0	0	0	0	6	0	0
14	0	0	0	2	0	56	0	0	0	0	0	6	0	0
16	0	0	0	2	0	28	0	0	6	0	0	2	0	0
13	0	20	0	20	0	14	0	0	246	0	0	6	0	0
15	0	0	0	6	0	28	0	0	4	0	0	18	0	0
22	0	0	0	4	0	16	0	0	6	0	0	2	0	0
19	0	0	0	60	0	38	0	0	4	0	0	0	0	0
18	0	80	0	2	0	6	0	0	0	0	0	8	0	0
17	0	12	0	4	0	18	0	0	6	0	0	4	0	0
34	0	12	0	4	0	4	18	0	8	0	0	2	0	6
3	0	100	0	0	0	46	0	16	0	0	0	28	0	0
6	0	0	0	0	0	30	0	0	0	0	0	0	0	0
4a	0	0	0	0	10	20	0	0	18	0	0	0	0	0
4 b	0	0	0	0	0	16	0	0	0	0	0	0	0	0
4 c	0	0	0	0	100	16	0	0	0	0	0	0	0	0
9	0	40	0	0	0	24	0	0	28	0	0	72	0	0
10	0	0	0	14	0	68	0	0	56	0	0	68	0	0
11	0	0	0	0	0	32	0	0	102	0	0	0	0	0

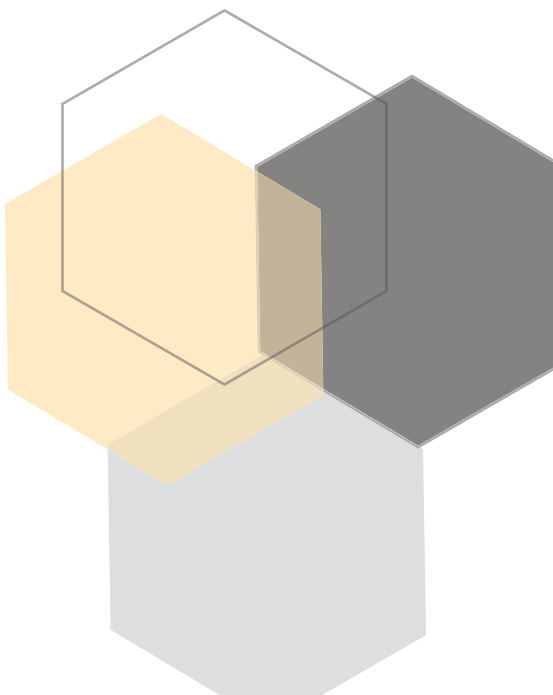
Numbers and letters in the left column are samples ID

Table S5. Hazard quotients (HQ pollen) values for fresh pollen samples analyzed.

29a	**33**	**24**	**28**	**12**	**26a**	**35**	**31**	**30**	**29b**	**36**	**32**	**21a**	**21b**	**21c**	**20**	**23**	**25**	**27**	**38**	**7**	**8**	**5**
23.5	167.2	107.5	357.1	44.6	57.6	113.9	23.5	525.2	100.0	111.4	122.8	2.2	129.4	3828.9	0.0	1.8	23.9	424.8	0.0	0.0	33.0	0.0
2	**1**	**37**	**26b**	**39**	**14**	**16**	**13**	**15**	**22**	**19**	**18**	**17**	**34**	**3**	**6**	**4a**	**4b**	**4c**	**9**	**10**	**11**	
3.9	3.9	141.4	480.0	81.4	15.9	10.0	215.9	17.6	11.0	117.5	671.8	111.5	108.6	938.1	6.5	46.7	3.5	420.1	339.7	43.9	11.0	

Table S6. Hazard quotients (HQ wax) values for beeswax samples analyzed.

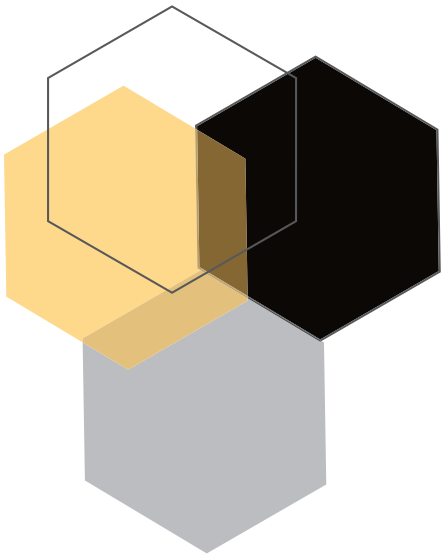
29a	**33**	**24**	**28**	**26a**	**35**	**31**	**30**	**29b**	**36**	**32**	**21a**	**21b**	**21c**	**20**	**23**	**25**	**27**	**38**	**7**	**8**	**5**
8751	326	182	66	5626	1747	15384	163	44299	5415	207	7568	1815	44544	21886	13885	301	4035	17728	2593	1425	2582
2	**1**	**37**	**26b**	**14**	**16**	**13**	**15**	**22**	**19**	**18**	**17**	**34**	**3**	**6**	**4a**	**4b**	**4c**	**9**	**10**	**11**	
6173	1000	333	4328	2900	5102	3748	3454	5968	9049	21201.05	9243	9431	1998	637.2	4552	45	3406	2589	732	2363	



CHAPTER 4:

Honey bee mortality together with pesticide residues in honey bees, beeswax and pollen were monitored in experimental apiaries located in different environments. Both scientific publications in this chapter contribute to comprehend the influence of the environment on the presence of dangerous pesticides in samples and the sudden honey bee mortality changes.

ARTICLE 03



INFLUENCE OF PESTICIDE USE IN FRUIT ORCHARDS DURING BLOOMING ON HONEY BEE MORTALITY IN 4 EXPERIMENTAL APIARIES.

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Influence of pesticide use in fruit orchards during blooming on honeybee mortality in 4 experimental apiaries

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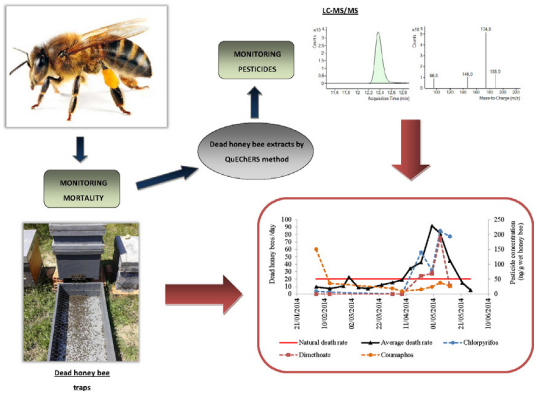
HIGHLIGHTS

- Pesticide residues and bee mortality were monitored in four apiaries for six months.
- QuEChERS extracts of bees were screened for 58 pesticides using LC–MS/MS.
- Honey bee mortality increased in blooming season until highest levels.
- Coumaphos at a residual concentration (50 ng/g) was not related to bee mortality.
- Chlorpyrifos and dimethoate concentrations were highly related to mortality peaks.

GRAPHICAL ABSTRACT



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ABSTRACT

Samples of dead honey bees (*Apis mellifera* L.) were collected periodically from 4 different locations during citrus and stone fruit trees blooming season to evaluate the potential impact of agrochemicals on honey bee death rate. For the determination of mortality, dead honey bee traps were placed in front of the experimental hives entrance located in areas of intensive agriculture in Valencian Community (Spain). A total of 34 bee samples, obtained along the monitoring period, were analyzed by means of QuEChERS extraction method and screened for 58 pesticides or their degradation products by LC–MS/MS. An average of four pesticides per honey bee sample was detected. Coumaphos, an organophosphate acaricide used against varroosis in the experimental hives, was detected in 94% of the samples. However, this acaricide was unlikely to be responsible for honey bee mortality because its constantly low concentration during all the monitoring period, even before and after acute mortality episodes. The organophosphates chlorpyrifos and dimethoate, as well as the neonicotinoid imidacloprid, were the most frequently detected agrochemicals. Almost 80% of the samples had chlorpyrifos, 68% dimethoate, and 32% imidacloprid. Maximum concentrations for these three compounds were 751, 403, 223 ng/g respectively.

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Influence of these pesticides on acute honey bee mortality was demonstrated by comparing coincidence between death rate and concentrations of chlorpyrifos, dimethoate and imidacloprid.

1. Introduction

Most of the flowering plants all over the world need animal pollination to survive (Ollerton et al., 2011). Insects pollinate more than a third of all crops and honey bees are usually the most abundant pollinators in cultivated areas, carrying out 85% of the effective insect pollination (Barclay and Moffett, 1984; Robinson et al., 1989). Latest estimates of the benefit of pollination in the world reach about 153 thousand million euros (Gallai et al., 2009) and nearly 80% can be attributed directly or indirectly to honey bees (Robinson et al., 1989). With a serious decline in wild honey and solitary bees, the importance of beekeeping and managed hives in sustaining biodiversity and crop pollination is increasing (Moritz et al., 2010; Calderone, 2012). Therefore, colonies of beekeepers in developed countries are assuming a strategic function to society and environment.

Beekeeping is living a murky panorama that many beekeepers and scientists have tried to clear up during last decade. Annual mortality of honey bee colonies is increasing in many developed countries and hives reach a weak state that often is hard to overcome (vanEngelsdorp and Meixner, 2010; Potts et al., 2010). Up to now, there is an agreement in considering honey bee decline as a result of multiple factors combination (Mullin et al., 2010; Moritz et al., 2010). Global effects of varroa parasite and associated viruses, impact of pesticides applied to cropland and deficient nutrition of honey bee colonies caused by lack of plant diversity, are the main factors implicated (vanEngelsdorp and Meixner, 2010; Spivak et al., 2011; Sanchez-Bayo and Goka, 2014).

Regarding pesticides, recent surveys show that honey bees are being exposed to high levels of pesticides used in crops and acaricides applied in hives. The most frequent residues of agrochemicals that honey bee acquire from treated crops are organophosphates and pyrethroids insecticides followed by fungicides (Johnson et al., 2010). Among miticides used against varroosis and detected in the honey bee samples, fluvalinate, amitraz degradation products, and coumaphos have been frequently detected (Ghini et al., 2004; Mullin et al., 2010; Lambert et al., 2013). Although neonicotinoids are not the main insecticides detected, they have become the subject of scientific debate for their impact on honeybees. These new insecticides — extensively used all over the world in the last two decades — are among the most toxic pesticides to bees. They are systemic and persistent, can be absorbed and transported throughout the plant, and remain toxic in vegetal tissues for months or even years (Krupke et al., 2012). Consequently, honey bees can experience chronic exposure over long-time periods (Johnson et al., 2010), coming into contact with sublethal doses when collect pollen, nectar, and other plant secretions. These sublethal doses can impair orientation abilities of honey bees, causing loss of foragers in the field that compromise colony viability (Henry et al., 2012; Blacquiere et al., 2012; Schneider et al., 2012; Fischer et al., 2014).

In general, the first sign of acute pesticide poisoning of honey bees is the appearance of large numbers of dead or dying bees at the colony entrances throughout the apiary. Honey bee is extremely sensitive to pesticides compared to other insects, because its noticeable deficiency in the number of genes encoding detoxification enzymes (Atkins, 1992). Forager honey bees with toxic and non-toxic contaminants return to the colony and if they die inside the hive, they are evacuated by cleaner honey bees and are susceptible of being collected in honey bee traps located in front of the hive entrance. With monitoring and chemical analysis, we can obtain the residues profile of dead honey bees (Porrini et al., 2003a).

This study aimed at establishing the occurrence of pesticide residues in honey bees and relating the concentrations to honey bee mortality rates. To analyze the impact of pesticides on mortality of honey bees, a rigorous counting of dead honey bees was made during blooming season of citrus and stone fruit trees. The QuECheRS technique was used for the extractions of pesticides and liquid chromatography–mass spectrometry (LC–MS/MS) for their analysis (Kasiotis et al., 2014). In the present study, four different locations from Valencian Community (Spain) surrounded mainly by citrus crops were monitored from January to June 2014 to detect pesticides presence in the dead honey bee samples. Acute mortality peaks were related to honey bee poisoning due to high concentrations of several pesticides in the samples.

2. Materials and methods

2.1. Chemicals

High purity (98–99.9%) standards of desired pesticides, namely, acetamiprid, acetochlor, alachlor, atrazine, atrazine-desethyl, atrazine-desisopropyl, azinphos-ethyl, azinphos-methyl, buprofezin, carbendazim, carbofuran, carbofuran-3-hydroxy, chlorfenvinphos, chlorpyrifos, coumaphos, diazinon, dichlofenthion, dimethoate, diuron, DMA, DMF, DMPF, ethion, fenitrothion, fenthion, fenthion-sulfone, fenthion-sulfoxide, fipronil, flumethrin, fluvalinate, hexythiazox, imazalil, imidacloprid, isoproturon, malathion, methiocarb, metolachlor, molinate, omethoate, parathion-ethyl, parathion-methyl, prochloraz, propanil, propazine, pyriproxyfen, simazine, tebuconazole, terbumeton, terbumeton-desethyl, terbuthylazine, terbuthylazine-desethyl, terbuthylazine-2-hydroxy, terbutryn, thiabendazole, thiamethoxam and tolclofos-methyl were acquired from Sigma-Aldrich (Steinheim, Germany). Fenoxon-sulfoxide and fenoxon-sulfone as 1 mL solution at a concentration of 10 $\mu g \cdot mL^{-1}$ in acetonitrile were from Dr. Ehrenstorfer (Augsburg, Germany).

Individual standard solutions were prepared in methanol at a concentration of 1000 $mg \cdot L^{-1}$. The working standard solution was prepared by mixing the appropriate amounts of individual standard solutions and diluting with methanol to a final concentration of 0.5 $mg \cdot L^{-1}$. All solutions were stored in 10 mL vials at 4 °C in the dark.

Magnesium sulfate was obtained from Alfa Aesar (Karlsruhe, Germany), ammonium formate, sodium hydroxide, sodium chloride, acetonitrile, and formic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Dichloromethane and methanol (gradient grade for liquid chromatography) were obtained from Panreac (Darmstadt, Germany). PSA, C18, and PTFE 13 mm × 0.22 μm filters were purchased from Análisis Vínicos S.L. (Tomelloso, Spain). High purity water was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA). Milli-Q water and methanol, both with ammonium formate 10 mM, were used as mobile phase in LC–MS/MS.

2.2. Samples collection

2.2.1. Area and season of study

Sampling apiaries (AP1 to AP4) were located in four settlements from Valencian Community in eastern Spain: Chiva, Montroi, Barxeta and Carcaixent (Fig. 1). Apiaries were situated in rural-cultivated areas where pesticides are extensively used. Apiary 2, where agricultural surface represents a 70% of the total area, was surrounded mainly by citrus and peach orchards together with dry farming lands. In the apiaries 1, 3 and 4, there was a clear predominance of citrus, scattered fruit trees orchards with khaki fruits or plums and natural vegetation, a

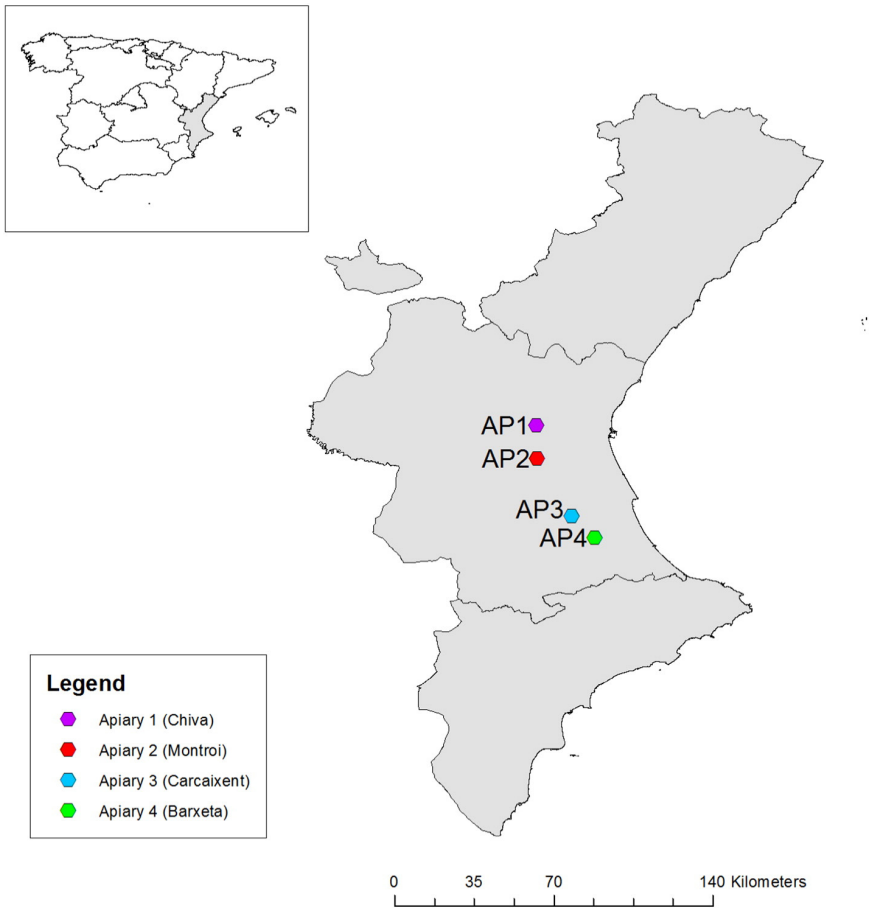

Fig. 1. Sampling apiaries in four townships from Valencian Community.

representative landscape of the local rural environment. The study was carried out from January to June in 2014, including blooming season.

2.2.2. Experimental hives

Experimental settlements consisted of two "Dadant" hives. At the beginning, the experimental hives contained a brood chamber (10 frames of measures 42 × 27 cm). Honey bee colonies were chosen for their high performance in terms of hive population, queen condition and colony health. Periodic inspections were made to ensure hive viability during the study. During nectar flow, supers were added when necessary.

2.2.3. Dead honey bee traps

To determine mortality with accuracy, traps were used to collect the dead honey bees. The trap chosen for the study was the underbasket proposed by Accorti et al. (1991) as modified by Porrini et al. (2003b), the effectiveness of which was tested and did not interfere with the role of undertaker bees. As schematized in Fig. 2, the trap does not form part of the hive and is located on the ground underneath the hive entrance. It consists basically of a wooden box with a chain mail on the top (Supplementary information Fig. S1 details dead honey bee traps used in this study). This metallic mail keeps the birds away and allows healthy honey bees that fall accidentally to get out. Dead honey bees were collected every week between January and June. If mortality grew up considerably, the collection frequency was increased to every 2–3 days and, if intoxication occurred, the immediate recovery of the dead honey bees permitted to delimit better mortality curves, minimize potential pesticide degradation and prevent pesticide wash off by the rain.

2.3. Extraction

A total of 34 honey bee samples were analyzed across all experimental period: 11 samples from Barxeta, 8 samples from Montroi, 8 samples from Chiva, and 7 samples from Carcaixent. Samples were transported in an insulated cooler and stored at −20 °C until analysis. A modified QuEChERS method was used for the extractions of pesticides from honey bees (Lambert et al., 2013; Krupke et al., 2012). Honey bee samples (5 g) were weighed into 50 mL centrifuge tubes and a volume of 7.5 mL of water and 10 mL of acetonitrile were added to the tubes

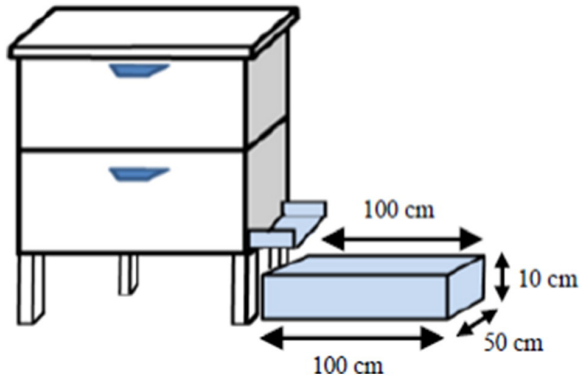

Fig. 2. Dead honey bee trap used for mortality monitoring. Side view modified by Porrini et al. (2003b).

containing the honey bees. After that, 6 g $MgSO_4$ and 1 g NaCl were added and the samples were vortexed immediately for 1 min. The extracts were then centrifuged for 5 min at 3000 rpm. A volume of 1 mL from the supernatant was sampled into another 15 mL centrifuge tube containing 50 mg C_{18}, 50 mg PSA, and 150 mg $MgSO_4$ and the samples were again vortexed for 1 min and centrifuged for 5 min at 3000 rpm. Finally, the supernatant was filtered using a PTFE 13 mm × 0.22 μm into the autosampler vials for LC–MS analysis.

2.4. Liquid chromatography–mass spectrometry (LC–MS/MS)

The chromatographic instrument was an HP1200 series LC equipped with an automatic injector, a degasser, a quaternary pump, and a column oven-combined with an Agilent 6410 triple quadrupole (QQQ) mass spectrometer with an electrospray ionization (ESI) interface (Agilent Technologies, Waldbronn, Germany). Data were processed using a MassHunter Workstation Software for qualitative and quantitative analysis (A GL Sciences, Tokio, Japan).

The chromatographic column was a Luna C18 (15.0 cm × 0.21 cm) with a 3 μm particle size (Phenomenex, Torrance, USA). The column temperature was kept at 30 °C and the volume injected was 5 μL. A binary mobile phase at flow rate of 0.3 mL min^{-1} with a gradient elution was used. Solvent A was Milli-Q water with 10 mM ammonium formate and solvent B was methanol also with 10 mM ammonium formate (detailed information in the supplementary material, text and Table S1. Chromatograms of the selected pesticides in Figs. S2–S3).

2.5. Method validation and quality control

The linearity of the MS/MS method was established with six calibration points, using external standards over a concentration range of 1–250 ng·mL^{-1} (equivalent to 20–500 ng·g^{-1} in honey bees as wet weight) (Supplementary material Table S2). The Peak area of target analytes was calculated using Mass Hunter software (Agilent). Each point was obtained as the mean of three injections. The data were fit to a linear least-squares regression curve with a 1/x weighting and was not forced through the origin. The regression coefficient was >0.99 with residuals <30%. Matrix effects were evaluated by comparing the slope of the previous calibration curve and the slope of that prepared in the extract of honey bees with six concentration levels of standard solutions..

The sensitivity of the method was estimated by establishing the limits of detection (LODs) and quantification (LOQs) (Table 1). LODs were calculated using standard solutions prepared in spiked honey bee samples that were free of pesticides. As it was difficult to find a sample without the selected pesticides, if one compound was initially in the honey bee samples (e.g. coumaphos), another honey bee sample free of the compound was used to establish LODs and LOQs for it. The LODs were determined as the lowest pesticide concentration whose qualified transition (SRM_2) presented a signal-to-noise ratio (S/N) ≥ 3. The LOQs were determined also in pure solvent and in spiked honey bees as the minimum detectable amount of analyte with S/N ≥ 10 for the quantifier (SRM_1) transition. All the LOQs were verified spiking the samples and analyzing them.

Recovery and precision, expressed as relative standard deviation (RSD, %), were determined by analyzing in quintuplicated the honey bees samples spiked at the LOQ and 50 ng g^{-1}.

Table 1
LOD and LOQ, recovery, precision and matrix effects of the analyzed pesticides. Recoveries values are the mean of five independent determinations at the LOQ and at 50 ng·g^{-1}.

Pesticide	LOD (ng/g)	LOQ (ng/g)	Recoveries [average (R) and RSD]				Matrix effects (%)
			At LOQ		50 ng/g		
			R (%)	RSD (%)	R (%)	RSD (%)	
Acetamiprid	1.3	3.9	86	13	92	12	−25
Acetochlor	1.3	3.9	91	19	95	15	−30
Alachlor	1.3	3.9	84	16	89	13	−35
Atrazine	1.3	3.9	83	16	91	16	−20
Atrazine-desethyl	2.5	7.5	80	26	85	17	−28
Atrazine-desisopropyl	2.5	7.5	84	10	89	15	−32
Azinphos-ethyl	1.3	3.9	88	17	93	12	−15
Azinphos-methyl	1.3	3.9	70	14	78	14	−15
Buprofezin	0.3	1	87	8	94	10	−18
Carbendazim	3	10	87	12	92	10	−35
Carbofuran	0.3	1	70	23	73	18	35
Carbofuran-3-hydroxy	3	10	92	19	90	15	10
Chlorfenvinphos	3	10	91	10	94	10	−40
Chlorpyrifos	0.3	1	90	15	95	11	−15
Coumaphos	1.3	3.9	82	14	87	12	−10
Diazinon	0.3	1	77	19	83	15	−30
Dichlofenthion	1.3	3.9	85	15	87	12	−22
Dimethoate	1.3	3.9	84	12	88	12	−27
Diuron	3	10	82	13	85	11	−38
DMA	0.3	1	80	5	84	7	−54
DMF	0.3	1	80	9	84	6	−28
DMPF	1.3	3.9	84	14	90	10	−33
Ethion	0.3	1	85	8	88	10	−42
Fenitrothion	1.3	3.9	82	20	83	18	−30
Fenoxon-sulfone	0.3	1	70	24	75	19	10
Fenoxon-sulfoxide	0.3	1	85	7	89	9	12
Fenthion	3	10	85	17	90	15	−5
Fenthion-sulfone	1.3	3.9	83	11	87	10	18
Fenthion-sulfoxide	0.3	1	75	23	80	18	15
Fipronil	0.3	1	81	8	82	8	−19
Flumethrin	1.3	3.9	84	4	86	8	−25
Fluvalinate	0.3	1	90	12	93	10	−28
Hexythiazox	0.3	1	83	14	85	12	−15
Imazalil	1.3	3.9	80	8	81	10	−30
Imidacloprid	0.3	1	87	19	91	15	−28
Isoproturon	1.3	3.9	83	11	86	10	−35
Malathion	1.3	3.9	85	6	88	9	−15
Methiocarb	3	10	90	5	95	7	−33
Methoalachlor	0.3	1	76	19	80	15	−22
Molinate	3	10	80	19	86	15	−21
Omethoate	0.3	1	78	24	82	19	−12
Parathion-ethyl	3	10	76	28	81	18	−16
Parathion-methyl	3	10	72	19	77	15	−18
Prochloraz	1.3	3.9	93	6	96	8	−24
Propanil	0.3	1	80	7	82	8	−38
Propazine	0.3	1	74	26	78	19	−22
Pyriproxifen	3	10	81	14	89	16	−50
Simazine	3	10	80	9	83	10	−60
Tebuconazole	1.3	3.9	88	6	91	8	−24
Terbumeton	1.3	3.9	80	9	82	10	−33
Terbumeton-desethyl	0.3	1	83	13	85	10	−28
Terbuthylazine	1.3	3.9	80	17	89	15	−38
Terbuthylazine-2-hydroxy	1.3	3.9	94	8	97	10	−40
Terbuthylazine-desethyl	1.3	3.9	75	14	82	10	−38
Terbutryn	1.3	3.9	84	12	87	10	−22
Thiabendazole	3	10	77	12	82	11	−25
Thiamethoxam	1.3	3.9	80	6	84	9	−30
Tolclofos-methyl	1.3	3.9	96	11	90	10	−20

3. Results

3.1. Validation of the analytical method

The QuEChERS extraction has been already proposed to assess pesticide residues in honey bees (Lambert et al., 2013; Krupke et al., 2012). However, the present study covers different compounds and applies a slightly different clean-up. Thus, the method was carefully validated.

Table 1 shows recoveries percentages (ranging from 70 to 96%) and precision values (≤20% for all analytes, except for atrazine-desethyl, carbofuran, fenoxon-sulfone, fenthion-sulfoxide, omethoate, parathion-ethyl, and propazine). The LODs were from 0.3 to 3 ng/g, whereas LOQs ranged from 1 to 10 ng/g. Matrix effects were in the range of −60% to 20% over the response of the standards prepared in solvent. The matrix effects were mostly suppressive (lower response compared to the standard), with the exception of carbofuran, 3-hydroxy

carbofuran, fenoxon sulfoxide, fenoxon sulfone, fenthion sulfoxide and fenthion sulfone, which showed an increase in the response. Both calibration curves, in methanol or in matrix extract, showed a linear response through the tested range (Supplementary information Table S2 details the equations of the calibration curves obtained in matrix). The analytical method is suitable for the monitoring of the selected pesticides in honey bee samples.

3.2. Monitoring of mortality

Mortality level was expressed in number of dead honey bees per day and colony. Average value of the two colonies of each apiary was used to draw mortality curves as shown in supplementary material Fig. S4.

Assuming that honey bee mortality before flowering period was only due to natural causes and according to the values proposed by Porrini et al. (2003b), a natural death rate of 20 bees/day was fixed. Between January and the beginning of March, the flowering period of peach and plum trees in Montroi, Barxeta, and Carcaixent — Figs. 3, 4 and 5 — mortality showed a slight increase possibly related with pesticide use in the vicinity of the experimental hives but not detected in the honey bees analysis.

The most relevant trait from the figures are the mortality peaks between March and May in all apiaries. During this period, the honey bees collected in the traps exceeded substantially the maximum natural death rate. Average values of mortality peaks ranged between 50 and 300 bees/day (Figs. 3 to 6), with the highest value of 500 bees/day in one colony of Barxeta apiary in the middle of April (Fig. S4). The increase of mortality took place during the citrus flowering and could be related to the insecticides applied to citrus orchards, where farmers were frequently seen spraying in the surrounding of the experimental apiaries. During May, at the end of citrus blooming season, honey bee mortality decreased beyond natural rate in all apiaries.

3.3. Monitoring of pesticides

A summary of the pesticide residues on honey bees are presented in Table 2 (detailed information of the four apiaries is provided in the Supplementary information Tables S3–S6). A total of eight pesticides were detected in the 34 honey bee samples analyzed. Coumaphos, an acaricide used against varroosis, was the most frequently detected, found in 94% of the samples. Residues of chlorpyrifos and dimethoate, common insecticides usually applied to citrus crops, were detected in 79% and 68% of the samples, showing the highest concentrations in honey bees of 751 ng/g and 403 ng/g respectively. Omethoate is a breakdown product of dimethoate and consequently was detected in a similar frequency. Imidacloprid residues were detected in 32% of the samples with a maximum concentration of 223 ng/g. Samples of dead honey bees collected in the traps had an average of four different pesticides per sample, with a maximum of seven. Chlorpyrifos and dimethoate were detected together in 68% of the cases and simultaneous detection of the three main agrochemicals implicated in honey bee mortality (chlorpyrifos, dimethoate, and imidacloprid) had a frequency of 29%..

Concentration curves were obtained for coumaphos, chlorpyrifos and dimethoate, the most frequent pesticides in honey bee samples throughout the monitoring period, as it is shown in Figs. 3, 4, 5, and 6. There is a clear coincidence between mortality peaks and increasing concentration of chlorpyrifos and dimethoate in honey bees in all apiaries. Residues of coumaphos were fairly constant and low during the monitoring period with average values ≤50 ng/g. With these results, coumaphos was not a relevant cause of honey bee mortality.

Barxeta apiary gave the highest concentration of organophosphate insecticides and the greatest honey bee death rates during citrus blooming (Fig. 3). Chlorpyrifos and dimethoate had a simultaneous increase that was followed by a high increase of honey bee death rate, suggesting a direct intoxication of forager honey bees. Almost the same occurred in Montroi apiary (Fig. 4). A peak of dimethoate concentration found in honey bees was related to the increasing honey bee mortality observed in Chiva (Fig. 6) in the middle of April. In contrast, pesticides levels in the honey bee samples of Carcaixent apiary were lower until middle of May, giving mortality values of 30 bees/day, close to natural death rate that probably reflected a minor use of agrochemicals (Fig. 5).

Coinciding with the end of citrus flowering, concentration of dimethoate and chlorpyrifos exhibited a second peak in Barxeta apiary that was not followed by an increment of mortality. A similar case occurred in the apiary of Chiva where an increase of dimethoate concentration was not related to any mortality peak. Due to the heavy loss of honey bee population detected in April, there were fewer honey bees on the field in May, but those achieving hives were highly poisoned. Pesticide use during May was more frequent and intense than any time of the blooming season. Dead honey bees in traps were decreasing while pesticides concentrations were still increasing. Therefore, insecticide applications during April were more harmful for the honey bee colonies, when nectar supply is maximum and there is a greater number of forager honey bees on citrus trees.

4. Discussion

To analyze results more accurately, the value of LD50, a value widely used to describe pesticide toxicity, was estimated in laboratory conditions, with caged honey bees that received one toxic topically or orally. Such conditions are better than field ones. Honey bee foragers, worker

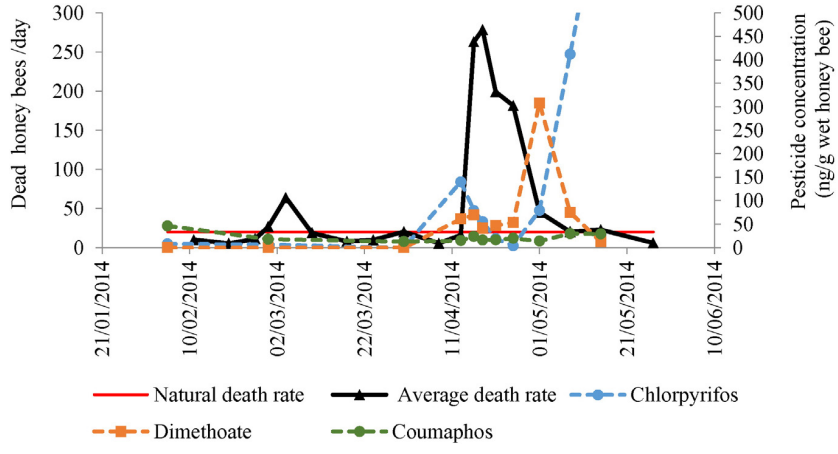

Fig. 3. Death rate and concentration of three main pesticides found in the honey bee samples from the apiary of Barxeta.

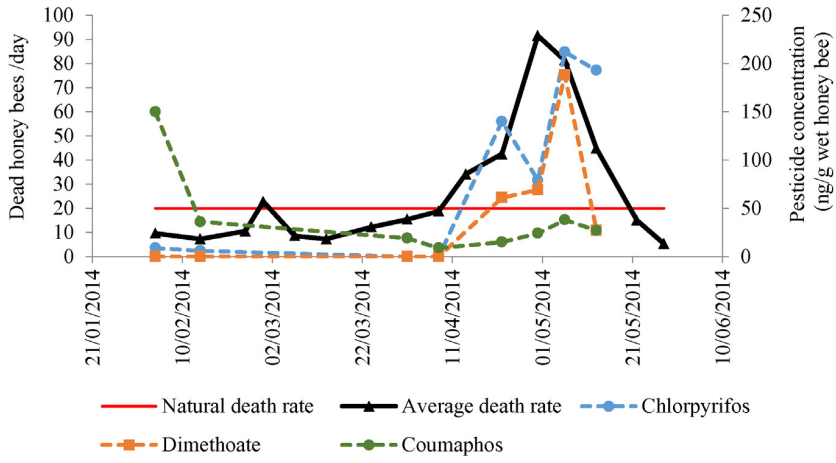

Fig. 4. Death rate and concentration of three main pesticides found in the honey bee samples from the apiary of Montroi.

bees in final phase of life, often exhausted by intense collecting activity, are directly exposed not only to one, but also to many pesticides. Under such conditions, with synergistic and/or cumulative effects of mixture of pesticides, including adjuvant substances, it is expected that colony viability could be affected (Gill et al., 2012), and LD50 should be lower for every toxic (Johnson et al., 2010; Sanchez-Bayo and Goka, 2014).

4.1. Coumaphos residues

In the experimental hives, the commercial product Checkmite was used once a year during previous years against varroosis. Coumaphos is the active acaricide of Checkmite and this active substance has been largely used to control *Varroa destructor*. Residues can be found in wax from many countries (Ghini et al., 2004; Bogdanov, 2006; Chauzat and Faucon, 2007; Mullin et al., 2010; Lambert et al., 2013). Coumaphos was the most frequent pesticide in the honey bee samples (Table 2) and its concentrations remained low and constant during all the monitoring period as stated before. Last treatment with Checkmite strips was removed from hives on November 2013, so the honey bee samples collected in this study were not in direct contact with coumaphos. Wax seems to be the contamination source and this is in consonance with its constant quantities in analytics made. Coumaphos residues found in honey bee samples are many times below LD50 for this compound (Ghini et al., 2004; Mullin et al., 2010). Furthermore, honey bees tolerate therapeutic doses of this organophosphate as a consequence of detoxicative P450 activity (Johnson et al., 2010). Thereby, coumaphos residues are unlikely to be responsible for relevant honey bee mortality. Effects of coumaphos on queen performance were not observed and brood production followed a normal pattern.

4.2. Chlorpyrifos residues

Of all agrochemical pesticides found in this study, chlorpyrifos was the most frequent, both in percentage and in number of positive cases (see Table 2). This organophosphate of high toxicity for honey bees is one of the most ubiquitous xenobiotic found in hive matrices like honey bee wax, pollen, and adult honey bees (Mullin et al., 2010; Lambert et al., 2013). As stated before, there was a clear coincidence between peaks of chlorpyrifos concentration and increasing death honey bee rate on April, particularly in Barxeta and Montroi apiaries, coinciding with citrus blooming (Figs. 3 and 4 respectively). April maximum concentration in both apiaries reached 140 ng/g. Assuming a mean weight of dead honey bees in traps of 0.06 g, this value equals to more than 8 ng/honey bee. If it is assumed a LD50 value for topical exposures of 60–110 ng/honey bee (Ghini et al., 2004), the maximum dose found in the dead honey bees would be approximately 7–14% of LD50. These values are significant if we consider forager conditions and a mean load of four pesticides on honey bees in the present study. When

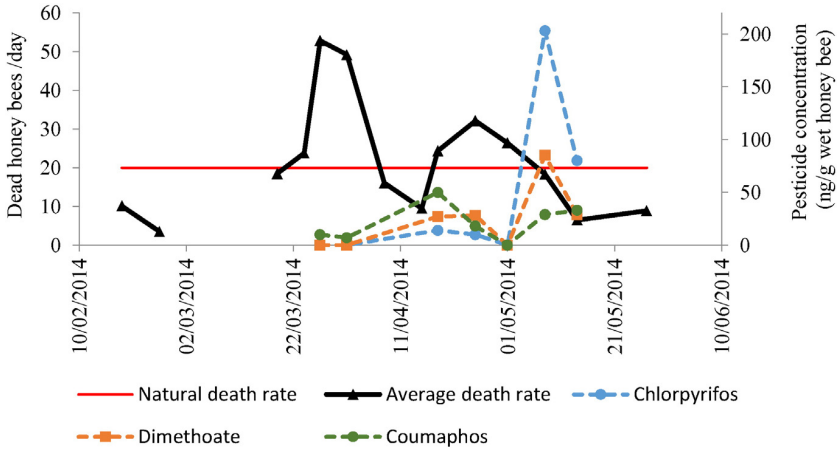

Fig. 5. Death rate and concentration of three main pesticides found in the honey bee samples from the apiary of Carcaixent.

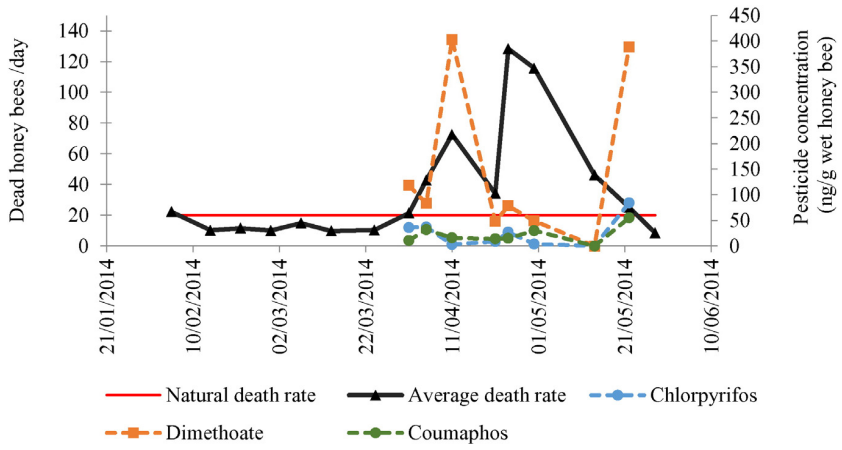

Fig. 6. Death rate and concentration of three main pesticides found in the honey bee samples from the apiary of Chiva.

collecting nectar and pollen from agricultural fields, honey bees are exposed to pesticides orally and topically by multiple routes (Krupke et al., 2012; Sanchez-Bayo and Goka, 2014). After days in dead honey bee traps certain quantity of each pesticide is lost by degradation and the concentration found in honey bee samples is always lower than the original dose of the pesticide exposed to the honey bee. It has to be mentioned the simultaneous effect of dimethoate which concentration also increased during the same intervals of the monitoring period. As a result, it can be concluded that honey bee mortality peak during April (in Barxeta and Montroi) was caused by cumulative effects of chlorpyrifos and dimethoate concentrations.

4.3. Dimethoate/Omethoate residues

These were the second and third agrochemical residues most frequently detected in honey bee samples (see Table 2). As chlorpyrifos, dimethoate is an organophosphate compound and its toxicity for honey bees is high, with a LD50 of 180 ng/honey bee (Ghini et al., 2004). The highest dimethoate concentration during April in Montroi apiary was 188 ng/g (Fig. 4). This value is about 5–10% of honey bees LD50 for dimethoate. As mentioned above, there is a simultaneous increase of chlorpyrifos and dimethoate concentration and death rate of honey bees in Barxeta and Montroi apiaries (Figs. 3 and 4 respectively), causing a cumulative intoxication of foragers. In the case of Chiva (Fig. 6), only dimethoate compound, with a maximum detection of 403 ng/g, that is approximately 24% of LD50, could be implicated in acute mortality of honey bees. Mortality was especially acute in Barxeta apiary, where death rates of almost 500 dead honey bee/day were reached (Supplmentary material Fig. S4).

Omethoate, in spite of being a dimethoate metabolite, it has also a high toxicity for honey bees and its effects are added to chlorpyrifos and dimethoate. Both pesticides are cataloged as very dangerous for honeybees and according to the UE regulation of plant protection products (Regulation 1107/2009), their use during blooming should be severely restricted in crops visited by insect pollinators. In fact, dimethotate spraying is only allowed on seedlings plants. Therefore, illegal use of this organophosphate insecticide according to the current legislation can be confirmed.

4.4. Imidacloprid residues

This neonicotinoid compound is the fourth insecticide most frequently detected in the extracts of honey bees. Its use has been severely restricted in the EU through the regulation 485/2013. One of its restrictions is a strict banning of imidacloprid use before and during blooming season of the crops foraged by honey bees during 2014 and 2015. In this regulation many harmful effects on bee colonies and wild pollinators of neocotinoids imidacloprid, thiamethoxam, and clothianidin were recognized. Imidacloprid LD50 for honey bees is about 3.9–40.9 ng/honey bee, one of the lowest of all insecticides (Iwasa et al., 2004; Schmuck et al., 2001). The mean concentration of imidacloprid in the samples analyzed was 53 ng/g, which is about 15–20% of LD50. The maximum value, detected in honey bees from Barxeta apiary, was 223 ng/g, around 74% of LD50 (detailed information in the supplementary information Table S3). These concentrations are above of those considered sublethal and could be responsible of honey bee losses or even acute intoxication of forager honey bees (Decourtye et al., 2005). However, low levels can produce sublethal effects during long periods without presence of dead honey bees at the entrance of the hive. Imidacloprid is applied to citrus crops by spraying or drip irrigation, it is a very persistent compound in the soil and due to its water solubility can contaminate puddles of water that are important honey bee sources of hydration in

Table 2
Global summary table of pesticides found in honey bee samples from all apiaries.

Pesticide	Number of samples	Positive cases	Percentage (%)	Maximum concentration (ng/g wet honey bee)	Minimum concentration (ng/g wet honey bee)	Mean concentration (ng/g wet honey bee)	SD
Coumaphos	34	32	94	150	7	28	25.4
Chlorpyrifos	34	27	79	751	3	100	160.0
Dimethoate	34	23	68	403	13	102	111.8
Omethoate	34	21	62	109	2	34	26.9
Imidacloprid	34	11	32	223	12	53	63.4
Carbendazim	34	11	32	616	3	141	195.4
Acetamiprid	34	8	24	44	25	32	6.7
Fluvalinate	34	3	9	91	10	52	40.6

agricultural surfaces (Samson-Robert et al., 2014), and it can be translocated throughout the plant, remaining toxic in vegetal tissues for months (Sanchez-Bayo, 2014). Because of this behavior, some residues from previous applications could have been in contact with honey bees, but the high concentrations detected in the present study are expected to be from illegal use of this neonicotinoid during citrus blooming, according to the European Regulation mentioned above.

4.5. Carbendazim residues

Although it was relatively frequent in the honey bee samples, this is a very low toxicity fungicide for honey bees. It could be highlighted a possible synergy between some fungicides and insecticides like imidacloprid (Thompson et al., 2014).

4.6. Acetamipirid residues

It is also a neocotinoid, but its toxicity is much lower than imidacloprid (Iwasa et al., 2004). Most positive samples appeared at the end of monitoring period, so it is expected that its influence on honey bee death rate was not relevant.

4.7. Fluvalinate residues

This compound has been largely used against varroosis all over the world and also in Spain. In fact, tau-fluvalinate (a subset of isomers of fluvalinate) is frequent in hive matrices (Ghini et al., 2004; Mullin et al., 2010; Lambert et al., 2013). The experimental hives used in the study were not treated with this acaricide for more than 10 years and is expected that honey bees acquired fluvalinate residues from contaminated wax combs, ultimate sink of varroacide products. Otherwise, while most pyrethroids are highly toxic to honeybees, fluvalinate is tolerated in high concentrations (Johnson et al., 2010).

5. Conclusions

The QuEChERS modified method for the extractions of honey bee samples followed by liquid chromatography mass spectrometry for their analysis is a good method to determine pesticides residues in honey bee samples. It can be concluded that chlorpyrifos and dimethoate were the main implicated pesticides in honey bee mortality episodes because of their high toxicity, high concentrations detected in the dead honey bee samples, and their coincidence with honey bee mortality peaks. Imidacloprid concentrations in the samples were probably involved in certain mortality episodes during the study and its effects on honey bee colonies were added to those caused by the organophosphates chlorpyrifos and dimethoate.

Coumaphos was unlikely to be responsible for mortality peaks due to its low and constant level during the course of the monitoring period. As showed in mortality results, honey bee losses during citrus blooming season cause a severe problem to local beekeepers. The immediate reduction of colony population compromise their viability and decrease honey yields. However, in spite of the important economic losses to beekeeping industry, harmful effects on other pollinators and wild life are expected in the surrounding areas of the treated crops.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.08.131.

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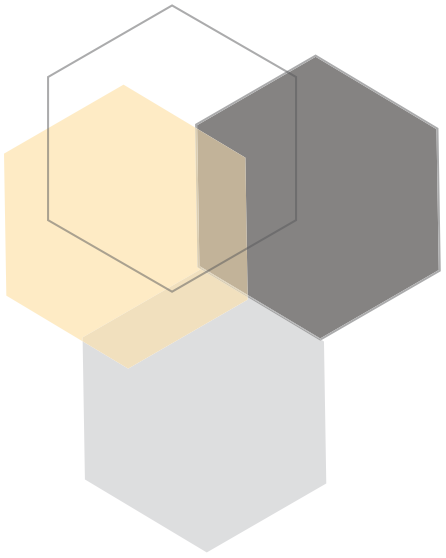
ARTICLE 03

SUPPLEMENTARY MATERIAL:

INFLUENCE OF PESTICIDE USE IN FRUIT ORCHARDS DURING BLOOMING ON HONEY BEE MORTALITY IN 4 EXPERIMENTAL APIARIES.



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A)

B)

Figure S1. Pictures of dead bee traps used in the study: A) front view, B) side view.

S1. Dynamic MRM conditions used for LC–MS/MS determination of pesticide residues.

The linear gradient was as follows: 0 min (50 % B), 10 min (83 % B), 12 min (83 % B), 12.5 min (98 % B), and 15.5 min (98 % B). Then, the mobile phase returns to the initial conditions with an equilibration time of 12 min.

Ionization and fragmentation settings were optimized by direct injection of pesticide standard solutions. MS/MS was performed in the SRM mode using ESI in positive mode. For each compound, two characteristic product ions of the protonated molecule $[M+H]^+$ were monitored, the first and most abundant one was used for quantification, while the second one was used as a qualifier. Collision energy and cone voltage were optimized for each pesticide (table S-1 supplementary material). Nitrogen was used as collision, nebulising and desolvation gas. The ESI conditions were: capillary voltage 4000 V, nebulizer 15 psi, source temperature 300 °C and gas flow 10 L min^{-1}. In order to maximize sensitivity, dynamic MRM was used, with MS_1 and MS_2 at unit resolution and cell acceleration voltage of 7 eV for all the compounds.

Table S1. Dynamic MRM conditions used for LC–MS/MS determination of pesticide residues.

Target Pesticide	t_R[a] (min)	Δ t_R [b]	Precursor Ion	SRM_1[c]	Frag[d] (V)	CE[e] (V)	SMR_2[f]	Frag[d] (V)	CE[e] (V)	SMR_2/SRM_1 (%) (%RSD)[g]
Acetamiprid	2.67	3.21	223	126	111	22	56	111	14	37.4 (12)
Acetochlor	10.07	2	270	224	120	10	148	120	10	46.8 (22)
Alachlor	10.07	2	270	238	80	15	162	80	10	50.4 (13)
Atrazine	6.52	2.63	216	132	120	15	174	120	20	17.3 (14)
Atrazine-desethyl	2.54	2.5	188	146	120	15	104	121	24	29.1 (15)
Atrazine-desisopropyl	1.75	2.08	174	96	120	15	132	120	15	78.6 (13)
Azinphos-ethyl	10.16	1.71	346	97	80	20	137	80	32	83.5 (12)
Azinphos-methyl	8.17	1.24	318	125	80	8	132	80	12	85.4 (11)
Buprofezin	14.5	1.1	306	201	120	10	116	120	15	64.6 (13)
Carbendazim	4.54	4.74	192	160	95	17	132	95	25	11.4 (14)
Carbofuran	4.37	2.91	222	123	120	10	165	70	15	98.0 (9,3)
Carbofuran-3-hydroxy	1.85	2.48	255	163	70	5	220	70	15	90.8 (9)
Chlorfenvinphos	11.74	1.61	359	155	120	10	127	120	15	63.8 (11)
Chlorpyriphos	15.33	2.23	350	350	92	13	198	97	13	78.6 (14)
Coumpahos	14.05	2.15	363	335	134	10	307	134	10	24.8 (10)
Diazinon	11.77	1.89	305	169	128	17	153	128	21	66.3 (12)
Dichlofenthion	14.68	2	315	259	120	10	287	120	5	44 (11)
Dimethoate	2.06	2.59	230	199	80	10	171	80	5	45.3 (12)
Diuron	7.5	1.25	233	72	120	20	160	120	20	3.2 (13)
DMA	2.33	25	122	107	111	18	77	111	42	3.0 (17)
DMF	5.14	4.5	150	132	111	10	107	111	15	41.6 (16)
DMPF	2.33	4.12	163	122	111	15	107	111	15	0.1 (15)
Ethion	14.88	1.23	385	199	80	5	171	80	15	35.3 (11)
Fenitrothion	10.03	1.18	278	125	140	15	109	121	12	95.5 (12)
Fenthion	11.51	1.83	279	247	114	5	169	114	13	76.6 (10)
Fenoxon sulfoxide	4.95	1.83	279	247	114	5	169	114	13	76.6 (11)

Target Pesticide	t_R[a] (min)	Δ t_R [b]	Precursor Ion	SRM_1[c]	Frag[d] (V)	CE[e] (V)	SMR_2[f]	Frag[d] (V)	CE[e] (V)	SMR_2/SRM_1 (%) (%RSD)[g]
Fenoxon sulfone	5.49	3	295	280	136	33	109	136	13	98.1 (14)
Fenthion sulfoxide	5.85	2.68	295	109	136	33	280	136	13	98.1 (14)
Fenthion sulfone	6.22	2.3	311	125	146	21	109	146	17	66.7 (11)
Fipronil	13.33	2.85	437	368	150	15	290	150	25	21.8 (11)
Flumethrin	18.53	1.85	527	267	50	10	239	50	10	48.3 (18)
Fluvalinate	18.11	1.81	503	208	50	10	181	50	26	73.4 (10)
Hexythiazox	15.11	1.15	353	228	120	20	168	120	10	67.4 (9)
Imazalil	11.4	1.71	297	159	120	20	201	120	15	56 (14)
Imidacloprid	1.61	1.96	256	209	80	10	175	80	10	75 (11)
Isoproturon	6.83	2.37	207	72	120	20	165	120	10	16.8 (12)
Malathion	9.36	1.96	331	99	80	10	127	80	5	98.5 (4)
Methiocarb	8.64	1.93	226	121	80	5	169	80	10	66.6 (11)
Metholachlor	10.49	2.04	284	252	120	15	176	120	10	10 (14)
Molinate	9.41	1.98	188	126	80	20	55	80	10	61.7 (11)
Omethoate	1.06	2.67	214	125	80	5	183	80	20	72.3 (12)
Parathion-ethyl	11.11	1.91	292	236	88	4	264	88	8	45.5 (13)
Parathion-methyl	8.17	1.5	264	125	120	20	232	110	5	34.5 (13)
Prochloraz	12.08	1.91	376	308	80	10	266	80	10	14.3 (9)
Propanil	8.6	2.01	218	162	120	20	127	120	15	92.4 (11)
Propazine	8.74	2	230	146	120	15	188	120	20	93.3 (14)
Pyriproxyfen	14.78	1.33	322	227	120	10	185	120	10	36.1 (12)
Simazine	4.53	1.76	202	124	120	20	132	120	20	93.8 (12)
Terbutryn	10.63	1.2	242	186	120	20	71	120	15	4.6 (14)
Tebuconazole	13.82	2.87	308	125	95	25	70	95	21	6.6 (11)
Terbumeton	10.98	2.89	226	170	95	17	114	95	25	13.8 (14)
Terbumeton-desethyl	6.69	3.76	198	142	90	13	86	90	25	31.7 (12)
Terbuthylazine	11.1	3.01	230	174	95	13	96	95	25	16,4 (13)
Terbuthylazine-2-hydroxy	6.92	3.28	212	156	95	13	86	95	25	28 (13)
Terbuthylazine-desethyl	6.98	2.81	202	146	95	13	79	95	25	13.2 (14)
Thiabendazole	5.06	3.5	202	175	95	25	131	95	25	29.1 (18)
Thiamethoxam	2	2.58	292	211	78	10	132	78	10	21.3 (11)
Tolclofos-methyl	12.13	1.71	301	125	115	12	269	120	15	73.8 (19)

[a] t_R = retention time.

[b] Δ t_R = delta retention time, that is the centered retention time window.

[c] SRM_1 = selected product ion for quantification.

[d] Frag = Fragmentor.

[e] CE = Collision energy.

[f] SRM_2 = selected product ion for qualification.

[g] (%RSD) = relative standard deviation of the ratio SRM_2/SRM_1, calculated from mean values obtained from the matrix-matched calibration curves.

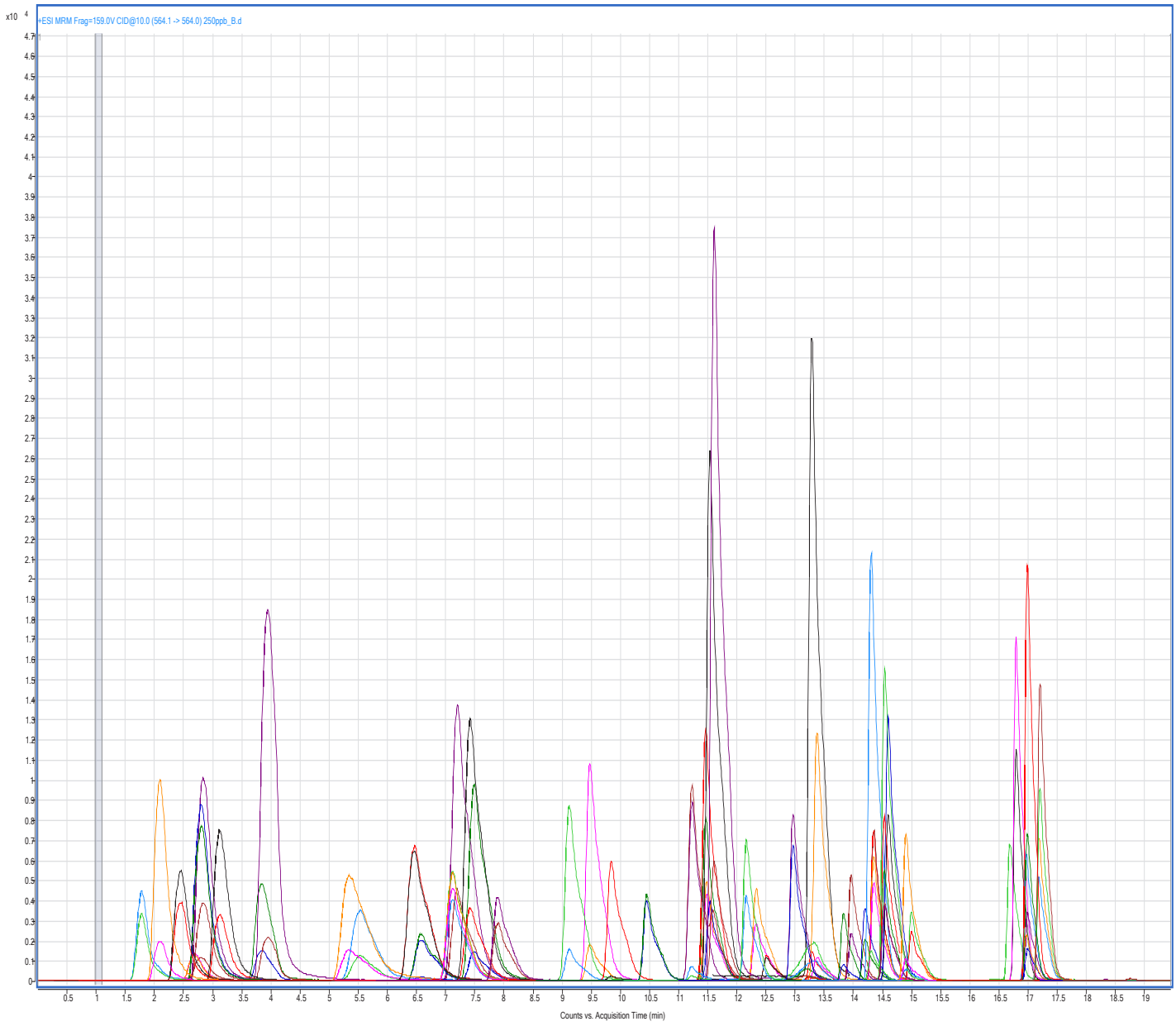


Figure S2. Chromatograms extracted from 250 mg/L standard of all pesticides analyzed.

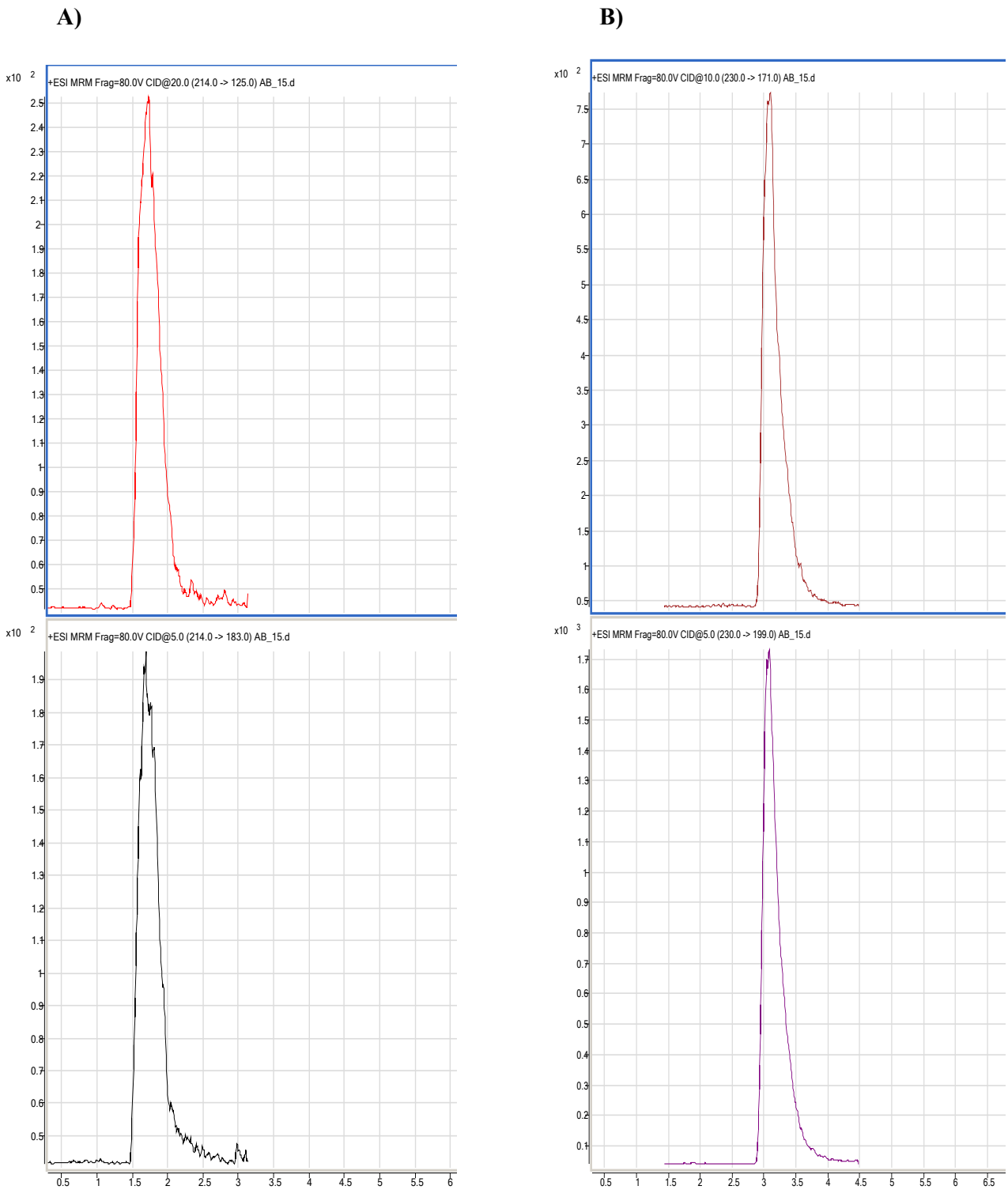


Figure S3. Chromatogram of a honey bee simple from Barxeta. Both ion products (125 and 183) of omethoate (214) are shown in A), both ion products (171 and 199) of dimethoate (230) are shown in B), and both ion products (97 and 198) of chlorpyrifos (350) are shown in C).

C)

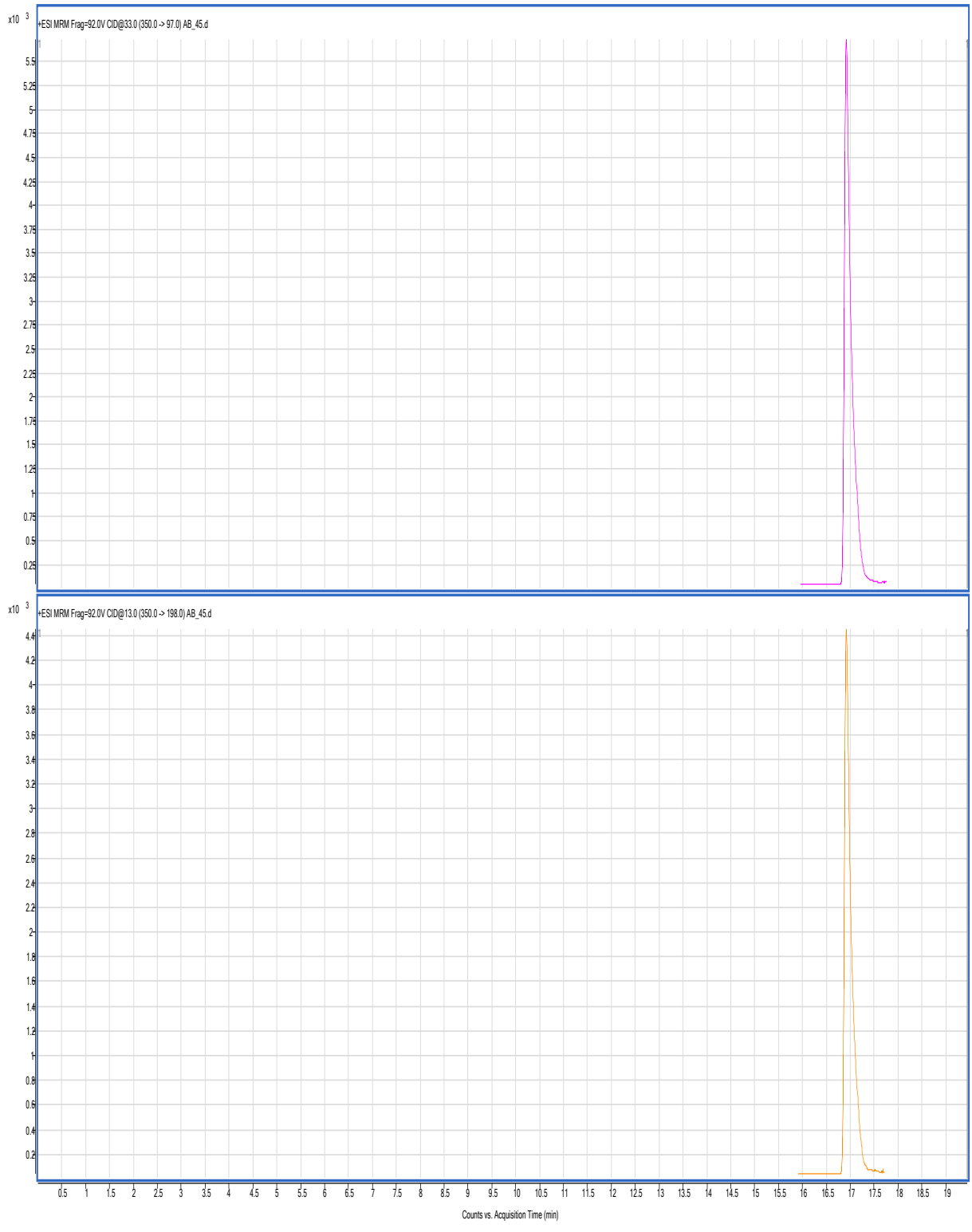

x10 3
+ESI MRM Frag=92.0V CID@33.0 (350.0 -> 97.0) AB_45.d
x10 3
+ESI MRM Frag=92.0V CID@13.0 (350.0 -> 198.0) AB_45.d
Counts vs. Acquisition Time (min)

Table S2. Linearity of the analyzed pesticides prepared in honeybee extracts (concentration range from LOQ to 250 ng/g).

Pesticide	Linearity	R^2
Acetamiprid	y=728.324x-3782.29	0.992
Acetochlor	y=56.84x+2.25	0.99
Alachlor	y=193.27x+1.08	0.99
Atrazine	y=710.81x+69.98	0.992
Atrazine-desethyl	y=492.95x+30.7	0.992
Atrazine-desisopropyl	y=93.01x+12.76	0.99
Azinphos-ethyl	y=645.51x+25.27	0.989
Azinphos-methyl	y=283.73x+23.81	0.992
Buprofezin	y=1235.99x+56.22	0.993
Carbendazim	y=2539.83x+373.63	0.992
Carbofuran	y=449.62x+6.6	0.99
Carbofuran-3-hydroxy	y=854.19x+19.03	0.993
Chlorfenvinphos	y=645.84x+13.94	0.985
Chlorpyrifos	y=354.05x+59.88	0.995
Coumaphos	y=746.46x-1773.25	0.995
Diazinon	y=896.56x+2.47	0.996
Dichlofenthion	y=159.24x+15.33	0.995
Dimethoate	y=643.3x+20.72	0.993
Diuron	y=513.03x+25.54	0.987
DMA	y=1.52x-0.13	0.995
DMF	y=364.62x-109.77	0.999
DMPF	y= 82.68x-173.54	0.998
Ethion	y=1691.33x+180.96	0.988
Fenitrothion	y=60.98x-0.04	0.99
Fenoxon- sulfoxide	y=903.37x+85.57	0.997
Fenoxon-sulfone	y=1248.64x+28.92	0.993
Fenthion	y=903.37x+85.57	0.997
Fenthion.-sulfone	y=431.48x+34.78	0.989
Fenthion-sulfoxide	y=1248.64x+28.92	0.995
Fipronil	y= 154.29x-709.75	0.993
Flumethrin	y= 6.90x+52.61	0.995
Fluvalinate	y= 364.18x-1581.14	0.997
Hexythiazox	y=895.33x+69.95	0.992
Imazalil	y=354.52x+16.41	0.994
Imidloprid	y=415.67x+33.42	0.99
Isoproturon	y=1114.98x+84.83	0.989
Malathion	y=515.75x+40.3	0.995

Metalachlor	y=808.59x+32.4	0.992
Methiocarb	y=861.93x+23.94	0.993
Molinate	y=143.53x+2.39	0.991
Omethoate	y=629.96x+38.68	0.989
Parathion-methyl	y=1430.03x-7774.97	0.996
Parathion-ethyl	y=447.21x+4.19	0.991
Prochloraz	y=734.23x+49.68	0.996
Propanil	y=85.79x-9.87	0.991
Propazine	y=588.89x+14.05	0.991
Pyriproxifen	y=382.46x+317.95	0.995
Simazine	y=213.64x+3.1	0.992
Tebuconazole	y=1724.59x+458.59	0.989
Terbumeton	y=2393.22x+181.28	0.989
Terbumeton-desethyl	y=1858.09x-3.55	0.994
Terbuthylazine	y=2790.5x+15.15	0.991
Terbuthylazine-2-hydroxy	y=1662.55x+134.95	0.992
Terbuthylazine-desethyl	y=936.25x+22.78	0.992
Terbutryn	y=2771.43x+15.39	0.989
Thiabendazole	y=1097.81x+22.88	0.99
Thiamethoxam	y= 638.39x-2474.93	0.996
Tolclofos-methyl	y=140.9x+4.25	0.992

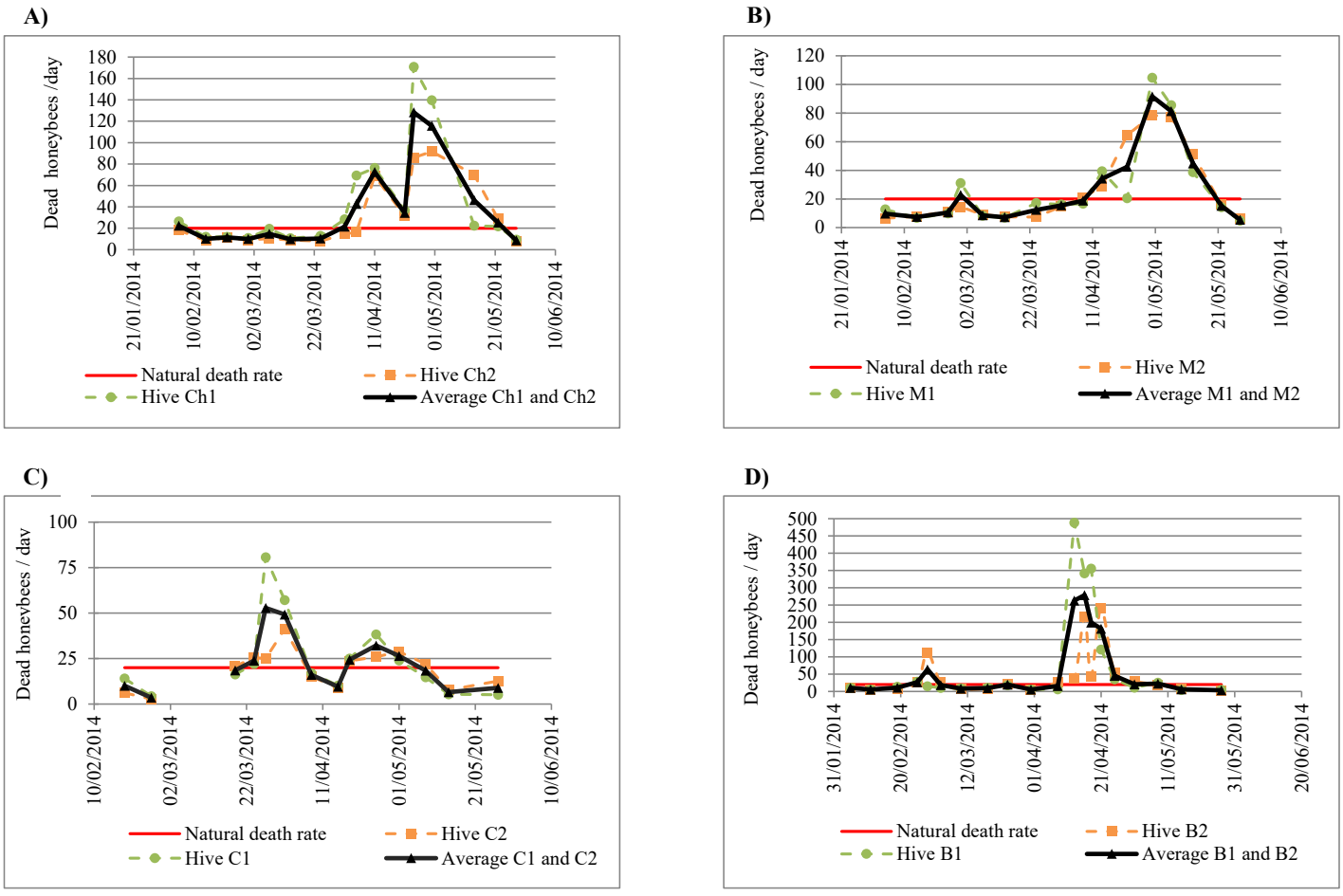


Figure S4. Mortality curves in the 4 apiaries: A) Chiva, B) Montroi C) Carcaixent and D) Barxeta.

Apiaries pesticide summary tables

Table S3. Pesticides found in honeybee samples from apiary of Barxeta (B1-B11).

Pesticide	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	Positive cases	%	Maximum concentration (ng/g wet honeybee)	Minimum concentration (ng/g wet honeybee)	Mean concentration (ng/g wet honeybee)	S.D.
	Samples concentration (ng/g wet honeybee)																
Coumpahos	46	18	13	15	24	16	17	20	14	30	29	11	100,0	46	13	22,0	9,84
Chlorpyriphos	8	7	0	140	79	55	19	4	79	412	751	10	90,9	751	4	141,3	234,48
Dimethoate	0	0	0	61	69	41	47	53	308	75	13	8	72,7	308	41	60,6	86,83
Omethoate	0	0	0	30	39	25	27	32	52	22	0	7	63,6	52	22	20,6	18,17
Imidacloprid	0	0	0	0	0	0	0	23	30	223	27	4	36,4	223	23	27,5	65,99
Carbendazim	0	0	0	0	0	0	0	0	0	0	0	0	0,0	0	0	0,0	0,00
Acetamiprid	0	0	0	0	0	44	25	0	0	27	37	4	36,4	44	25	12,1	17,47
Fluvalinate	0	0	0	0	0	0	0	0	0	0	0	0	0,0	0	0	0,0	0,00

Table S4. Pesticides found in honeybee samples from apiary of Carcaixent (C1-C7).

Pesticide	C1	C2	C3	C4	C5	C6	C7	Positive cases	%	Maximum concentration (ng/g wet honeybee)	Minimum concentration (ng/g wet honeybee)	Mean concentration (ng/g wet honeybee)	S.D.
	Samples concentration (ng/g wet honeybee)												
Coumpahos	10	7	50	18	0	29	33	6	85,7	50	7	21,0	17,40
Chlorpyriphos	0	0	14	10	0	203	80	4	57,1	203	10	43,9	75,76
Dimethoate	0	0	27	28	0	85	29	4	57,1	85	27	24,1	30,27
Omethoate	0	0	11	26	0	25	25	4	57,1	26	11	12,4	12,69
Imidacloprid	0	0	0	0	0	0	0	0	0,0	0	0	0,0	0,00
Carbendazim	0	9	0	0	0	7	31	3	42,9	31	7	6,7	11,37
Acetamiprid	0	0	0	0	0	28	26	2	28,6	28	26	7,7	13,19
Fluvalinate	0	56	0	0	0	0	0	1	14,3	56	56	8,0	21,17

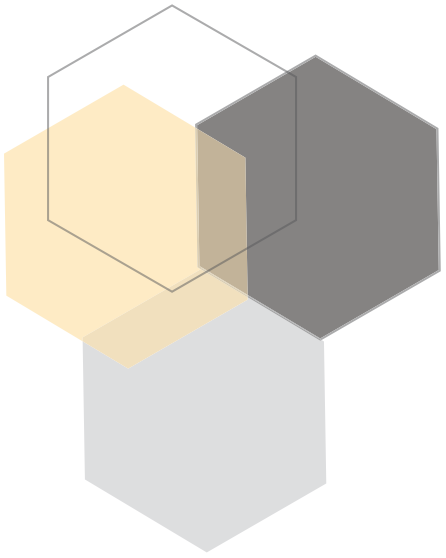
Table S5. Pesticides found in honeybee samples from apiary of Montroi (M1-M8).

Pesticide	M1	M2	M3	M4	M5	M6	M7	M8	Positive cases	%	Maximum concentration (ng/g wet honeybee)	Minimum concentration (ng/g wet honeybee)	Mean concentration (ng/g wet honeybee)	S.D.
	Samples concentration (ng/g wet honeybee)													
Coumpahos	150	36	19	9	15	24	38	27	8	100,0	150	9	39,8	45,63
Chlorpyriphos	9	6	0	0	140	79	212	193	6	75,0	212	79	79,9	90,33
Dimethoate	0	0	0	0	61	69	188	27	4	50,0	188	61	43,1	65,11
Omethoate	0	0	0	0	30	26	40	2	4	50,0	40	2	12,3	16,82
Imidacloprid	0	0	0	13	0	0	88	36	3	37,5	88	13	17,1	31,31
Carbendazim	0	0	0	0	0	0	3	0	1	12,5	3	3	0,4	1,06
Acetamiprid	0	0	0	0	0	0	0	33	1	12,5	33	33	4,1	11,67
Fluvalinate	0	0	0	0	0	0	0	0	0	0,0	0	0	0,0	0,00

Table S6. Pesticides found in honeybee samples from apiary of Chiva (Ch1-Ch8).

Pesticide	Ch1	Ch2	Ch3	Ch4	Ch5	Ch6	Ch7	Ch8	Positive cases	%	Maximum concentration (ng/g wet honeybee)	Minimum concentration (ng/g wet honeybee)	Mean concentration (ng/g wet honeybee)	S.D.
	Samples concentration (ng/g wet honeybee)													
Coumpahos	11	32	16	14	15	30	0	55	7	87,5	55	11	21,6	16,93
Chlorpyriphos	36	37	3	9	27	4	0	84	7	87,5	84	3	25,0	28,17
Dimethoate	118	83	403	48	78	49	0	388	7	87,5	403	48	145,9	157,81
Omethoate	12	0	108	28	27	19	0	109	6	75,0	109	12	37,9	44,86
Imidacloprid	0	12	0	17	0	15	0	94	4	50,0	94	12	17,3	31,88
Carbendazim	7	125	616	381	187	151	0	34	7	87,5	616	7	187,6	213,00
Acetamiprid	0	0	0	0	0	0	0	36	1	12,5	36	36	4,5	12,73
Fluvalinate	91	10	0	0	0	0	0	0	2	25,0	91	10	12,6	31,86

ARTICLE 04



A TWO-YEAR MONITORING OF PESTICIDE HAZARD IN-HIVE: HIGH HONEY BEE MORTALITY RATES DURING INSECTICIDE POISONING EPISODES IN APIARIES LOCATED NEAR AGRICULTURAL SETTINGS.

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A two-year monitoring of pesticide hazard in-hive: High honey bee mortality rates during insecticide poisoning episodes in apiaries located near agricultural settings

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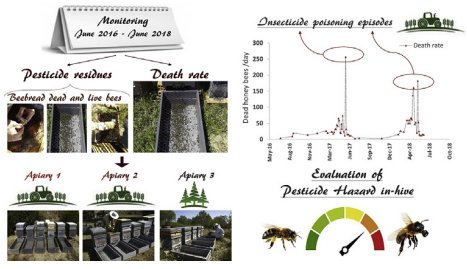
HIGHLIGHTS

- Pesticide residues in live honey bees were low and ranged from 2 to 56 ng g $^{-1}$.
- Relevant pesticide hazard in beebread was produced by insecticides used in crops.
- Beeswax was contaminated by miticides from present and past uses in beekeeping.
- Honey bee insecticide poisoning occurred in apiaries located near farmlands.
- Chlorpyrifos, dimethoate and imidacloprid were related to high mortality rates.

GRAPHICAL ABSTRACT



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ABSTRACT

Pesticide residues in beebread, live and dead honey bees, together with honey bee death rate were monitored from June 2016 to June 2018 in three apiaries, located near agricultural settings and in wildlands. Dead honey bees were only collected and analyzed when significant mortality episodes occurred and pesticide content in beeswax of each experimental apiary was evaluated at the beginning of the study. Samples were extracted by a modified QuEChERS procedure and screened for pesticides residues by liquid chromatography mass spectrometry (LC-MS/MS). Pesticide hazard in the samples was evaluated through the hazard quotient approach (HQ). Beebread was widely contaminated with coumaphos and amitraz degradate 2, 4-dimethylphenylformamide (DMF), miticides detected in 94 and 97% of samples respectively. However, insecticides sprayed during citrus bloom like chlorpyrifos (up to 167 ng g $^{-1}$) and dimethoate (up to 34 ng g $^{-1}$) were the main responsible of the relevant pesticide hazard in this matrix. Pesticide levels in live bees were mostly residual, and pesticide hazard was low. Beeswax of the apiaries, contaminated by miticides, revealed a low pesticide hazard to honey bee colonies. Acute mortality episodes occurred only in the two apiaries located near agricultural settings. Dead bees collected during these episodes revealed high levels (up to 2700 ng g $^{-1}$) of chlorpyrifos, dimethoate,

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omethoate and imidacloprid. HQ calculated in dead bees exceeded up to 37 times the threshold value considered as elevated hazard to honey bee health.

1. Introduction

Insect pollination increases yield of many crops (Andrikopoulos and Cane, 2018; Fijen et al., 2018; Perrot et al., 2018), and a 35% of fruit, vegetable and seed global production depends directly on pollinators (Klein et al., 2007). While global demand of pollinators in food production is increasing (Aizen and Lawrence, 2009), wild pollinators are disappearing from intensively farmed landscapes (Kosior et al., 2007; Garibaldi et al., 2011), and honey bee colonies are experiencing concerning loss rates (Potts et al., 2010; Kulhanek et al., 2017; Brodschneider et al., 2018). The increasing use of pesticides, habitat loss and lack of floral diversity, together with pathogens, is likely to be the explanation of pollinator loss documented worldwide (Goulson et al., 2015; Grassl et al., 2018).

Honey bees are exposed to multiple pesticides applied to crops, which are transferred to the hive by forager bees, due to that bees have been used as bioindicator of pesticides in agroecosystems (Porrini et al., 2014; Niell et al., 2017). In addition, honey bees are also in contact with acaricides used in beekeeping against *Varroa*, and analysis of beebread and beeswax have revealed contamination by several pesticide groups (Mullin et al., 2010; Calatayud-Vernich et al., 2018). As a result, bees are exposed to cocktails of pesticides inside and outside the hive (Traynor et al., 2016) that affect not only bee individuals but also colony viability. Risks may vary from acute toxicity that produces mortality in the short or middle term, to sub lethal effects in the long-term (Sanchez-Bayo and Goka, 2016). Acute and chronic exposure effects on bee health to a single or multiple pesticides are well documented, and can impair food transfer, sperm viability, alter learning and odour processes, enhance gene suppression, cause immune and nutritional stress, and cause mortality (Bevk et al., 2012; Andrione et al., 2016; Chaimanee et al., 2016; Gregore et al., 2018; Reeves et al., 2018; Siviter et al., 2018). Furthermore, high mortality rates of honey bees caused by insecticides used in plant protection have been reported around Europe (Calatayud-Vernich et al., 2015; Kiljanek et al., 2016a, 2017; Martinello et al., 2017).

Considering honey bees as the primary pollinator in agricultural landscapes, it is important to understand the magnitude of pesticide incidence in honey bee apiaries. The present study could be considered as a continuation of our previous pilot study **Calatayud-Vernich et al. (2016)**, in which pesticide concentration in dead bees samples and mortality of honeybees were monitored in different locations during blooming season. This study introduces innovative aspects since it reports results of a longer monitoring period, and analyze the most relevant matrices of beekeeping, the study was 2 years long, and the experimental apiaries were located not only near agricultural settings, but also in forest areas in order to compare whether high mortality episodes appear in both types of apiaries environment. Pesticide hazard was assessed not only in dead honeybees when acute mortality took place, but also periodically in live honey bees and beebread. Beeswax pesticide content was also analyzed to understand the contribution of this matrix to overall pesticide hazard in-hive.

2. Material and methods

2.1. Experimental apiaries

The three experimental apiaries were located in the east coast of Spain, in a typical Mediterranean climatic area. Apiary 1 and 2 were placed in intensive agriculture areas, while apiary 3 surroundings were predominantly wildlands with scattered rainfed crops like olive and carob trees. Apiary 1 was surrounded mainly by citrus orchards and apiary 2 was surrounded by citrus but also by other fruit trees like nectarines (Fig. 1).

Experimental apiaries consisted of five Dadant hives (10 frames of measures 42 × 27 cm). Colony health was evaluated throughout the study by periodic sanitary inspections. Analysis of pathogens including Deformed Wing Virus (DWV), Acute Paralysis Virus group (IAPV), Black Queen Cell Virus (BQCV), Chronic Bee Paralysis Virus (CBPV) and *Nosema ceranae* were carried out following standard molecular biology approaches for reverse transcription quantitative real-time polymerase-chain reaction (RT-qPCR) (Herrero et al., 2019). Primer pairs used to detect and quantify each pathogen were either published elsewhere or designed *de novo* for this study (Table S9). Colonies were replaced if strength or viability was compromised. Screened bottom boards were used to monitor varroa infestation, and amitraz (Apitraz commercial product) was the only miticide applied in-hive against varroosis from September to December during the study.

2.2. Monitoring mortality

During two years, from June 2016 to June 2018, honey bee mortality was monitored in the three apiaries (Table S2-S3-S4 Supplementary material). Mortality was calculated for each of the five colonies in the three apiaries, and the average value of the five colonies of each apiary was used to plot mortality curves. When significant mortality episodes occurred, collection of dead bees was carried out more frequently. A natural threshold death rate of 20 honey bees per day and colony was assumed according to the values proposed by Porrini et al. (2003). In spring season, there is a natural population growth in honey bee colonies, thus death rate should be considered moderately above 20 dead bees/day.

Death rate was quantified by collecting dead honey bees through basket traps (Accorti et al., 1991; Porrini et al., 2003). Traps consisted of a wooden box with a chain mail on top, placed under the hive entrance.

2.3. Sampling

2.3.1. Live and dead honey bees

Live bees (38 samples) from inside of the hives were collected periodically from the lateral combs to avoid recently born bees, and were a pool of bees from the five hives of each apiary (Table 1). Dead bees (17 samples) were collected when acute mortality signs appeared in the apiaries, this is piles of dead or dying bees at colony entrance. Dead bees were collected from front-door traps and pooled per apiary. The samples were transported to the laboratory

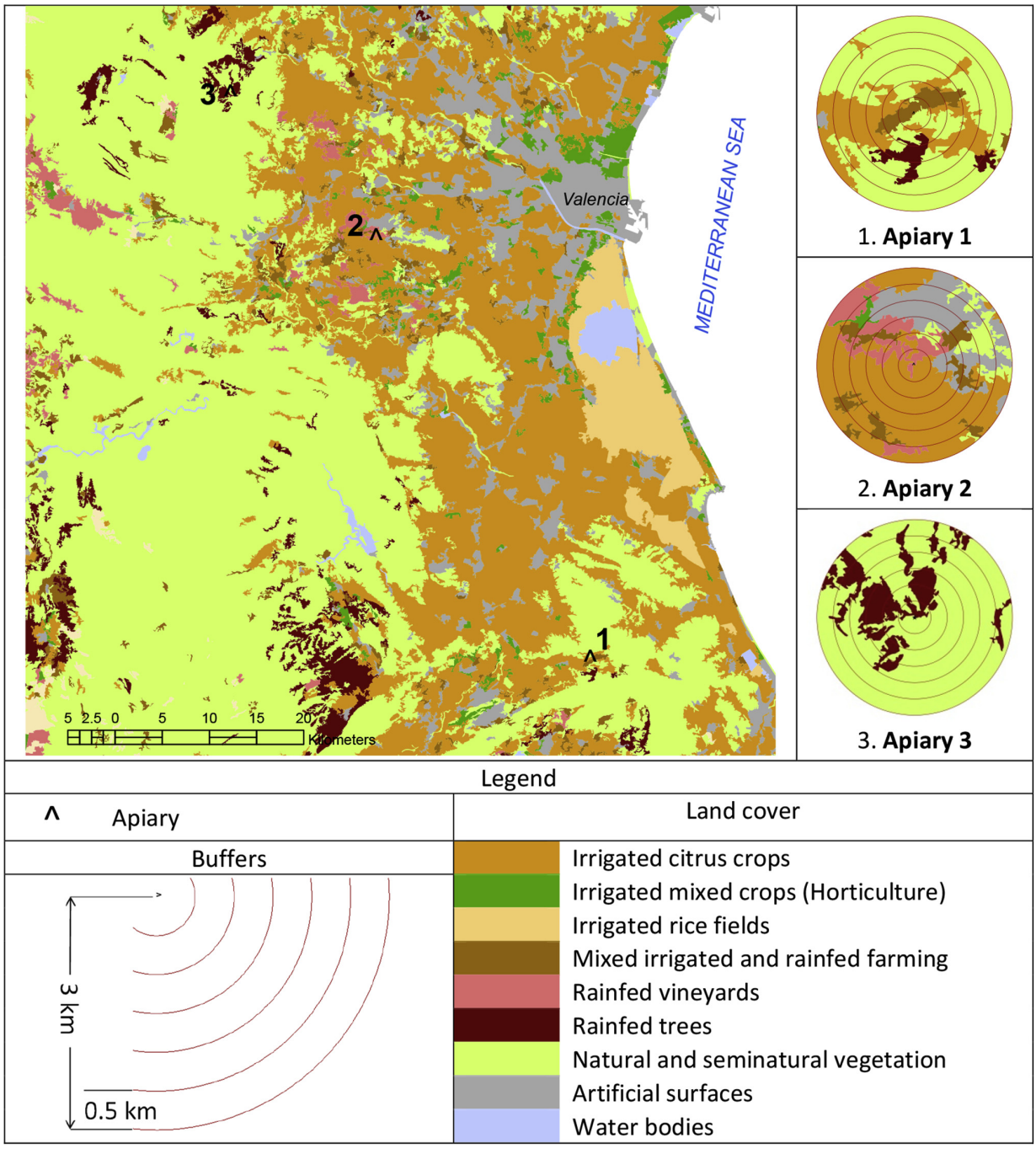

Fig. 1. Location of the experimental apiaries and land cover uses.

in an insulated cooler and stored at −20 °C until pesticide analysis.

2.3.2. Hive matrices

Beebread (33 samples) was collected periodically along with live honey bee samples. Beebread was collected from inside of a comb portion with a disposable wooden stick, and all beebread samples were pooled per location.

Three beeswax samples were collected and analyzed at the beginning of the monitoring period to be used as reference values for pesticide concentrations in wax from each apiary. Beeswax was obtained by cutting a portion of the comb free of beebread, honey or brood. The beeswax from each of the five colonies was mixed in a unique wax sample representative of each apiary.

2.4. Chemicals and reagents

High purity standards (98–99.9%) of the 60 selected pesticides together with the degradate products of amitraz; 2,4-dimethylaniline (DMA), 2,4-dimethylphenylformamide (DMF) and N-(2,4-dimethylphenyl)-N′-methylformamidine (DMPF) were from

Table 1
Sampling outline.

		Sample composition	Apiary 1 (Nº samples)	Apiary 2 (Nº samples)	Apiary 3 (Nº samples)	Time frame	Sampling dates
Honey bees	**Live bees**	5 g (c. 80 bees)	13	13	12	From June 2016 to June 2018	Each 1.5 or 2 months
	Dead bees	5 g (c. 80 bees)	11	6	–		During acute mortality episodes
Hive matrices	**Beebread**	5 g	11	11	11		Each 1.5 or 2 months
	Beeswax	2 g	1	1	1	June 2016	At the beginning of the study

Sigma-Aldrich (Steinheim, Germany) (listed in supplementary material Table S1). Individual standard solutions were prepared in methanol at a concentration of 1000 mg·L-1. The working standard solutions were prepared by mixing the appropriate amounts of individual standard solutions and diluting them with methanol to a final concentration of 1 and 10 mg L^{-1}. Solutions were stored in 15 mL vials at 4 °C in the dark. Magnesium sulfate was obtained from Alfa Aesar (Karlsruhe, Germany), ammonium formate, sodium chloride, acetonitrile and formic acid were purchased from Sigma-Aldrich (Steinheim, Germany). PSA and C18 sorbents, and PTFE (13 mm × 0.22 mm) filters were purchased from Análisis Vínicos S.L. (Tomelloso, Spain). Methanol was obtained from VWR chemicals (Radnor, Pennsylvania). Deionized water was from a MilliQ SP Reagent Water System (Millipore, Bedford, MA, USA).

2.5. Analysis

Methodology used in the present study has been widely used to detect pesticide residues in beekeeping matrices (Herrera-López et al., 2016; Daniele et al., 2017). The samples were extracted by a slightly modified QuEChERS procedure and screened for 63 pesticides and its degradation products by liquid chromatography mass spectrometry (LC-MS/MS). The QuEChERS protocol using acetonitrile as extraction solvent and primary-secondary amine (PSA) and C18 as cleaner sorbents was applied to honey bees, beebread and beeswax samples (see Supplementary material for detailed information). Beeswax extraction procedure adapted from Niell et al. (2014), and methods used for beebread and honey bee extractions were validated in previous studies (Calatayud-Vernich et al., 2015, 2017, 2018). The chromatographic instrument was an HP1200 series LC equipped with an automatic injector, a degasser, a quaternary pump and a column oven-combined with an Agilent 6410 triple quadrupole (QQQ) mass spectrometer with an electrospray ionization (ESI) interface (Agilent Technologies, Waldbronn, Germany). Data were processed using a MassHunter Workstation Software for qualitative and quantitative analysis (A GL Sciences, Tokyo, Japan).

2.6. Hazard quotients (HQ)

Pesticide hazard to honey bees was calculated through the hazard quotient (HQ) scores (HQ = pesticide concentration in ng·g^{-1} ÷ pesticide topical/oral LD50 as μg/bee) proposed by Stoner and Eitzer (2013). This is, the sum of all pesticide residue concentrations detected (ng·g^{-1}) divided by their respective contact or oral LD50 in μg/bee for each residue in a given sample. The HQ score provides an estimate based on percentages of LD50 equivalents present in beebread, wax, and in honey bees themselves. Honey bees and beebread samples had a relevant HQ score when it was greater than 50, and the HQ score was considered as elevated when it was greater than 1000. In beeswax, pesticides are embedded in a lipophilic matrix and not all residues are in contact with honey bees. Only a fraction of the pesticide load is exposed to the individuals of the colony, so HQ in beeswax samples was considered as relevant when it was greater than 250. Samples with $HQ_{beeswax}$ > 5000 were considered to have an elevate pesticide load (Traynor et al., 2016). Pesticides LD50 used for the hazard quotient were taken from Sanchez-Bayo and Goka (2014), and University of Hertfordshire Pesticide Properties Database (Hertfordshire, 2018). Amitraz concentrations in the samples were calculated through its main breakdown products DMF and DMPF (Korta et al., 2001). Amitraz parent compound ecotoxicological data was used to HQ calculations when detected.

2.7. Data spatial integration and GIS information treatment analysis

Spatial distribution analysis was performed using GIS techniques with ARCGIS (V. 10.5). All digital layers were geographically positioned following national and regional mapping standards: Spatial reference system ETRS89 and Universal Transverse Mercator projection. Initial information consisted of a vector line layer with an update land use-cover for the year 2018 following a simplification of CORINE Land Cover nomenclature (Kosztra and Büttner, 2018). The original CORINE land cover nomenclature based in three levels was adapted into a single semantic legend considering the major land cover classes. Geometric and land cover type extraction was performed using the 2018 orthophoto provided by the Spanish Institute of Geography. As a result, nine land use cover groups were stablished, namely: Irrigated citrus crops, irrigated mixed crops, irrigated rice fields, mixed irrigated and rainfed farming, rainfed vineyards, rainfed trees, natural and seminatural vegetation, artificial surfaces and water bodies. Finally, ring maps were constructed from the point layer containing the location of the different apiaries. The buffer criteria applied was the creation of six circles of 0.5, 1, 1.5, 2, 2.5 and 3 km which center was each experimental apiary, with the assumption that the ring of 3 km radius would represent a typical honey bee foraging distance and would constitute a potential area of influence for incoming pesticides used in plant protection. Map overlay techniques were applied to land uses map and the rings to obtain the potential area of influence with land uses for each apiary and each buffer distance. Summarize relative values (percentages) for each land cover ring were obtained (Supplementary material S10).

3. Results and discussion

3.1. Monitoring pesticide hazard in-hive

3.1.1. Beeswax

Pesticide content in beeswax was assessed at the beginning of the study, and expected to be similar throughout the duration of the study, as several pervious studies already showed that pesticide levels were similar between wax from different seasons due to pesticides stability in this matrix and its low replacement rate (Calatayud-Vernich et al., 2017, 2018). Pesticides analysis of beeswax evidenced the high contamination of this matrix by miticides. Coumaphos and chlorfenvinphos were detected

simultaneously in the three apiaries. Coumaphos, not used as varroa treatment in the apiaries for many years, remain embedded in this matrix. It was found at concentrations of 880, 1935 and 5085 ng g $^{-1}$ in apiaries 3, 2 and 1, respectively. Chlorfenvinphos detections were 35, 295 and 320 ng g $^{-1}$ in apiaries 2, 1 and 3. This compound was not used in the experimental apiaries, so pesticide residues in wax come from the beeswax recycling process, where a mixed pool of wax from multiple beekeepers is melted to make new foundations sheets. These levels suggested the non-authorized use of this product in beekeeping (Regulation (EC), 2013). Previous surveys in Italy and Spain have also evidenced the use of this compound in beekeeping through detections in beeswax (Boi et al., 2016; Calatayud-Vernich et al., 2017; Perugini et al., 2018). Although Amitraz was the acaricide used in the experimental apiaries, amitraz degradate DMF was only detected in beeswax from apiary 3 with a concentration of 190 ng g $^{-1}$. This is explained by the DMF lower stability and affinity for beeswax (LogP = −1.1) (Hertfordshire, 2018). HQ $_{beeswax}$ scores were low (53 and 182) for apiaries 2 and 3, but relevant (326) for apiary 1. HQ $_{beeswax}$ calculated in this study was lower than those calculated in previous studies that showed average HQ in beeswax over 6000 points, and considered elevated (Calatayud-Vernich et al., 2018).

3.2. Beebread

Samples of beebread (n = 33) contained 17 different pesticide residues among miticides, insecticides, fungicides and herbicides (Table 2). Five samples from apiaries 1 and 2, located in agricultural landscapes, contained more than eight different pesticide residues simultaneously. An average of five pesticides per sample was detected in both apiaries, while beebread from apiary 3 was less contaminated with an average of three pesticides per sample (Supplementary material TableS5). Apiaries 1 and 2, located in areas with intensive agriculture surroundings, exhibited average HQ $_{beebread}$ between six and seven times higher than apiary 3, located in wildlands and with less agricultural settings in the surroundings. Apiary 3 exhibited a low pesticide hazard in more than 90% of samples. Beebread from apiaries 1 and 2 exhibited relevant pesticide hazard in more than 50% of samples. Therefore, apiaries surroundings influenced beebread HQ scores (Colwell et al., 2017). Amitraz and coumaphos were detected in most of the samples, 97 and 94% respectively. Both miticides had the highest mean concentrations, 71.2 and 31.6 ng g^{-1}, respectively. However, contributions to HQ $_{beebread}$ were low and did not exceeded 38 points (Table 2). Miticides not used in the apiaries like fluvalinate, chlorfenvinphos and acrinathrin were detected with mean concentrations below 2 ng g^{-1}, and their contributions to HQ $_{beebread}$ were low (<5 points) and did not pose substantial hazard to colonies health with the exception of acrinathrin, which showed low but also relevant contributions (>300 points) to hazard quotients in apiary 2. Hexythiazox was detected in 24% of samples with a mean concentration of 1 ng g^{-1}. So, while hexythiazox is used in fruit trees fields, and is likely to be transported to the hive through foraging activity, the main source of beebread contamination by miticides appears to be the wax matrix. Beeswax in our experimental apiaries was contaminated with amitraz degradate DMF, coumaphos and chlorfenvinphos, and previous surveys of Spanish beeswax have showed that acrinathrin and fluvalinte were also found in this matrix at high levels (Calatayud-Vernich et al., 2018).

Chlorpyrifos and dimethoate (organophosphates insecticides) were detected in 45 and 24% of the samples, and mean concentrations were 16.2 and 3.4 ng g^{-1}. Both compounds are the most used in citrus crops during bloom, and so, they were detected at high levels in beebread from apiaries 1 and 2. Chlorpyrifos is the most frequently detected insecticide in hive matrices worldwide, and levels in pollen and beebread have reached level of concern for bee health (Mullin et al., 2010; Tosi et al., 2017). In apiary 1, chlorpyrifos was responsible of the highest contributions (up to 696 points) to pesticide hazard found in 2016 and 2017, while dimethoate showed a relevant contribution to HQ $_{beebread}$ (200 points) during nectar flow in 2018 (Fig. 2). In apiary 2, both insecticides had substantial contributions to pesticide hazard during bloom in 2018. Dimethoate, applied in scattered olive trees orchards close to apiary 3, appeared in three beebread samples from this apiary. HQ $_{beebread}$ scores from apiary 3 were low with the exception of one sample in June 2016, with a relevant contribution of dimethoate to HQ $_{beebread}$ (82 points). As olive trees are rarely visited by honey bees, dimethoate found in beebread from apiary 3 came most likely from non-cultivated plants in olive field margins contaminated by spray drift. Contamination by pesticides of non-

Table 2
Summary of pesticide residues detected in beebread samples.

Beebread (n = 33)							
Pesticide	Oral LD_{50}(μg·bee^{-1})	Use	Detection (%)	Range (ng·g^{-1})	Mean [a] (ng·g^{-1})	HQ score	
						Lowest	Highest
DMF (Amitraz)[b]	50	Miticide	32 (97%)	2–496	71.2	<0.1	20
Coumaphos	4.6	Miticide	31 (94%)	4–174	31.6	0.9	38
Chlorpyrifos	0.24	Insecticide	15 (45%)	2–167	16.2	8	696
Carbendazim	50	Fungicide	10 (30%)	2–29	2.0	<0.1	0.6
Acetamiprid	14	Insecticide	9 (27%)	1–19	1.7	0.1	1
Fluvalinate	45	Miticide	9 (27%)	1–20	1.5	<0.1	0.4
Dimethoate	0.17	Insecticide	8 (24%)	2–34	3.4	12	200
Hexythiazox	200	Miticide	8 (24%)	1–14	1.1	<0.1	<0.1
Chlorfenvinphos	0.55	Miticide/Insecticide	6 (18%)	1–2	0.2	2	4
Acrinathrin	0.12	Miticide/Insecticide	6 (18%)	3–40	2.0	29	333
Pyriproxyfen	100	Insecticide	4 (12%)	1–5	0.5	<0.1	<0.1
Imidacloprid	0.0037	Insecticide	4 (12%)	1	0.1	270	270
DMPF (Amitraz)[b]	50	Miticide	3 (9%)	8–22	1.4	<0.1	20
Methiocarb	0.08	Insecticide	3 (9%)	2–28	1.4	25	350
Tebuconazole	83.05	Fungicide	2 (6%)	1–3	0.1	<0.1	<0.1
Buprofezin	164	Insecticide	1 (3%)	2	<0.1	<0.1	<0.1
Terbuthylazine	22.6	Herbicide	1 (3%)	2	<0.1	<0.1	<0.1

[a] If a compound was not detected in a sample, concentration value was considered as 0.
[b] DMF and DMPF are the degradation products of the amitraz pesticide.

cultivated habitats adjacent to agricultural areas can represent a high pesticide risk to honey bees (Botias et al., 2015, 2016; Long and Krupke, 2016; McArt et al., 2017). Imidacloprid and Methiocarb, detected in 12 and 9% of samples respectively, were involved in relevant HQ $_{beebread}$ scores (up to 350 points). Methiocarb was detected in beebread samples, from apiary 2, collected in May and August 2017. Imidacloprid was found in beebread from both apiaries collected during citrus bloom in 2017 and 2018, and in February 2018. Low levels of this neonicotinoid, as detected in this study, were proved to alter honey bee physiology and reduce foraging motivations in other pollinator species (Lamsa et al., 2018; Cook, 2019). Acetamiprid and pyriproxyfen were detected in 27 and 12% of samples respectively, with mean concentrations below 2 ng g^{-1}. Insecticide buprofezin, together with herbicide terbuthylazine were found in less than 10% of samples and mean concentrations did not exceeded 1.4 ng g^{-1}. Fungicides Carbendazim and tebuconazole, detected in 30% and 6% of beebread samples, contributed less than one point to HQ $_{beebread}$ scores in positive samples for these compounds. In general, fungicides toxicity to honey bees is considered low, and in the HQ approach used in this study, indirect effect of fungicides on the colony are not contemplated. However, fungicides reduce the population of beneficial symbiotic fungi present in pollen that are crucial in the maturation of pollen into beebread. Therefore, nutritional value of beebread contaminated by fungicides is adversely affected and honey bee colony weakened (Yoder et al., 2012; Steffan et al., 2017).

3.2.1. *Live honey bees*

Live honey bees samples (n = 38) were less contaminated in both, number and quantity of pesticide residues. Ten samples (26%) were free of pesticides and an average of one pesticide per sample was detected (Supplementary material TableS6). Honey bees were contaminated mostly by compounds used in beekeeping against varroosis. Chlorpyrifos and dimethoate insecticides, involved in poisoning episodes, were only detected in one and two samples, respectively, but contributions to pesticide hazard were relevant in

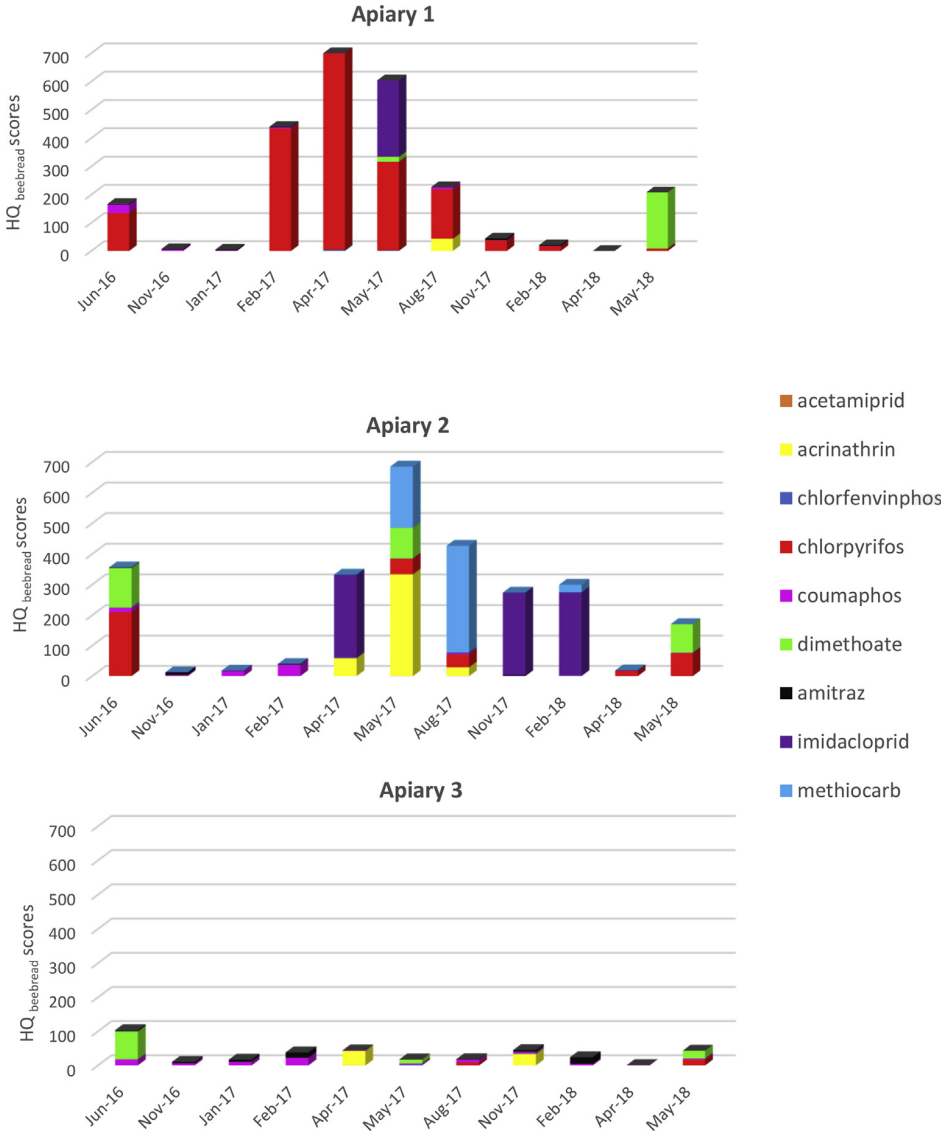

Fig. 2. Evolution of Hazard Quotients (HQ) calculated in beebread samples in the three monitored apiaries. Contribution of each pesticide detected to HQ scores is illustrated. Pesticides contributing less than 0.5 points to HQ scores are not included in the figures.

the three samples. Coumaphos was detected in 50% of samples, mostly at residual concentrations (Table 3). This miticide remains trapped in wax matrix and can contaminate honey bees years after its last application to the colonies. Amitraz, detected in the samples through its degradate DMF, was the miticide applied for *varroa* control in the colonies from September to December in 2017 and 2018. Results showed how hazard posed by amitraz decreased gradually since the application date (Fig. 3). Furthermore, amitraz contributions to HQs in the samples were insignificant because this product is relatively safe for bees compared to other synthetic acaricides (Gashout et al., 2018). Carbendazim fungicide and fluvalinate acaricide were detected in one sample at residual concentrations. On five occasions, the date of collection of dead and live bees coincided. Whereas dead bees from mortality traps were highly contaminated, analysis of live in-hive bees showed a remarkably low pesticide load (Supplementary Material Table S8). Guard bees that prevent the entry of poisoned bees with abnormal behaviors to the colony, the hygienic behavior of honey bees - like the fast intervention of undertaker bees in removing poisoned dead bees from inside the hive-, and honey bees' detoxifying enzymes are probably the main reasons that could explain the reduced pesticide load of live in-hive bees compared to dead bees collected outside the hive.

3.3. High mortality rates during pesticide poisoning episodes of honey bees

Mortality traps underestimate death rates of honey bee colonies because deaths outside the hives are not quantified. Furthermore, honey bees with high doses of pesticides that die while foraging, or disoriented poisoned bees unable to find the way back to the colonies are not analyzed, thus underestimating the magnitude of poisoning episodes occurred in the apiaries. Nevertheless, poisoning symptoms were observed in apiaries 1 and 2, located near agricultural settings. Honey yield of the bee colonies affected by poisoning events was significantly reduced, and population of forager bees decreased, thus debilitating the colonies, but not killing them. Apiary 3, surrounded by wildlands and with less agricultural pressure, was free of pesticide poisoning episodes. Death rate in apiary 3 followed a natural pattern throughout the monitoring period. Mortality was around 20 dead bees/day during periods of low activity, summer (July–August) and winter (December–January), and higher during periods of high activity like citrus (April–May) and rosemary (February–March) blooming seasons (Fig. 3). During flowering, hive population grows and honey bees intensify foraging flights, thus reducing their lifespan. As a result, there is a natural growth in mortality.

In apiary 1 and 2, elevated pesticide hazard appeared during and immediately after spraying and decreased after application periods, as also reported by Beyer et al. (2018). Dead honey bees collected in mortality traps were mostly contaminated by dimethoate (76.5%), its metabolite omethoate (52.9%) and chlorpyrifos (41.2%), confirming the high exposure of foragers (Supplementary material TableS7). Chlorpyrifos (found up to 2700 ng g^{-1}) and dimethoate (up to 338 ng g^{-1}) were detected in dead honey bees with the highest mean concentrations, 232.9 and 89.9 ng g^{-1}, respectively (Table 3). Fluvalinate (35.3%) was found at residual concentrations in most of the samples (6–10 ng g^{-1}). Imidacloprid neonicotinoid was found in two samples (11.8%), at 22 and 476 ng g^{-1} in apiary 2. Amitraz degradate DMF (5.9%), hexythiazox (17.6%), and coumaphos (5.9%), together with the insecticides pyriproxifen and acetamiprid (11.8%), were detected in the samples and contribution to pesticide HQ were insignificant.

3.3.1. Apiary 1

Considering a natural death rate of 20 dead bees/day, three important acute mortality peaks occurred during the monitoring period. The highest mortality peaks were found in May 2017 (up to 256 dead bees/day) and May 2018 (up to 160 and 180 dead bees/day) during citrus bloom, and dead bees were poisoned with the organophosphate insecticides chlorpyrifos and dimethoate (Fig. 4), as also occurred in previous studies (Calatayud-Vernich et al., 2015; Kiljanek et al., 2017). Both compounds were also identified as responsible of poisoned honey bees from other European countries

Table 3
Summary of pesticide residues detected in live and dead honey bee samples.

Live honey bees (n = 38)							
Pesticide	Contact LD_{50}(μg·bee^{-1})	Use	Detection (%)	Range (ng·g^{-1})	Mean[a] (ng·g^{-1})	HQ score	
						Lowest	Highest
Coumaphos	20	Miticide	21 (55.3%)	2–34	5.2	0.1	2
DMF (Amitraz)[b]	50	Miticide	16 (42.1%)	2–56	11.5	<0.1	2
Dimethoate	0.12	Insecticide	2 (5.3%)	12–36	1.3	100	300
Chlorpyrifos	0.072	Insecticide	1 (2.6%)	22	0.6	306	306
Carbendazim	50	Fungicide	1 (2.6%)	3	<0.1	<0.1	<0.1
Fluvalinate	8.7	Miticide	1 (2.6%)	2	<0.1	0.2	0.2

Dead honey bees (n = 17)							
Pesticide	Contact LD_{50} (μg·bee^{-1})	Use	Detection (%)	Range (ng·g^{-1})	Mean[a] (ng·g^{-1})	HQ score	
						Lowest	Highest
Dimethoate	0.12	Insecticide	13 (76.5%)	4–338	89.9	33	2817
Omethoate	0.05	Insecticide	9 (52.9%)	10–48	13.8	200	960
Chlorpyrifos	0.072	Insecticide	7 (41.2%)	2–2702	232.9	28	37528
Fluvalinate	8.7	Miticide	6 (35.3%)	6–180	19.4	0.7	21
Hexythiazox	200	Miticide	3 (17.6%)	4–266	16.2	<0.1	1
Pyriproxyfen	100	Insecticide	2 (11.8%)	4–558	33.1	<0.1	6
Imidacloprid	0.061	Insecticide	2 (11.8%)	22–476	29.3	361	7803
Acetamiprid	7.9	Insecticide	2 (11.8%)	6–14	1.2	0.8	2
DMF (Amitraz)[b]	50	Miticide	1 (5.9%)	47	2.8	0.9	0.9
Coumaphos	20	Miticide	1 (5.9%)	2	0.1	0.1	0.1

[a] If a compound was not detected in a sample, concentration value was considered as 0.
[b] DMF and DMPF are the degradation products of the amitraz pesticide.

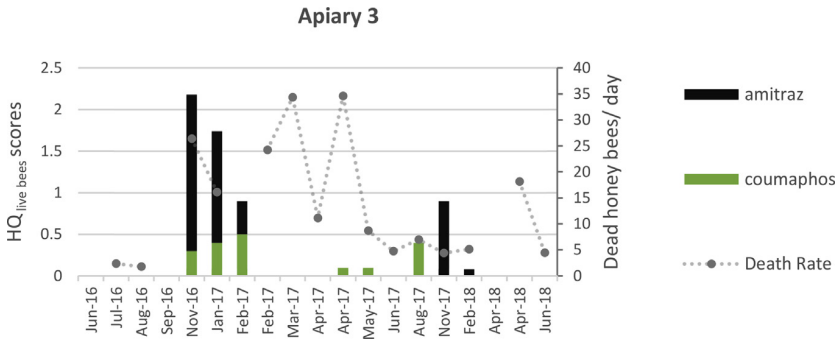

Fig. 3. Evolution of death rate and contribution of each pesticide detected to Hazard Quotients (HQ) scores in live honey bees from apiary 3.

(Barnett et al., 2007; Porrini et al., 2014; Kiljanek et al., 2016b). HQ $_{\text{dead bees}}$ in May 2018 and 2017 exceeded from 3 to 37 times the threshold value considered as elevated hazard to honey bee health, respectively. At the beginning of April 2017, mortality started to rise up to 65 dead bees/day. During this increase, we collected one dead bee sample that was free of pesticides. Two weeks later, chlorpyrifos, dimethoate and omethoate were detected in dead bees and were responsible of the elevated HQ $_{\text{bees}}$ (>15000 points). During March–April 2018, mortality was slightly above natural death rate (up to 65 dead bees/day), and pesticide analysis revealed that two dead bee samples collected during this period were free of pesticides. In spite of a good spring buildup of bee population, black bees with hairless syndrome, a typical sign of chronic bee paralysis virus (CBPV), were detected in traps, and virus analysis of live bees revealed an infection by CBPV that could be responsible of rise in mortality during this period. Presence of hairless black bees ceased in the middle of April, and in early May 2018 (up to 160 dead bees/day), dead bee samples were contaminated by dimethoate contributing to a relevant hazard to bees (HQ $_{\text{bees}} = 67$ points). However, such pesticide hazard is unlikely to be the only factor involved in the high mortality observed, so the undetected presence of others pesticides not included in our methodology, the degradation of dimethoate in traps, and a higher pesticide susceptibility of exhausted forager bees at the end of bloom, could be contributing to this acute mortality event.

3.3.2. *Apiary 2*

Poisoning symptoms were observed during nectarine (February 2017) and citrus bloom (April–May 2017 and 2018). Dead honey bees collected in February 2017 were contaminated with

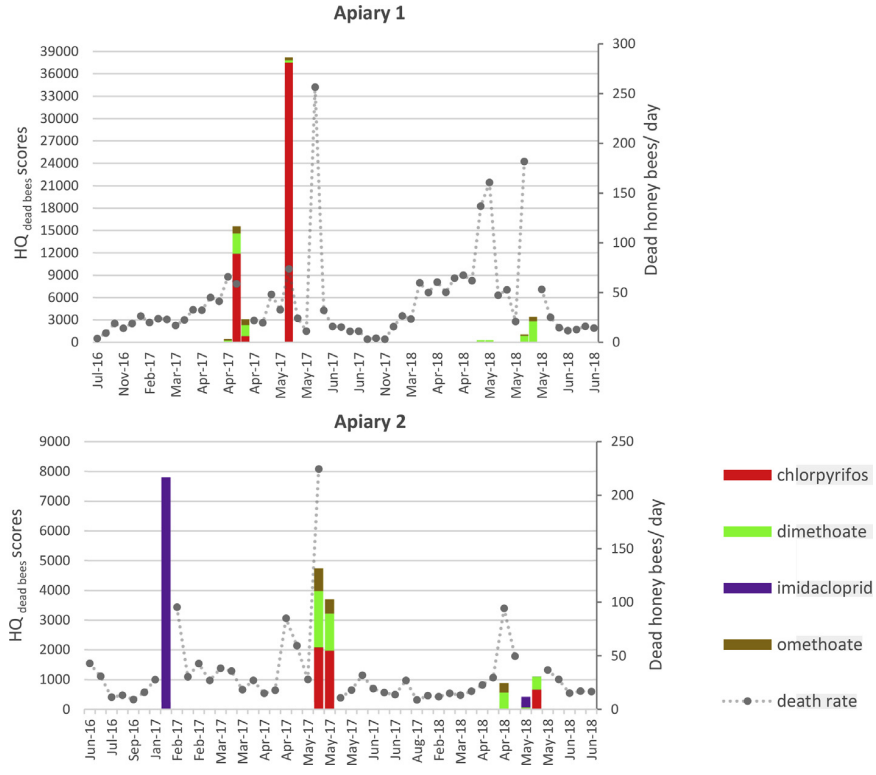

Fig. 4. Evolution of death rate and contribution of each pesticide detected to Hazard Quotients (HQ) scores in dead bees samples collected during acute mortality episodes. Pesticides contributing less than 21 points to HQ scores are not included in the figures.

imidacloprid, used in nectarine orchards near to the apiary. Sprayings of this neonicotinoid during bloom was banned in 2013, and since 2018, the use outdoors is completely prohibited by European Union (EU regulation 2018/783). Therefore, detections of this neonicotinoid suggest a violation of EU regulation. Levels detected of this compound and its high toxicity to honey bees were responsible of the rise in mortality (up to 95 dead bees/day). Contribution to HQ_{bees} was elevated and exceeded 7000 points (Fig. 4). Death rate increased the second half of April, and in May 2017 mortality reached the highest value (>200 dead bees/day). As occurred in apiary 1, chlorpyrifos, dimethoate and omethoate insecticides were sprayed in citrus orchards during blooming season, thus poisoning forager honey bees. Analysis of dead bees revealed that these compounds were responsible of the elevated pesticide hazard found in honey bee samples (HQ_{bees} > 4700 points). In April 2018, mortality increased up to 95 dead bees/day, forager bees were poisoned with the compounds fraudulently applied during citrus bloom (chlorpyrifos, dimethoate and omethoate). Imidacloprid was also found in poisoned bees during this mortality peak and had a relevant contribution (360 points) to pesticide hazard calculated in one sample collected during this mortality episode. Furthermore, two samples from apiary 2 contained 120 and 180 ng g^{-1} of fluvalinate, such concentrations were not residual and could not be acquired by honey bees through contact with contaminated beeswax. Both samples were collected during May 2017, so fluvalinate residues came most likely from citrus spraying with this compound. Fluvalinate, only detected in one live honey bee sample at 2 ng g^{-1}, and with a residual mean concentration lower than 0.1 ng g^{-1}, support this explanation (Table 3).

4. Conclusions

Beeswax was contaminated exclusively with acaricides used in beekeeping, and exhibited products not used in the apiaries for years, thus pointing out the stability of pesticides in this matrix. Miticides used in beekeeping were the most frequent pesticides in beebread from the three apiaries, whereas insecticides were responsible of the highest contributions to pesticide hazard. Live honey bees collected from inside the colonies were remarkably less contaminated. Pesticide poisoning episodes only took place in the two apiaries located near agricultural settings, and dead honey bees analyzed revealed high levels of chlorpyrifos, dimethoate and imidacloprid, used in the surrounding crops. In view of our results, the use of less contaminated sources of beeswax is needed to dilute pesticides accumulated in wax and prevent future pesticide transferences from this matrix to honey bees and beebread. Sustainable management practices like reducing applications of persistent pesticides in-hive and the use of organic acids against *varroa* should be implemented in beekeeping in order to reduce miticides levels in honey bee colonies. It is important to consider the location of the apiaries to avoid poisoning events, and reduce pesticide hazard in honey bee colonies. Nevertheless, reliance on pesticides of modern agriculture should be reconsidered, and wild and managed pollinators should be valued as essential components in agroecosystems in order to develop a more sustainable management of the agroenvironments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.05.170.

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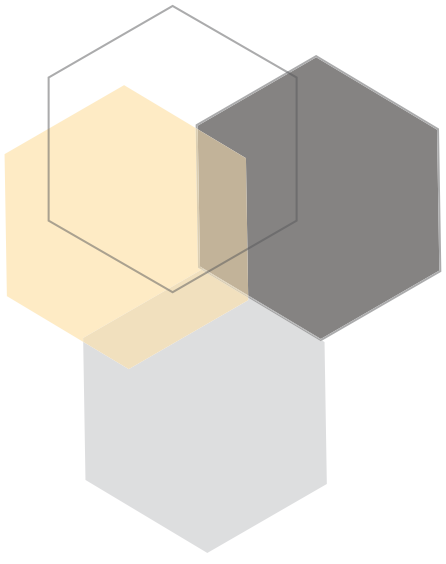
ARTICLE 04

SUPPLEMENTARY MATERIAL:

A TWO-YEAR MONITORING OF PESTICIDE HAZARD IN-HIVE: HIGH HONEY BEE MORTALITY RATES DURING INSECTICIDE POISONING EPISODES IN APIARIES LOCATED NEAR AGRICULTURAL SETTINGS.



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MATERIAL AND METHODS

LC-MS/MS conditions

The chromatographic column was a Luna C18 (15.0 cm × 0.21 cm) with a 3 µm particle size (Phenomenex, Torrance, USA). The column temperature was kept at 30 °C and the volume injected was 5 µL. A binary mobile phase at flow rate of 0.3 $mL \cdot min^{-1}$ with a gradient elution was used. Solvent A was Milli-Q water with 10 mM ammonium formate, and solvent B was methanol with 10 mM ammonium formate. The linear gradient was as follows: 0 min (50 % B), 10 min (83 % B), 12 min (83 % B), 12.5 min (98 % B) and 15.5 min (98 % B). Then, the mobile phase returns to the initial conditions with an equilibration time of 12 min.

Ionization and fragmentation settings were optimized by direct injection of pesticide standard solutions. MS/MS was performed in the SRM mode using ESI in positive mode. For each compound, two characteristic product ions of the protonated molecule [M+H] + were monitored, the first and most abundant one was used for quantification, while the second one was used as a qualifier. Collision energy and cone voltage were optimized for each pesticide. Nitrogen was used as collision, nebulising and desolvation gas. The ESI conditions were: capillary voltage 4000 V, nebulizer 15 psi, source temperature 300 °C and gas flow 10 $L \cdot min^{-1}$. In order to maximize sensitivity, dynamic MRM was used, with MS1 and MS2 at unit resolution and cell acceleration voltage of 7 eV for all the compounds.

Analysis of honey bees, beebread and beeswax

Honeybee and beebread samples (5g) were weighed into 50 mL centrifuge tubes and a volume of 7.5 mL water and 10 mL of acetonitrile were added to the tubes containing the bees. After that, 6 g $MgSO_4$ and 1 g NaCl were added and the samples were vortexed immediately for 1 min. The extracts were then centrifuged for 5 min at 3000 rpm. A volume of 1 mL from the supernatant was sampled into another 15 mL centrifuge tube containing 50 mg C18, 50 mg PSA and 150 mg $MgSO_4$ and the samples were again vortexed for 1 min and centrifuged for 5 min at 3000 rpm. Finally, the supernatant was filtered using a PTFE 13mm x 0.22 µm into the autosampler vials for LC-MS analysis.

Beeswax (2 g) was weighed into 50 mL centrifuge tubes and 10 mL of acetonitrile were added. The tubes were closed and placed in a water bath at – 80 °C. Once the beeswax had melted, the tubes were vortexed vigorously for 30 s and placed again in the water bath to melt. This step was repeated four times to ensure adequate pesticide extraction. For beeswax precipitation, centrifugation tubes were left to cool to room temperature and put into the freezer (-18 °C) overnight. For the extract cleaning, a volume of 2 mL was sampled into a 15 mL centrifuge tube containing 50 mg C18 and 50 mg primary-secondary amine (PSA). The mixture was shaken for 15 s and centrifuged at 3000 rpm for 5 minutes. Finally, the supernatant was filtered using a PTFE 13 mm x 0.22 µm into the autosampler vials for LC-MS analysis and pH was adjusted to ca. 5 by adding a 5% formic acid solution in acetonitrile (v/v) (10 µL/mL extract).

Table S1. Dynamic MRM conditions used for LC-MS/MS determination of pesticide residues.

Target Pesticide	t_R[a] (min)	Δt_R [b]	Precursor Ion	SRM_1[c]	Frag[d] (V)	CE[e] (V)	SMR_2[f]	Frag[d] (V)	CE[e] (V)	SMR_2/SRM_1 (%) (%RSD)[g]
Acetamiprid	3.21	2.97	223	126	111	22	56	111	14	37.4 (12)
Acetochlor	14	2	270	224	120	10	148	120	10	46.8 (22)
Acrinathrin (adduct) *	18.4	2	559.16	208.1	76	10	181.1	76	30	56.3 (3)
Alachlor	13.63	2	270	238	80	15	162	80	10	50.4 (13)
Atrazine	9.56	2.63	216	132	120	15	174	120	20	17.3 (14)
Atrazine-desethyl	4.06	2.5	188	146	120	15	104	121	24	29.1 (15)
Atrazine-desisopropyl	2.7	2.08	174	96	120	15	132	120	15	78.6 (13)
Azinphos-ethyl	13.8	1.71	346	97	80	20	137	80	32	83.5 (12)
Azinphos-methyl	11.3	1.24	318	125	80	8	132	80	12	85.4 (11)
Bifenthrin	18.38	1.84	440.2	181.1	94	6	166	94	46	35.1 (1)
Buprofezin	17.46	1.1	306	201	120	10	116	120	15	64.6 (13)
Carbendazim	4.16	4.74	192	160	95	17	132	95	25	11.4 (14)
Carbofuran	6.96	2.91	222	123	120	10	165	70	15	98.0 (9,3)
Carbofuran-3-hydroxy	3.87	2.48	255	163	70	5	220	70	15	90.8 (9)
Chlorfenvinphos	14.8	1.61	359	155	120	10	127	120	15	63.8 (11)
Chlorpyriphos	17.3	2.23	350	97	92	13	198	97	13	78.6 (14)

Target Pesticide	t_R[a] (min)	Δ t_R [b]	Precursor Ion	SRM_1[c]	Frag[d] (V)	CE[e] (V)	SMR_2[f]	Frag[d] (V)	CE[e] (V)	SMR_2/SRM_1 (%) (%RSD)[g]
Chlothianidin	2.33	2	250	169	86	9	132	89	5	53.8 (19)
Coumaphos	15.4	2.15	363	335	134	10	307	134	10	24.8 (10)
Diazinon	14.86	1.89	305	169	128	17	153	128	21	66.3 (12)
Dichlofenthion	17.13	2	315	259	120	10	287	120	5	44 (11)
Dimethoate	3.22	2.59	230	199	80	10	171	80	5	45.3 (12)
Diuron	10.7	1.25	233	72	120	20	160	120	20	3.2 (13)
DMA (amitraz)	2.92	2.5	122	107	111	18	77	111	42	3.0 (17)
DMF (amitraz)	5.88	4.5	150	132	111	10	107	111	15	41.6 (16)
DMPF (amitraz)	2.88	4.12	163	122	111	15	107	111	15	0.1 (15)
Ethion	17.63	1.23	385	199	80	5	171	80	15	35.3 (11)
Etofenprox	18.23	3	394.2	359.2	66	10	177.1	66	10	42.2 (3)
Fenitrothion	13.35	1.18	278	125	140	15	109	121	12	95.5 (12)
Fenthion	14.63	1.83	279	247	114	5	169	114	13	76.6 (10)
Fenthion sulfone	8.7	2.3	311	125	146	21	109	146	17	66.7 (11)
Fenthion sulfoxide	7.65	2.68	295	109	136	33	280	136	13	98.1 (14)
Fipronil	14.6	2.9	437	368	150	15	290	150	25	21.8 (11)
Flumethrin (adduct) *	19	2	527.1	267	66	10	239	66	18	59.3 (35)
Fluvalinate	18.3	1.81	503	208	50	10	181	50	26	73.4 (10)

Target Pesticide	t_R[a] (min)	Δ t_R [b]	Precursor Ion	SRM_1[c]	Frag[d] (V)	CE[e] (V)	SMR_2[f]	Frag[d] (V)	CE[e] (V)	SMR_2/SRM_1 (%) (%RSD)[g]
Hexythiazox	17.84	1.15	353	228	120	20	168	120	10	67.4 (9)
Imazalil	15.18	1.71	297	159	120	20	201	120	15	56 (14)
Imidacloprid	2.46	1.96	256	209	80	10	175	80	10	75 (11)
Isoproturon	10.3	2.37	207	72	120	20	165	120	10	16.8 (12)
Lambda-cyhalothrin (adduct) *	18.1	2	467.1	225	66	10	141	66	46	26.1 (32)
Malathion	12.5	1.96	331	99	80	10	127	80	5	98.5 (4)
Methiocarb	11.86	1.93	226	121	80	5	169	80	10	66.6 (11)
Metolachlor	13.67	2.04	284	252	120	15	176	120	10	10 (14)
Molinate	12.64	1.98	188	126	80	20	55	80	10	61.7 (11)
Omethoate	1.69	2.67	214	125	80	5	183	80	20	72.3 (12)
Parathion-ethyl	14.25	1.91	292	236	88	4	264	88	8	45.5 (13)
Parathion-methyl	12.06	1.5	264	125	120	20	232	110	5	34.5 (13)
Prochloraz	15.18	1.91	376	308	80	10	266	80	10	14.3 (9)
Propanil	11.9	2.01	218	162	120	20	127	120	15	92.4 (11)
Propazine	11.61	2	230	146	120	15	188	120	20	93.3 (14)
Pyriproxyfen	17.63	1.33	322	227	120	10	185	120	10	36.1 (12)
Simazine	7.04	1.76	202	124	120	20	132	120	20	93.8 (12)
Spinosyn A	16.85	2.3	732.5	142.1	190	25	98.1	190	65	23.4 (2)

Target Pesticide	t_R[a] (min)	Δ t_R [b]	Precursor Ion	SRM_1[c]	Frag[d] (V)	CE[e] (V)	SMR_2[f]	Frag[d] (V)	CE[e] (V)	SMR_2/SRM_1 (%) (%RSD)[g]
Spinosyn D	17.41	1.74	746.5	142.1	190	25	98.1	190	69	22.9 (3)
Tebuconazole	14.6	2.87	308	125	95	25	70	95	21	6.6 (11)
Terbumeton	11.88	2.89	226	170	95	17	114	95	25	13.8 (14)
Terbumeton-desethyl	7.68	3.76	198	142	90	13	96	95	25	31.7 (12)
Terbuthylazine	11.97	3.01	230	174	95	13	96	95	25	16.4 (13)
Terbuthylazine-2-hydroxy	7.91	3.28	212	156	95	13	86	95	25	28 (13)
Terbuthylazine-desethyl	8	2.81	202	146	95	13	79	95	25	13.2 (14)
Terbutryn	14.1	1.2	242	186	120	20	71	120	15	4.6 (14)
Thiabendazole	5.65	3.5	202	175	95	25	131	95	25	29.1 (18)
Thiamethoxam	3.09	2.58	292	211	78	10	132	78	10	21.3 (11)
Tolclofos-methyl	16.9	1.71	301	125	115	12	269	120	15	73.8 (19)

[a] t_R = retention time.

[b] Δ t_R = delta retention time, that is the centered retention time window.

[c] SRM_1 = selected product ion for quantification.

[d] Frag = Fragmentor.

[e] CE = Collision energy.

[f] SRM_2 = selected product ion for qualification.

[g] (%RSD) = relative standard deviation of the ratio SRM_2/SRM_1, calculated from mean values obtained from the matrix-matched calibration curves.

[*] = Adducts of target pesticides [Ion mass + NH_4^+]; non adduct target pesticides [Ion mass + H^+]

Table S2. Dath rate of apiary 1.

Sampling period		30/06/2016	25/08/2016	01/09/2016	08/11/2016	28/12/2016	09/02/2017	17/02/2017	10/03/2017	16/03/2017	20/03/2017	25/03/2017	30/03/2017	04/04/2018	08/04/2017
		04/07/2016	01/09/2016	06/09/2016	17/11/2016	04/01/2017	17/02/2017	25/02/2017	16/03/2017	20/03/2017	25/03/2017	30/03/2017	04/04/2017	08/04/2017	12/04/2017
Dead bees /day	Hive 1	4.8	15.4	25.6	18.2	13.1	17.8	7.0	25.3	21.8	15.6	16.0	18.2	25.8	42.8
	Hive 2	3.8	11.0	32.8	17.6	17.4	23.1	16.3	20.5	21.3	12.2	12.2	22.2	28.8	35.0
	Hive 3	3.5	10.4	17.6	16.1	25.0	30.0	20.6	22.7	17.5	12.0	15.6	41.2	32.5	45.8
	Hive 4	4.0	4.9	5.6	6.4	27.1	26.3	26.3	23.7	21.8	26.4	25.0	18.0	14.3	24.0
	Hive 5	3.3	5.4	14.0	12.7	12.1	35.0	30.0	27.0	33.3	19.2	44.0	64.0	60.0	78.8
Average death rate		**3.9**	**9.4**	**19.1**	**14.2**	**19.0**	**26.4**	**20.0**	**23.8**	**23.1**	**17.1**	**22.6**	**32.7**	**32.3**	**45.3**
Sampling period		**12/04/2017**	**17/04/2017**	**20/04/2017**	**24/04/2017**	**29/04/2017**	**02/05/2017**	**05/05/2017**	**08/05/2017**	**11/05/2017**	**17/05/2017**	**22/05/2017**	**26/05/2017**	**29/05/2017**	**03/06/2017**
		17/04/2017	**20/04/2017**	**24/04/2017**	**29/04/2017**	**02/05/2017**	**05/05/2017**	**08/05/2017**	**11/05/2017**	**17/05/2017**	**22/05/2017**	**26/05/2017**	**29/05/2017**	**03/06/2017**	**09/06/2017**
Dead bees /day	Hive 1	36.8	54.3	96.0	20.0	31.7	45.0	34.3	99.0	30.3	15.6	170.8	27.7	15.4	12.0
	Hive 2	35.6	59.3	20.5	17.0	24.7	64.0	48.0	92.3	14.8	12.4	283.3	48.3	24.0	35.8
	Hive 3	34.4	73.3	49.5	28.6	18.0	54.7	14.0	31.3	23.2	4.0	491.8	5.7	8.0	7.8
	Hive 4	33.2	69.3	63.8	20.8	14.0	46.0	26.0	58.0	26.0	7.8	195.8	46.7	14.2	4.0
	Hive 5	66.4	73.0	64.5	23.4	10.0	31.0	41.7	88.7	27.2	17.0	141.8	32.3	18.6	17.5
Average death rate		**41.3**	**65.9**	**58.9**	**22.0**	**19.7**	**48.1**	**32.8**	**73.9**	**24.3**	**11.4**	**256.7**	**32.1**	**16.0**	**15.4**
Sampling period		**09/06/2017**	**15/06/2017**	**08/07/2017**	**29/07/2017**	**17/11/2017**	**08/02/2018**	**22/02/2018**	**07/03/2018**	**26/03/2018**	**29/03/2018**	**04/04/2018**	**09/04/2018**	**13/04/20184**	**17/04/2018**
		15/06/2017	**21/06/2017**	**12/07/2017**	**02/08/2017**	**23/11/2017**	**13/02/2018**	**24/02/2018**	**13/03/2018**	**29/03/2018**	**04/04/2018**	**09/04/2018**	**13/04/2018**	**17/04/2018**	**19/04/2018**
Dead bees /day	Hive 1	10.3	10.2	4.3	5.8	2.6	11.8	32.5	10.5	17.7	21.2	27.2	20.5	22.3	80.0
	Hive 2	18.8	15.7	3.8	3.5	3.4	11.2	27.0	8.0	38.3	21.7	31.0	28.0	34.5	35.0
	Hive 3	12.3	14.3	3.0	6.3	4.6	12.4	20.0	29.0	93.3	65.8	56.6	42.5	68.5	52.5
	Hive 4	6.8	8.8	2.5	3.0	2.4	21.6	17.5	28.3	45.0	36.7	27.8	29.5	72.5	55.0
	Hive 5	7.3	7.5	2.0	2.5	3.0	22.0	36.0	41.3	105.0	105.0	160.8	131.3	125.8	115.0
Average death rate		**11.1**	**11.3**	**3.1**	**4.2**	**3.2**	**15.8**	**26.6**	**23.4**	**59.9**	**50.1**	**60.7**	**50.4**	**64.7**	**67.5**
Sampling period		**19/04/2018**	**23/04/2018**	**27/04/2018**	**01/05/2018**	**07/05/2018**	**12/05/2018**	**18/05/2018**	**22/05/2018**	**25/05/2018**	**28/05/2018**	**30/05/2018**	**04/06/2018**	**11/06/2018**	**15/06/2018**
		23/04/2018	**27/04/2018**	**01/05/2018**	**07/05/2018**	**12/05/2018**	**18/05/2018**	**22/05/2018**	**25/05/2018**	**28/05/2018**	**30/05/2018**	**04/06/2018**	**11/06/2018**	**15/06/2018**	**21/06/2018**
Dead bees /day	Hive 1	49.3	147.0	150.0	20.7	36.8	9.0	38.5	34.0	12.7	12.5	8.0	9.4	5.5	7.5
	Hive 2	39.8	101.3	90.8	36.7	36.0	17.3	46.0	39.3	14.3	8.0	10.2	10.6	27.5	20.0
	Hive 3	60.8	150.8	209.5	13.0	18.0	12.0	259.0	45.0	6.0	6.0	14.8	10.0	11.5	14.0
	Hive 4	53.5	137.5	212.5	122.2	133.4	51.7	345.5	114.0	80.0	28.0	10.8	14.7	17.8	18.2
	Hive 5	107.0	148.3	140.8	44.5	40.2	15.0	220.8	34.3	13.0	19.5	15.2	19.9	18.5	12.2
Average death rate		**62.1**	**137.0**	**160.7**	**47.4**	**52.9**	**21.0**	**182.0**	**53.3**	**25.2**	**14.8**	**11.8**	**12.9**	**16.2**	**14.4**

Table S3. Dath rate of apiary 2.

Sampling period		04/06/2016	09/06/2016	18/06/2018	22/08/2016	30/08/2016	27/10/2016	30/12/2016	08/02/2017	10/02/2017	15/02/2017	23/02/2017	06/03/2017	11/03/2017	20/03/2017
		09/06/2016	18/06/2016	01/07/2016	30/08/2016	07/09/2016	03/11/2016	04/01/2017	10/02/2017	15/02/2017	23/02/2017	06/03/2017	11/03/2017	20/03/2017	30/03/2017
Dead bees /day	Hive 1	40.6	32.8	9.5		21.9		31.2	156.0	35.2	37.1	19.0	58.0	35.9	6,8
	Hive 2	107.0	76.3	21.9		7.9	17.1	28.6	57.0	54.6	85.0	48.4	34.4	53.8	31,0
	Hive 3	22.8	13.7	7.4	14.4	4.8	13.9	29.4	67.0	15.6	23.1	15.4	36.6	29.8	12,3
	Hive 4	31.4	18.2	12.5	13.8	5.9	14.9	19.6	58.0	19.0	28.8	16.5	16.8	27.4	13,2
	Hive 5	12.6	13.2	5.5	11.3	5.4	17.9	29.6	139.0	27.2	39.4	35.4	45.6	31.8	28,3
Average death rate		**42.9**	**30.8**	**11.4**	**13.1**	**9.2**	**15.9**	**27.7**	**95.4**	**30.3**	**42.7**	**26.9**	**38.3**	**35.7**	**18.3**
Sampling period		**30/03/2017**	**03/04/2017**	**07/04/2017**	**18/04/2017**	**21/04/2017**	**25/04/2017**	**02/05/2017**	**08/05/2017**	**18/05/2017**	**22/05/2017**	**29/05/2017**	**05/06/2017**	**15/06/2017**	**10/07/2017**
		03/04/2017	**07/04/2017**	**18/04/2017**	**21/04/2017**	**25/04/2017**	**02/05/2017**	**05/05/2017**	**18/05/2017**	**22/05/2017**	**29/05/2017**	**05/06/2017**	**15/06/2017**	**24/06/2017**	**17/07/2017**
Dead bees /day	Hive 1	30.8	10.0	16.7	82.0	69.5	36.9	131.0	12.3	13.5	5.0	8.1	18.3	21.0	46.9
	Hive 2	26.8	10.3	19.8	76.0	53.0	17.3	192.7	13.7	23.3	23.4	9.7	12.8	9.7	8.9
	Hive 3	28.0	18.0	8.7	77.3	46.8	31.0	382.3	9.4	28.0	87.1	41.9	22.0	21.3	26.6
	Hive 4	14.3	10.5	11.6	132.0	30.8	27.7	195.0	8.4	18.3	33.6	24.9	12.9	5.2	37.6
	Hive 5	35.5	25.8	32.2	57.7	97.3	26.7	221.0	9.6	7.0	10.3	12.0	12.4	10.9	14.9
Average death rate		**27.1**	**14.9**	**17.8**	**85.0**	**59.5**	**27.9**	**224.4**	**10.7**	**18.0**	**31.9**	**19.3**	**15.7**	**13.6**	**26.9**

Sampling period		28/07/2018	06/02/2018	09/02/2018	15/03/2018	20/03/2018	27/03/2018	04/04/2018	09/04/2018	14/04/2018	01/05/2018	16/05/2018	22/05/2018	30/05/2018	08/06/2018	15/06/2018
		02/08/2017	09/02/2018	15/02/2018	20/03/2018	27/03/2018	04/04/2018	09/04/2018	14/04/2018	17/04/2018	07/05/2018	22/05/2018	30/05/2018	08/06/2018	15/06/2018	21/06/2018
Dead bees /day	Hive 1	7.4	12.3	18.7	23.6	17.6	36.5	52.0	64.0	197.7	35.8	29.3	38.4	10.3	11.7	10.5
	Hive 2	8.4	5.0	14.0	7.6	13.6	14.1	23.6	28.0	45.0	95.0	43.8	57.3	23.9	54.0	
	Hive 3	9.6	19.3	7.5	12.6	9.7	10.3	11.4	19.0	146.0	22.0	34.5	20.3	14.2	6.6	16.2
	Hive 4	7.8	10.3	8.0	14.8	8.9	6.8	6.4	24.6	54.7	14.8	12.2	9.1	12.7	1.7	5.8
	Hive 5	10.6	16.3	10.5	16.4	16.6	16.9	20.6	12.6	28.3	80.3	63.8	14.8	14.7	10.6	33.8
Average death rate		**8.8**	**12.7**	**11.7**	**15.0**	**13.3**	**16.9**	**22.8**	**29.6**	**94.3**	**49.6**	**36.7**	**28.0**	**15.2**	**16.9**	**16.6**

Table S4. Dath rate of apiary 3.

Sampling period		29/06/2016	23/08/2016	27/10/2016	27/12/2016	21/02/2017	24/02/2017	20/03/2017	03/04/2017	08/05/2017	05/06/2017	29/07/2017	17/11/2017	12/02/2018	15/04/2018	01/06/2018
		04/07/2016	30/08/2016	03/11/2016	03/01/2017	24/02/2017	11/03/2017	03/04/2017	07/04/2017	12/05/2017	24/06/2017	01/08/2017	22/11/2017	15/02/2018	28/04/2018	08/06/2018
Dead bees /day	Hive 1	2.2	1.6	20.0	14.6	16.3	18.3	2.5	12.5	4.5	2.2	7.0	3.8	5.0	10.2	1.9
	Hive 2	3.8	1.9	32.0	16.0	29.3	40.8	16.8	57.5	13.3	1.2	7.0	5.8	5.7	35.6	10.7
	Hive 3	1.2	2.1	23.6	24.9	20.3	32.7	14.3	28.8	14.5	11.4	7.0	4.4	4.0	8.1	4.3
	Hive 4	0.6	2.0	38.3	13.3	19.7	46.0	17.3	61.3	7.3	6.5	7.0	4.0	4.3	10.6	2.0
	Hive 5	4.2	1.4	18.3	12.0	35.7	34.1	5.0	13.3	4.0	2.4	7.0	4.0	6.7	26.4	3.4
Average death rate		**2.4**	**1.8**	**26.4**	**16.1**	**24.3**	**34.4**	**11.2**	**34.7**	**8.7**	**4.8**	**7.0**	**4.4**	**5.1**	**18.2**	**4.5**

Table S5. Pesticide residues in beebread from apiary 1, 2 and 3

APIARY 1											
Pesticides	**01/06/2016**	**17/11/2016**	**04/01/2017**	**25/02/2017**	**04/04/2017**	**11/05/2017**	**02/08/2017**	**23/11/2017**	**13/02/2018**	**17/04/2018**	**22/05/2018**
Acetamiprid	7	0	0	0	1	7	0	0	0	0	0
acrinathrin	0	0	0	0	0	0	5	0	0	0	0
buprofezin	0	0	0	0	0	2	0	0	0	0	0
carbendazim	0	3	0	0	0	0	0	0	0	0	0
chlorfenvinphos	0	0	0	0	2	1	0	0	0	0	0
Chlorpyrifos	32	0	0	104	167	75	42	9	4	0	2
coumaphos	142	20	10	20	17	5	36	6	4	0	0
dimethoate	0	0	0	0	0	3	0	0	0	0	34
DMF	42	64	70	52	15	3	20	145	88	16	0
DMPF	16	0	0	0	0	0	0	0	0	0	0
fluvalinate	10	0	0	0	1	2	0	0	0	0	0
hexythiazox	0	3	0	0	0	0	0	4	2	0	0
imidacloprid	0	0	0	0	0	1	0	0	0	0	0
pyriproxyfen	0	0	0	5	0	0	0	0	0	0	0

APIARY 2											
Pesticides	**01/06/2016**	**17/11/2016**	**04/01/2017**	**18/02/2017**	**03/04/2017**	**08/05/2017**	**04/08/2017**	**24/11/2017**	**15/02/2018**	**24/04/2018**	**30/05/2018**
Acetamiprid	19	0	0	0	1	6	7	0	0	0	0
acrinathrin	0	0	0	0	7	40	3	0	0	0	0
carbendazim	29	7	4	6	8	3	0	0	2	0	0
chlorfenvinphos	0	0	0	0	1	0	0	0	1	0	0
Chlorpyrifos	50	0	0	0	0	12	10	0	0	4	18
coumaphos	70	17	78	174	7	5	22	4	8	6	6
dimethoate	22	0	0	0	0	17	0	0	0	0	16
DMF	36	217	22	48	6	4	26	66	12	18	2
DMPF	8	0	0	0	0	0	0	0	0	0	0
fluvalinate	20	0	0	0	1	2	0	0	0	0	2
hexythiazox	14	0	0	0	0	0	0	2	1	2	8
imidacloprid	0	0	0	0	1	0	0	1	1	0	0
methiocarb	0	0	0	0	0	16	28	0	2	0	0
pyriproxyfen	0	0	0	0	5	5	0	0	1	0	0
tebuconazole	0	0	0	0	3	0	0	0	0	0	0
terbuthylazine	0	0	0	0	0	0	0	0	2	0	0

APIARY 3											
Pesticides	**01/06/2016**	**03/11/2016**	**03/01/2017**	**21/02/2017**	**07/04/2017**	**12/05/2017**	**01/08/2017**	**22/11/2017**	**15/02/2018**	**18/04/2018**	**08/06/2018**
Acetamiprid	0	0	0	0	8	1	0	0	0	0	0
acrinathrin	0	0	0	0	5	0	0	4	0	0	0
carbendazim	0	3	0	0	2	0	0	0	0	0	0
chlorfenvinphos	0	0	0	0	0	1	0	1	0	0	0
Chlorpyrifos	0	0	0	0	0	0	2	0	0	0	4
coumaphos	80	28	48	104	8	17	40	22	20	4	14
dimethoate	14	0	0	0	0	2	0	0	0	0	4
DMF (Amitraz)	66	100	162	394	2	4	24	125	496	2	2
DMPF (Amitraz)	22	0	0	0	0	0	0	0	0	0	0
fluvalinate	8	0	0	0	0	2	0	0	0	0	0
tebuconazole	0	0	0	0	1	0	0	0	0	0	0

Table S6. Pesticide residues in ive honey bees from apiary 1, 2 and 3

	Apiary 1												
Pesticides	**01/06/2016**	**01/09/2016**	**17/11/2016**	**04/01/2017**	**25/01/2017**	**07/04/2016**	**20/04/2017**	**11/05/2017**	**02/08/2017**	**23/11/2017**	**13/02/2018**	**17/04/2018**	**22/05/2018**
carbendazim	0	0	0	0	0	0	0	0	0	0	0	0	0
Chlorpyrifos	0	0	0	0	0	0	0	0	0	0	0	0	0
coumaphos	0	0	6	6	6	2	2	2	8	0	0	0	0
dimethoate	0	0	0	0	0	12	0	0	0	0	0	0	36
Amitraz	0	0	110	67	110	0	0	0	0	16	10	0	0
fluvalinate	0	0	0	0	0	0	0	2	0	0	0	0	0
	Apiary 2												
Pesticides	**01/06/2016**	**22/08/2016**	**03/11/2016**	**04/01/2017**	**18/02/2017**	**10/03/2017**	**03/04/2017**	**08/05/2017**	**04/08/2017**	**24/11/2017**	**15/02/2018**	**24/04/2018**	**30/05/2018**
carbendazim	0	0	3	0	0	0	0	0	0	0	0	0	0
Chlorpyrifos	0	0	0	0	0	0	0	0	22	0	0	0	0
coumaphos	0	34	6	33	24	22	2	2	8	0	0	0	0
dimethoate	0	0	0	0	0	0	0	0	0	0	0	0	0
Amitraz	0	87	35	108	12	0	0	0	0	63	10	0	0
fluvalinate	0	0	0	0	0	0	0	0	0	0	0	0	0
	Apiary 3												
Pesticides	**01/06/2016**	**01/09/2016**	**03/11/2016**	**03/01/2017**	**21/02/2017**	**07/04/2016**	**12/05/2017**	**01/08/2017**	**22/11/2017**	**15/02/2018**	**18/04/2018**	**08/06/2018**	
carbendazim	0	0	0	0	0	0	0	0	0	0	0	0	
Chlorpyrifos	0	0	0	0	0	0	0	0	0	0	0	0	
coumaphos	0	0	6	8	10	2	2	8	0	0	0	0	
dimethoate	0	0	0	0	0	0	0	0	0	0	0	0	
Amitraz	0	0	94	67	20	0	0	0	45	4	0	0	
fluvalinate	0	0	0	0	0	0	0	0	0	0	0	0	

Table S7. Pesticide residues in dead honey bees from apiary 1, 2

	Apiary 1										
Pesticides	**04/04/2017**	**20/04/2017**	**24/04/2017**	**25/04/2017**	**11/05/2017**	**29/03/2018**	**09/04/2018**	**27/04/2018**	**01/05/2018**	**22/05/2018**	**23/05/2018**
Acetamiprid	0	14	0	0	6	0	0	0	0	0	0
Chlorpyrifos	0	0	856	60	2702	0	0	0	0	0	0
coumaphos	0	0	0	2	0	0	0	0	0	0	0
dimethoate	0	30	326	178	34	0	0	8	8	104	338
Amitraz	0	0	0	0	0	0	0	0	0	0	47
fluvalinate	0	0	0	0	10	0	0	0	0	0	0
hexythiazox	0	0	0	0	266	0	0	0	0	0	0
omethoate	0	10	48	38	20	0	0	0	0	10	30
pyriproxyfen	0	0	0	0	558	0	0	0	0	0	0

	Apiary 2					
Pesticides	**01/02/2017**	**05/05/2017**	**08/05/2017**	**17/04/2018**	**08/05/2018**	**17/05/2018**
Chlorpyrifos	0	150	142	0	2	48
dimethoate	0	228	150	68	4	52
fluvalinate	0	120	180	6	6	8
hexythiazox	0	0	0	0	6	4
imidacloprid	476	0	0	0	22	0
omethoate	0	38	24	16	0	0
pyriproxyfen	4	0	0	0	0	0

Table S8. Summary of pesticide residues in live and dead honey bees collected the same day.

	Concentration detected ($ng \cdot g^{-1}$)	
Pesticide	**Live honey bees**	**Dead bees**
	04/07/2017	
Coumaphos	2	2
Dimethoate	12	0
	04/20/2017	
Acetamiprid	0	14
Coumaphos	2	0
Dimethoate	0	30
Omethoate	0	10
	05/11/2017	
Acetamiprid	0	6
Chlorpyrifos	0	2702
Coumaphos	2	0
Dimethoate	0	34
Fluvalinate	2	10
Hexythiazox	0	266
Omethoate	0	20
Pyriproxyfen	0	558
	05/12/2018	
Dimethoate	0	28
Omethoate	0	8
	05/22/2018	
Dimethoate	36	104
Omethoate	0	10

Table S9. Squences of the primers used for detection and quantification of pathogens in honeybee samples.

Name	Sequence (5' - 3')	Target	Reference
DWV-F	GCGCTTAGTGGAGGAAATGAA	Deform Wing Virus	(Di Prisco et al. 2016)
DWV-R	GCACCTACGCGATGTAAATCTG		
IAPVF	CCATGCCTGGCGATTCAC	Israeli Acute Paralysis Virus	(Niu et al. 2014)
IAPVR	CTGAATAATACTGTGCGTATC		
BQCV-qF7893	AGTGGCGGAGATGTATGC	Black Queen Cell Virus	(Locke et al. 2012)
BQCV-qB8150	GGAGGTGAAGTGGCTATATC		
NOS-FOR	TGCCGACGATGTGATATGAG	*Nosema ceranae*	(Higes et al. 2006)
NOS-REV	CACAGCATCCATTGAAAACG		
CBPV_F	CCCAAAACCTGGAAGTCATC	Chronic Bee Paralysis Virus	This work (Herrero et al., 2019)
CBPV_R	AATCTGGCAAGGTTGACTGG		

Di Prisco, G., D. Annoscia, M. Margiotta, R. Ferrara, P. Varricchio, V. Zanni, E. Caprio, F. Nazzi, and F. Pennacchio. 2016. A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. Proc Natl Acad Sci U S A 113: 3203-3208.

Herrero, S., S. Coll, R. M. González-Martínez, S. Parenti, A. Millán-Leiva, and J. González-Cabrera. 2019. Identification of new viruses specific to the honey bee mite *Varroa destructor*. bioRxiv: 610170.

Higes, M., R. Martin, and A. Meana. 2006. *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. J Invertebr Pathol 92: 93-95.

Locke, B., E. Forsgren, I. Fries, and J. R. de Miranda. 2012. Acaricide treatment affects viral dynamics in *Varroa destructor* infested honey bee colonies via both host physiology and mite control. Appl Environ Microbiol 78: 227-235.

Niu, J., K. Cappelle, J. R. de Miranda, G. Smagghe, and I. Meeus. 2014. Analysis of reference gene stability after Israeli acute paralysis virus infection in bumblebees *Bombus terrestris*. J Invertebr Pathol 115: 76-79.

Table S10. Land cover propotions for each distance ring.

Apiary 1

	Percentages					
Land cover type	**500m**	**1000m**	**1500m**	**2000m**	**2500m**	**3000m**
Artificial surfaces	0.00	0.00	0.00	0.00	0.00	0.50
Irrigated mixed crops (Huerta)	0.00	0.00	0.00	0.00	0.00	0.02
Irrigted citrus crops	17.65	27.24	32.32	0.31	0.27	22.93
Mixed irrigated and rainfed farming	47.59	30.50	18.06	0.11	0.07	5.06
Natural and seminatural vegetation	34.76	37.37	41.31	0.51	0.61	67.11
Rainfed trees	0.00	4.89	8.31	0.07	0.05	4.40

Apiary 2

	Percentages					
Land cover type	**500m**	**1000m**	**1500m**	**2000m**	**2500m**	**3000m**
Artificial surfaces	0.00	6.31	11.32	18.96	21.54	19.80
Irrigated mixed crops (Huerta)	0.00	0.00	0.00	0.25	1.30	1.05
Irrigted citrus crops	70.46	64.09	56.08	52.17	49.48	51.94
Mixed irrigated and rainfed farming	0.00	0.44	7.22	9.20	9.95	8.26
Natural and seminatural vegetation	0.00	2.77	4.20	3.92	6.08	7.00
Rainfed vineyards	29.54	26.38	21.18	15.50	11.64	11.95

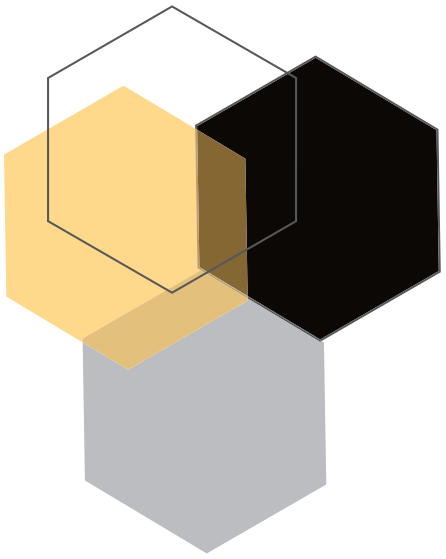
Apiary 3

	Percentages					
Land cover type	**500m**	**1000m**	**1500m**	**2000m**	**2500m**	**3000m**
Rainfed trees	29.85	33.69	35.08	26.42	21.22	18.14
Natural and seminatural vegetation	70.15	66.31	64.92	73.58	78.78	81.86

CHAPTER 5:

Compare the pesticide content among different sources of beeswax used in beekeeping: beeswax cappings, foundation, old combs and virgin beeswax. Furthermore, a preliminary study carried out during the research stay in the University of Maryland (United States of America) about beeswax cleaning by solvent extraction of pesticides is presented. This chapter contains two scientific publications:

ARTICLE 05



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Occurrence of pesticide residues in Spanish beeswax

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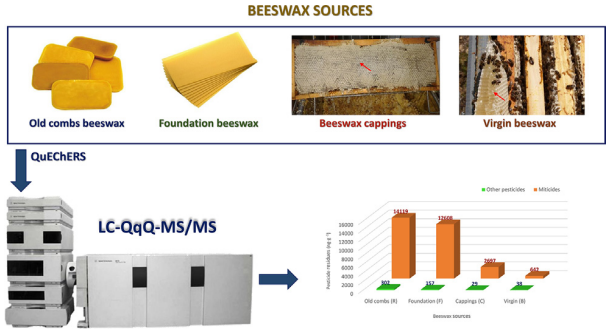
HIGHLIGHTS

- Pesticide levels in foundation, old combs, cappings or virgin wax were compared.
- QuEChERS extracts screened for 58 pesticides by LC-QqQ-MS/MS.
- Acaricides were the main source of beeswax contamination, >95%.
- Insecticides and fungicides were less frequent and at lower concentrations.
- Cappings and virgin wax were markedly less contaminated.

GRAPHICAL ABSTRACT



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ABSTRACT

Beeswax from Spain was collected during 2016 to determine pesticide residues incidence. The 35 samples were divided in foundation, old combs, cappings or virgin beeswax to compare pesticide content between groups. Wax was screened for 58 pesticides or their degradation products by QuEChERS extraction and liquid chromatography mass spectrometry (LC-MS/MS). Beeswax was uniformly contaminated with acaricides and, to a much lesser extent, with insecticide and fungicide residues. Virgin followed by cappings were less contaminated than foundation and old combs beeswax. The miticides applied in-hive had a contribution to average pesticide load higher than 95%. Compounds widely used as acaricides, as coumaphos (100%), fluvalinate (86%) and amitraz (83%), were the pesticides most frequently detected with maximum concentrations of 26,858, 3593 and 6884 ng·g^{-1}, respectively. Chlorfenvinphos, acrinathrin and flumethrin, also acaricides, were detected in 77, 71 and 54%, respectively. Frequencies of pesticides used in crops were 40% for chlorpyrifos, 29% for dichlofenthion, 9% for malathion, 6% for fenthion-sulfoxide and 3% for azinphos-methyl, carbendazim, ethion, hexythiazox, imazalil and pyriproxyfen. Pesticide assessment in beeswax could be an excellent monitoring tool to establish veterinary treatments applied by beekeepers and environmental contaminants exposure of honey bees.

1. Introduction

Beeswax is the comb architecture element manufactured by honey bees (*Apis mellifera* L.) themselves that is literally the walls, home, nursery, pharmacy, storage pantry and dance floor for the numerous inhabitants of the colony (Schmidt and Buchmann, 1992). When visiting flowers, honey bees collect nectar rich in carbohydrates (i.e. the honey sugars fructose, glucose and sucrose) and utilize them for wax formation into their specialized wax-secreting epidermal glands found on the ventral side of the worker bees' abdomen in a high energy demanding process (Bogdanov, 2004).

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Beeswax is a very complex mixture of lipophilic compounds which major components are hydrocarbons and lipids up to an 80% (Tulloch, 1980). Of all beehive products, beeswax has the lowest replacement rate and can remain in the hive for many years, thus leading to a greater accumulation of different non-polar xenobiotics applied in beekeeping and agriculture (Chauzat and Faucon, 2007; Mullin et al., 2010; Lambert et al., 2013). Due to that, beeswax is the most contaminated beehive product and has already been used as a bioindicator of environmental pollution (Porrini et al., 2003; Tsigouri et al., 2004; Lodesani et al., 2003; Orantes-Bermejo et al., 2010; Johnson et al., 2010).

Since the worldwide spread of the parasite *Varroa destructor* (Anderson & Trueman), beekeepers started to use acaricides to control mite population, avoiding damage threshold to the colonies. Nowadays, acaricides (e.g. coumaphos and fluvalinate) applied against varroa mite are the main source of beeswax contamination in both, frequency and concentration. In USA, coumaphos and fluvalinate residues showed the highest frequency (98.1% for both pesticides) and the highest average levels in beeswax samples, 3300 and 7474 $ng \cdot g^{-1}$, respectively (Mullin et al., 2010). Europe surveys have also revealed the extensive use of these acaricides. In France, coumaphos and fluvalinate were found in 46.7% and 52.2% of the samples, and reached average levels of 648 and 220 $ng \cdot g^{-1}$, respectively (Chauzat et al., 2011). In Italy, coumaphos (83%) and fluvalinate (75%) were also the most frequently detected pesticide residues in beeswax samples (Lodesani et al., 2003). Belgium beeswax results also confirms the high presence of these two acaricides (Ravoet et al., 2015). In Spain, one of the EU largest honey producer and the European country with the highest beehives census (Agriculture and rural development - European Commission, 2017), results showed a high incidence of fluvalinate (>93%) (Orantes-Bermejo et al., 2010; Serra-Bonvehi and Orantes-Bermejo, 2010; García et al., 2017). Among insecticides residues found in beeswax samples, the organophosphate chlorpyrifos was the most frequently detected in North American apiaries (63.2%) (Mullin et al., 2010). Other frequently detected contaminants were the pyrethroids cypermethrin, fenpropathrin, esfenvalerate and bifenthrin (12–18%) together with some fungicides (Mullin et al., 2010; Chauzat et al., 2011).

The use of veterinary agricultural treatments in beehives and its environment implies a risk of contamination of the honey bees and related apicultural matrices (wax, honey, pollen, royal jelly and propolis) and their analysis have also shown a widespread contamination (Mullin et al., 2010; Chauzat et al., 2011; Lambert et al., 2013; Kasiotis et al., 2014). In addition of recycled beeswax used in beekeeping, beeswax is found in myriad products: lipsticks, facial creams, pill coatings, salves, chewing gum, candles, floor and furniture polishes, and waterproofing materials. As beeswax is used in cosmetics and pharmaceuticals it should contain minimal amounts of contaminants (Bogdanov, 2004). Therefore, studying residues in beeswax is relevant not only to beekeeping issues but also to economic, environment and to public health purposes.

Recently, advances in analytical methods have improved sensitivity and sample throughput that were the problems of previous studies to tackle this subject. Long and tedious solid liquid extraction procedures involving a great number of additional clean-up steps that takes several days have been progressively replaced by simple, generic and rapid QuEChERS platforms. As well, application of liquid chromatography-mass spectrometry (LC-MS/MS) has ensure optimum sensitivity and selectivity to analyze pesticide residues in a complicated matrix because its apolar character and the high hydrocarbons content (Niell et al., 2014; Calatayud-Vernich et al., 2016a; Calatayud-Vernich et al., 2016b; Herrera López et al., 2016).

In view of these concerns, this study aimed at comparing pesticide residues in foundation, old combs, cappings and virgin wax, to discuss implications for the beekeeping management practices and health of the honey bee colonies taken into account the pesticide residues levels and frequency, as well as whether they come from veterinary treatment or the surrounding environment. Among the 58 pesticides included in this study, the most relevant were the pyrethroids achrinathrin, cyhalothrin, flumethrin, and tau-fluvalinate, the organophosphates chlorpyrifos, coumaphos and chlorfenvinphos, and acaricide amitraz. The target analytes were chosen based on their potential toxicity to honey bees and/or their widespread use in plant protection or in the beehive against varroosis. The sample preparation method was based on QuEChERS extraction with subsequent determination by liquid chromatography coupled to a triple quadrupole mass spectrometry (LC-MS/MS).

2. Materials and methods

2.1. Chemicals

High purity (98–99.9%) standards of the 55 selected pesticides together with the transformation products of amitraz; 2,4-dimethylaniline (DMA), 2,4-dimethylphenylformamide (DMF) and N-(2,4-dimethylphenyl)-*N*′-methylformamidine (DMPF) were acquired from Sigma-Aldrich (Steinheim, Germany) (listed in Table 1). Individual standard solutions were prepared in methanol at a concentration of 1000 $mg \cdot L^{-1}$. The working standard solutions were prepared by mixing the appropriate amounts of individual standard solutions and diluting with methanol to a final concentration of 1 and 10 $mg \cdot L^{-1}$. All solutions were stored in 15 mL vials at 4 °C in the dark.

Magnesium sulfate was obtained from Alfa Aesar (Karlsruhe, Germany), ammonium formate, sodium hydroxide, sodium chloride, acetonitrile and formic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol was obtained from VWR chemicals (Radnor, Pennsylvania). PSA and C18 sorbents, and PTFE (13 mm × 0.22 μm) filters were purchased from Análisis Vínicos S.L. (Tomelloso, Spain). High purity water was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA). Milli-Q water and methanol, both with ammonium formate 10 mM, were used as mobile phase in LC-MS/MS.

2.2. Origin and characterization of the samples

A total of 35 beeswax samples were collected from different relevant beekeeping areas in Spain during 2016 (Fig. 1). Four different beeswax sources were analyzed: beeswax foundation from commercial suppliers as a mixture of beeswax from many beekeepers (F1–F11); beeswax cappings (virgin wax covering on sealed honeycombs) rendered by particular beekeepers (C1–C12); beeswax from recycled old combs from the brood chamber of commercial hives from particular beekeepers (R1–R10); and virgin wax combs recently built (<7 days) by honey bees in empty spaces of commercial beehives were used as an assumed contrast less contaminated beeswax reference (B1–B2) (Fig. S1 Supplementary material). Except foundation, beeswax samples were acquired from migratory beekeepers that alternate wild flowering plants as rosemary, thyme and heather, with crops blooming, principally citrus and sunflower, but also canola, almond, plums and other fruit trees orchards that require entomophilous pollination.

Method for rendering the beeswax of R group was the centrifugal extraction, in which old combs, placed into a metal basket, are melted by steam (over 70 °C) in a centrifugal wax extractor spinning at >1500 rpm. Metal basket perforated walls eliminate solid impurities while liquid phase containing melted beeswax flows into the lower part of the tank. After solidification, pieces of beeswax blocks from particular beekeepers were collected as R source samples. C group samples were obtained during honey extraction process, when wax cappings are removed from ripe honeycombs. After that, beeswax is subjected to a melting and cleaning procedure similar to the process for rendering R beeswax. Combined steam and press extraction manufacturing method is usually used in suppliers companies (F samples) and it consists on a tank of boiling water where old combs are placed and melted. Afterwards, a piston exerts pressure for about an hour to separate solid

Table 1
LOD and LOQ, recovery, precision (RSD) and matrix effects of the analyzed pesticides. Recoveries values are the mean of five independent determinations at 10, 50 and 100 ng·g^{-1}.

Pesticides	LOD (ng·g^{-1})	LOQ (ng·g^{-1})	Recoveries [average (R) and RSD]						Matrix effects (%)
			10 ng·g^{-1}		50 ng·g^{-1}		100 ng·g^{-1}		
			R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)	
Acetamiprid	0.8	2.5	118	1	85	1	84	1	20
Acetochlor	3.3	10.0	56	30	96	1	76	4	−40
Acrinathrin	4.2	12.5	–	–	71	14	107	2	−25
Alachlor	4.2	12.5	–	–	51	8	66	7	−12
Atrazine	0.8	2.5	74	3	52	7	62	1	−22
Atrazine-desethyl	0.8	2.5	74	4	72	3	77	1	1
Atrazine-desisopropyl	0.8	2.5	86	1	75	6	82	5	−2
Azinphos-ethyl	0.8	2.5	95	3	71	3	77	8	−5
Azinphos-methyl	0.8	2.5	113	9	73	6	73	11	−48
Buprofezin	0.4	1.3	71	3	70	6	71	1	−17
Carbendazim	0.4	1.3	95	3	76	1	70	1	−2
Carbofuran	0.4	1.3	63	3	87	3	88	1	9
Carbofuran-3-hydroxy	0.8	2.5	120	15	91	2	91	1	−10
Chlorfenvinphos	0.4	1.3	91	16	92	1	84	5	−39
Chlorpyrifos	0.4	1.3	88	3	89	1	82	8	−19
Coumaphos	0.3	1.0	83	2	81	2	84	5	−29
Diazinon	0.4	1.3	73	4	71	4	70	4	−27
Dichlofenthion	0.3	1.0	51	3	70	1	82	1	−24
Dimethoate	0.8	2.5	71	10	75	4	78	1	9
Diuron	0.4	1.3	70	1	83	2	84	3	−14
DMA (amitraz)	1.7	5.0	70	29	89	34	81	6	−10
DMF (amitraz)	0.3	1.0	107	4	112	1	91	1	2
DMPF (amitraz)	4.2	12.5	–	–	24	12	20	4	15
Ethion	0.4	1.3	83	2	82	3	82	3	−25
Fenitrothion	4.2	12.5	–	–	70	5	71	5	−27
Fenthion	0.8	2.5	70	2	68	3	73	5	−52
Fenthion-sulfone	0.3	1.0	92	3	87	4	87	3	−9
Fenthion-sulfoxide	0.3	1.0	71	1	72	4	72	2	5
Fipronil	1.7	5.0	70	22	70	4	79	2	−65
Flumethrin	4.2	12.5	–	–	91	4	95	6	−42
Fluvalinate	0.3	1.0	86	19	96	13	108	11	−35
Hexythiazox	0.4	1.3	85	1	82	5	82	6	−25
Imazalil	0.3	1.0	57	23	75	4	82	10	−29
Imidacloprid	4.2	10.0	120	17	72	8	71	1	−21
Isoproturon	0.3	1.0	70	6	83	6	81	2	−3
Lambda-cyhalothrin	4.2	12.5	–	–	73	5	83	3	−23
Malathion	0.3	1.0	89	2	81	2	80	2	−33
Methiocarb	0.3	1.0	78	14	80	9	78	2	−16
Metolachlor	0.3	1.0	72	2	75	3	78	5	9
Molinate	0.3	1.0	77	2	73	3	73	1	−5
Omethoate	1.7	5.0	87	12	82	2	78	2	−15
Parathion-ethyl	0.8	2.5	73	1	72	1	73	3	−54
Parathion-methyl	0.8	2.5	86	19	84	17	84	1	19
Prochloraz	0.3	1.0	72	6	71	10	74	1	−52
Propanil	0.3	1.0	79	2	74	2	76	1	−15
Propazine	0.3	1.0	48	3	50	4	46	5	−36
Pyriproxyfen	0.7	2.0	91	11	77	8	76	1	−13
Simazine	1.7	5.0	74	10	81	1	81	1	−11
Tebuconazole	0.8	2.5	66	5	71	7	71	1	−10
Terbumeton	0.3	1.0	51	1	54	2	55	1	−1
Terbumeton-desethyl	0.3	1.0	59	3	60	2	60	1	3
Terbuthylazine	0.3	1.0	75	5	76	1	74	4	−28
Terbuthylazine-desethyl	0.3	1.0	78	6	82	1	80	2	−1
Terbuthylazine-2-hydroxy	3.3	10.0	7	32	8	6	8	2	−12
Terbutryn	0.3	1.0	52	6	54	5	55	2	−5
Thiabendazole	1.7	5.0	50	2	56	7	52	3	15
Thiamethoxam	4.2	10.0	70	33	71	3	72	3	−62
Tolclofos-methyl	1.7	5.0	77	20	78	3	73	4	−63

impurities. Beeswax runs to the top by decantation while liquid phase runs at the lower part of the tank. Foundation wax pressed into sheets used as templates for comb production were acquired as F samples. B1 and B2 samples were obtained directly from the hive without previous beeswax treatment. Samples were transported to the laboratory in a clean, and insulated cooler, and stored in individual plastic containers at −20 °C until their extraction procedure. The concentration values detected in the samples were the mean of two independent determinations (detailed information of the two determinations for each beeswax group is provided in the Supplementary information Tables S3 to S10).

2.3. Extraction procedure

A QuEChERS ("Quick Easy Cheap Effective Rugged Safe") approach for the beeswax sample preparation adapted from Niell et al. (2014) was used. Beeswax (2 g) was weighed into 50 mL centrifuge tubes and 10 mL of acetonitrile were added, the tubes were closed and placed

Fig. 1. Location of the 22 sampling points in Spain distributed in the Valencian Community (8, 10 to 21), Castille and León (1, 2, 3 and 5), Cantabria (6 and 7), Andalusia (4), Region of Murcia (9) and Balearic Islands (22). Beeswax samples (B, C, F and R) distribution among the sampling points is illustrated.

in a water bath at −80 °C. Once the beeswax had melted, the tubes were vortexed vigorously for 30 s and placed again in the water bath to melt. This step was repeated four times to ensure adequate pesticide extraction. For beeswax precipitation, centrifugation tubes were left to cool to room temperature and put into the freezer (−18 °C) overnight. For the extract cleaning, a volume of 2 mL was sampled into a 15 mL centrifuge tube containing 50 mg C_{18} and 50 mg primary-secondary amine (PSA), the mixture was shaken for 15 s and centrifuged at 3000 rpm for 5 min. Finally, the supernatant was filtered using a PTFE 13 mm × 0.22 μm into the autosampler vials for LC-MS analysis and pH was adjusted to ca. 5 by adding a 5% formic acid solution in acetonitrile (v/v) (10 μL/mL extract).

2.4. Liquid chromatography-mass spectrometry (LC-MS/MS)

The chromatographic instrument was an HP1200 series LC equipped with an automatic injector, a degasser, a quaternary pump and a column oven-combined with an Agilent 6410 triple quadrupole (QQQ) mass spectrometer with an electrospray ionization (ESI) interface (Agilent Technologies, Waldbronn, Germany). Data were processed using a MassHunter Workstation Software for qualitative and quantitative analysis (A GL Sciences, Tokio, Japan).

The chromatographic column was a Luna C18 (15.0 cm × 0.21 cm) with a 3 μm particle size (Phenomenex, Torrance, USA). The column temperature was kept at 30 °C and the volume injected was 5 μL. A binary mobile phase at flow rate of 0.3 mL·min^{-1} with a gradient elution was used. Solvent A was Milli-Q water with 10 mM ammonium formate and solvent B was methanol with 10 mM ammonium formate. The linear gradient was as follows: 0 min (50% B), 10 min (83% B), 12 min (83% B), 12.5 min (98% B), and 15.5 min (98% B). Then, the mobile phase returns to the initial conditions with an equilibration time of 12 min.

Ionization and fragmentation settings were optimized by direct injection of pesticide standard solutions. MS/MS was performed in the SRM mode using ESI in positive mode. For each compound, two characteristic product ions of the protonated molecule $[M + H]^+$ were monitored, the first and most abundant one was used for quantification, while the second one was used as a qualifier. Collision energy and cone voltage were optimized for each pesticide (Table S1 Supplementary material). Nitrogen was used as collision, nebulising and desolvation gas. The ESI conditions were: capillary voltage 4000 V, nebulizer 15 psi, source temperature 300 °C and gas flow 10 L·min^{-1}. In order to maximize sensitivity, dynamic MRM was used, with MS_1 and MS_2 at unit resolution and cell acceleration voltage of 7 eV for all the compounds.

2.5. Method validation and quality control

The method was evaluated regarding sensitivity, accuracy, precision and robustness according to SANTE guidance document on analytical quality control and validation procedures for pesticides (SANTE/11945/2015, 2016).

The linearity of the MS/MS method was established with seven calibration points, using external standards over a concentration range of 10–500 ng·mL^{-1} (Supplementary material Table S2). The peak area of target analytes was calculated using Mass Hunter software (Agilent).

Each point was obtained as the mean of three injections. The data were fit to a linear least-squares regression curve with a 1/x weighting, and not forced through the origin. The R-squared was >0.99 with residuals <30%. Matrix effects were evaluated by comparing the slope of the previous calibration curve and the slope of that prepared in the extract of beeswax with seven concentration levels of standard solutions. To validate the method and to quantify the samples, matrix matched standards (prepared in beewax) were used.

The sensitivity of the method was estimated by establishing the limits of detection (LODs) and quantification (LOQs) (Table 1). LODs were calculated using standard solutions prepared in spiked beeswax samples that were free of pesticides. As it was difficult to find a sample without the selected pesticides, if one compound was initially in the beeswax samples (e.g. coumaphos), another beeswax sample free of the compound was used to establish LODs and LOQs for it. The LODs were determined as the lowest pesticide concentration whose qualified transition (SRM_2) presented a signal-to-noise ratio (S/N) ≥ 3. The LOQs were determined also in spiked beeswax samples as the minimum detectable amount of analyte with S/N ≥ 10 for the quantifier (SRM_1) transition. All the LOQs were verified spiking the samples and analyzing them. Recovery, as accuracy, and precision, expressed as relative standard deviation (RSD), were determined by analyzing quintuplicate beeswax samples spiked at 10, 50 and 100 $ng \cdot g^{-1}$.

2.6. Beeswax and toxicity

In order to evaluate toxicity in wax matrix, the hazard quotient (HQ_{wax} = pesticide concentration in ppb ÷ pesticide topical LD_{50} as μg/bee) proposed by Stoner and Eitzer (2013) was calculated for the pesticides detected in the samples. LD_{50} used for the hazard quotient were from Sanchez-Bayo and Goka, 2014, and University of Hertfordshire Pesticide Properties Database. Dichlofenthion and fenthion sulfoxide pesticides were excluded from the hazard quotient because no honey bee ecotoxicological data was available (see Supplementary material Table S14). Samples with HQ_{wax} > 5000 were considered to have an elevate pesticide load (Traynor et al., 2016).

2.7. Statistical analysis

IBM SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Analysis of variance (ANOVA) and Tukey's multiple range test at $\alpha = 0.05$ were performed to detect differences in the variables between treatments. In the cases where the homogeneity and/or normality of the data could not be assumed, the Kruskal–Wallis and Mann–Whitney non parametric test ($P \leq 0.05$) were applied.

3. Results

3.1. Validation of the analytical method

Recovery values ranged from 50 to 120% with the exception of terbuthylazine-2-hydroxy that was not present in any sample. RSDs were <20% except for acetochlor, DMA (amitraz), imazalil, fipronil, terbuthylazine-2-hydroxy and thiamethoxam at the 10 $ng \cdot g^{-1}$ spiking level (Table 1). The 50 $ng \cdot g^{-1}$ spiked beeswax recoveries were from 50 to 112% with DMPF and terbuthylazine-2-hydroxy exception. RSDs were <20% except for DMA. Recoveries at 100 $ng \cdot g^{-1}$ ranged from 52 to 108% except for DMPF (amitraz) and terbuthylazine-2-hydroxy. RSDs were <20 for all pesticides (Fig. 2). The LODs were from 0.3 to 4.2 $ng \cdot g^{-1}$, whereas LOQs ranged from 1 to 12.5 $ng \cdot g^{-1}$. The Matrix effects were in the range of −65 to 20% over the response of the standards prepared in solvent. The matrix effects were mostly suppressive (lower response compared to the standard), with the exception of acetamiprid, atrazine-desethyl, carbofuran, dimethoate, DMF (amitraz), DMPF, fenthion-sulfoxide, metolachlor, parathion-methyl, terbumethon-desethyl and thiabendazole which showed an increase in the response. Both calibration curves, in acetonitrile or in matrix extract, showed a linear response through the tested range (Supplementary information Table S2 details the equations of the calibration curves obtained in matrix).

3.2. Beeswax and pesticide residues

A summary of the pesticides found is showed in Tables 2 and 3. Pesticide residues of 16 different compounds were detected. Four or more pesticides were found in 86%, five or more in 74%, and six or more in 63% of the 35 samples analyzed. Pesticide content found in virgin wax was >18 times lower than those exhibited in F and R and 4 times lower than C beeswax source (Fig. 3). Four pesticides showed statistical significant differences between capping, foundation and old combs beeswax (Table 3). Concentrations of chlorfenvinphos showed significant differences between capping and foundation. Levels of coumaphos and flumethrin showed significant differences between capping and the other beeswax groups. Levels of DMF show significant differences between foundation and the old combs.

3.2.1. Virgin combs beeswax (B)

Two contrast virgin wax samples were analyzed to establish a less contaminated beeswax source, and six pesticides residues were found (Table 2). Coumaphos residues, found in both samples, had the highest mean concentration of 550 $ng \cdot g^{-1}$. DMF and chlorfenvinphos were also in both beeswax samples and their concentrations were 34.3 and 32.5 $ng \cdot g^{-1}$, respectively.

3.2.2. Cappings beeswax (C)

In cappings samples, 9 pesticides and an average of 4.1 pesticides per sample was detected (Table S13). Pesticide frequencies ranged from 33 (Chlorpyrifos and Chlorfenvinphos) to 100% (Coumaphos) except for hexythiazox, flumethrin and pyriproxyfen, found in 8% of the samples (Table 3). DMF was detected in almost 92% of samples, fluvalinate and acrinathrin in 67 and 58%, respectively. Coumaphos had the highest concentration (6880 $ng \cdot g^{-1}$) and the highest mean concentration (1420 $ng \cdot g^{-1}$), followed by miticides DMF, fluvalinate and acrinathrin with a mean concentration of 286.3, 353.8 and 626.7 $ng \cdot g^{-1}$, respectively.

3.2.3. Foundation beeswax (F)

A total of 11 pesticide residues were found. An average load of 7 pesticides per beeswax sample was detected and the less contaminated sample had five different pesticide residues (Table S12). Frequencies of pesticide residues ranged from 50 to 100% with the exception of malathion (27%), azinphos-methyl (9%) and fenthion-sulfoxide (9%) (Table 3). The most frequent residues were coumaphos, chlorfenvinphos and fluvalinate with a frequency of 100%. Pyrethroids acrinathrin and flumethrin, and amitraz degradation product DMF, were detected in 81.8% of samples. Dichlofenthion and chlorpyrifos were detected in 63.6 and 54.5% of samples respectively. Coumaphos had the highest concentration of 17,371 $ng \cdot g^{-1}$, with the highest average content of 9486 $ng \cdot g^{-1}$, followed by chlorfenvinphos and fluvalinate, which respective mean concentrations were 1490.5 and 1085.3 $ng \cdot g^{-1}$. Acrinathrin and flumethrin had a mean concentration of 414.8 and 90.5 $ng \cdot g^{-1}$, and maximum concentrations were 2585 and 170.1 $ng \cdot g^{-1}$, respectively.

3.2.4. Old combs beeswax (R)

Residues of 11 pesticides were found and an average of 6.5 pesticides per sample was detected (Table S11). Pesticide residues frequencies ranged from 30 to 100% with the exception of carbendazim, fenthion-sulfoxide and imazalil detected in 10% of samples (Table 3). Acaricides coumaphos, fluvalinate and chlorfenvinphos were the most frequently detected pesticides (100%). Detection of pyrethroids acrinathrin and flumethrin was 90% and respective frequencies for dichlofenthion,

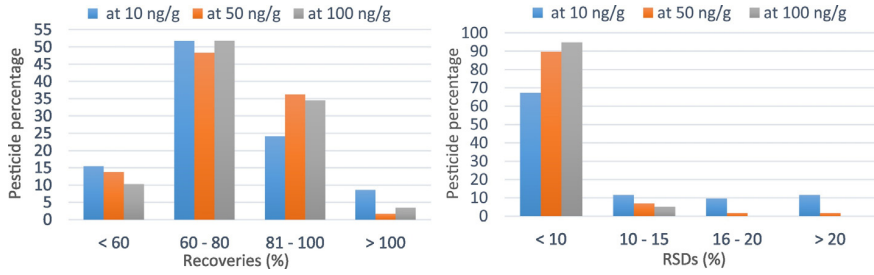

Fig. 2. Percentage of pesticides according to the range of recoveries and RSDs of the validated method.

chlorpyrifos and DMF were 30, 40 and 70%. Coumaphos had the highest mean content (11,431 $ng \cdot g^{-1}$) with the maximum concentration of 26,858 $ng \cdot g^{-1}$, that was the highest concentration of all pesticides analyzed. Fluvalinate and chlorfenvinphos mean concentrations were 472.7 and 428.8 $ng \cdot g^{-1}$, with a maximum value of 746.2 and 796.6 $ng \cdot g^{-1}$, respectively. Acrinathrin mean concentration was 250.8 $ng \cdot g^{-1}$, and flumethrin mean concentration in the samples reached 87.2 $ng \cdot g^{-1}$. Residues of DMF were found in the second highest mean (1493.7 $ng \cdot g^{-1}$) and maximum concentration (6885 $ng \cdot g^{-1}$) quantities.

3.3. Beeswax and toxicity

Hazard quotient ranged from 13 to 17,600 and 13 out of 35 samples had an elevated toxicity to honey bees with values over 5000 (Fig. 4). In Foundation and old combs, samples with an elevated HQ_{wax} represented >50%, and the average HQ_{wax} in both groups was 6283 and 5775. Virgin and capping average HQ_{wax} was 423 and 4188, respectively. Acrinathrin, flumethrin and chlorpyrifos were the main contributors to the scores $HQ_{wax} > 5000$. Four samples with $HQ_{wax} > 15{,}000$ were detected, and the main contributors to the highest scores were acaricide acrinathrin in F3, C7 and C8 samples, and insecticide chlorpyrifos in R6.

4. Discussion

4.1. Validation of the analytical method

The QuEChERS extraction procedure followed by LC-MS/MS has been already proposed to assess pesticide residues in beeswax (Niell et al., 2014; Herrera López et al., 2016). Previously, more complex and tedious protocols were used to assess pesticide content in beeswax by UHPLC-MS/MS (Jabot et al., 2015). Appropriate results in terms of accuracy and sensitivity, low cost and quickness make QuEChERS a suitable procedure for determining pesticides in beeswax matrix and also to other beekeeping related matrices (Calatayud-Vernich et al., 2016b). Furthermore, the pesticide detection based on LC-MS/MS analysis, used in the present study, has been proved in previous work to be more appropriate for wider scope and better sensitivity than GC–MS/MS (Alder et al., 2006).

Table 2
Mean of the virgin beeswax samples (B1 and B2) and the total mean concentration of pesticide residues found in beeswax from virgin combs (B).

Pesticide	B 1 ($ng \cdot g^{-1}$)	B 2 ($ng \cdot g^{-1}$)	B mean concentration ($ng \cdot g^{-1}$ beeswax)
Coumaphos	794.2	306.4	550.3
DMF (amitraz)	52.2	16.3	34.3
Chlorfenvinphos	41.0	23.9	32.5
Chlorpyrifos	55.2	0.0	27.6
Fluvalinate	49.6	0.0	24.8
Ethion	0.0	21.3	10.65

Average total pesticide load ($ng \cdot g^{-1}$ beeswax): 680.15.

Validation data for some pesticides did not fulfill the analytical requirements of the SANTE guideline (SANTE/11945/2015, 2016). Recoveries for some pesticides are outside the range of 70–120% in which is not necessary correct by the recovery. Terbuthylazine-2-hydroxy and the metabolite of amitraz (DMPF) provided recoveries <25% at any of the studied concentrations because they are quite unstable and then, results provided would not be quantitative. Alachlor, atrazine, propazine, terbumeton, terbumeton deethyl, terbutryn and thiabenzole gave recoveries >50% but <70% at all the concentrations studied. Then, their values in samples were corrected by the recovery. Acetochlor, carbofuran, imazalil and tebuconazol also provided recoveries >50 and

Table 3
Summary of pesticide detections in old combs (R), foundation (F) and capping (C) beeswax.

Pesticide	Frequency (%)	Maximum concentration ($ng \cdot g^{-1}$ beeswax)	Minimum concentration ($ng \cdot g^{-1}$ beeswax)	Mean concentration[a] ($ng \cdot g^{-1}$ beeswax)
Cappings beeswax (C)				
Coumaphos	100.0	6880.0	90.0	1420.0**a**
DMF (amitraz)	91.7	1065.0	75.0	286.3**ab**
Fluvalinate	66.7	3065.0	25.0	353.8
Acrinathrin	58.3	2595.0	25.0	626.7
Chlorpyrifos	33.3	260.0	5.0	23.3
Chlorfenvinphos	33.3	50.0	5.0	7.5**a**
Hexythiazox	8.3	45.0	45.0	3.8
Flumethrin	8.3	35.0	35.0	2.9**a**
Pyriproxyfen	8.3	25.0	25.0	2.1
Average total pesticide load ($ng \cdot g^{-1}$ beeswax): 2726.4				
Foundation beeswax (F)				
Coumaphos	100.0	17,370.7	25.0	9486.2**b**
Chlorfenvinphos	100.0	5284.8	433.9	1490.5**b**
Fluvalinate	100.0	3593.3	374.9	1085.3
Acrinathrin	81.8	2584.9	96.3	414.8
Flumethrin	81.8	170.1	48.0	90.5**b**
DMF (amitraz)	81.8	118.9	15.9	40.9**a**
Dichlofenthion	63.6	96.2	28.9	38.6
Chlorpyrifos	54.5	327.2	19.4	69.7
Malathion	27.3	189.7	67.5	39.8
Azinphos-methyl	9.1	75.1	75.1	6.8
Fenthion-sulfoxide	9.1	44.4	44.4	2.0
Average total pesticide load ($ng \cdot g^{-1}$ beeswax): 12,765.0				
Old combs beeswax (R)				
Coumaphos	100	26,858	431.4	11,431.1**b**
Fluvalinate	100	746.2	289.6	472.7
Chlorfenvinphos	100	796.6	219.1	428.8**ab**
Acrinathrin	90	802	30.7	250.8
Flumethrin	90	120.1	24.5	87.2**b**
DMF (amitraz)	70	6884.6	15.8	1493.7**b**
Chlorpyrifos	40	978	6.8	129.2
Dichlofenthion	30	962.9	59.3	108.6
Carbendazim	10	113.6	113.6	11.4
Imazalil	10	50.9	50.9	5.1
Fenthion-sulfoxide	10	31.6	31.6	3.2

Average total pesticide load ($ng \cdot g^{-1}$ beeswax): 14,421.7.
[a] Different letters indicate statistical significant differences between the mean of the different pesticides among the groups.

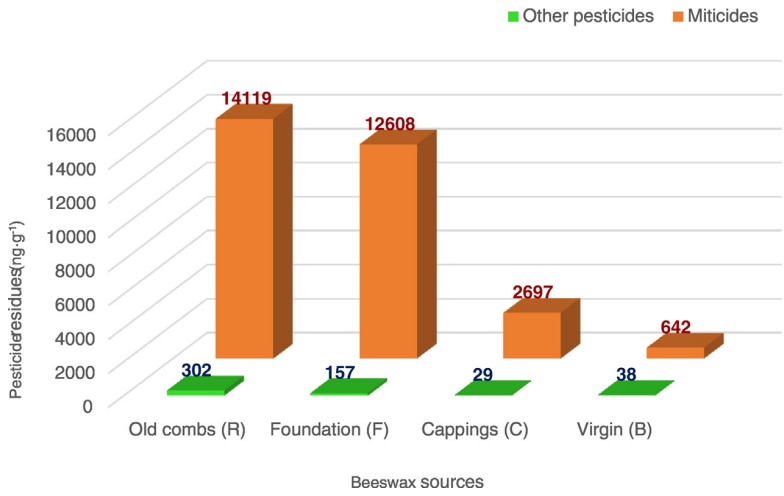

Fig. 3. Average total load of miticides used in beekeeping and other pesticides ($ng \cdot g^{-1}$) of the different beeswax sources (old combs, foundation, cappings and virgin).

<70% at the lowest concentration (10 $ng \cdot g^{-1}$). Furthermore, four compounds, acetochlor, imazalil, terbuthylazine-2-hydroxy and thiametoxan provided RSDs >20% only at the lowest concentration.

The present study covers 46 compounds than differ from those tested by Niell et al. (2014) multiresidue method. An additional difference is that this method does not use internal standards but use matrix matched standards. A filtration step was also added before the injection in order to prevent LC system occlusion. Recovery, precision and matrix effects were similar in both studies. Sensitivity was improved by the methodology here proposed compared to Niell et al. (2014). Herrera López et al. (2016) using citrate buffered QuEChERS evaluated the extraction of 120 pesticides in beeswax, reporting less matrix effects, slightly higher recoveries and similar precision compared to Niell et al. (2014) and the present study. Herrera López et al. (2016) sensitivity was moderately lower compared to LOQs presented here. Our method is simpler, cheaper and more rapid, and the differences on the method performance are small and within the range of precision.

4.2. Beeswax and pesticide residues

To give a representative profile of pesticide contaminants in beeswax, samples were acquired from diverse wax manufacturers (F) and beekeepers (R, B and C) operating in different regions of the country. The samples of beeswax analyzed in the present study have revealed high levels of miticides. Insecticides and fungicides residues were less frequent and quantities were in most cases lower. Comparison of wax groups denoted an accused difference among beeswax nature (Fig. 3). Intra-group and among groups differences were observed when comparing diverse origin of the samples due to veterinary treatments or pesticides used in the surrounding environment in each studied region

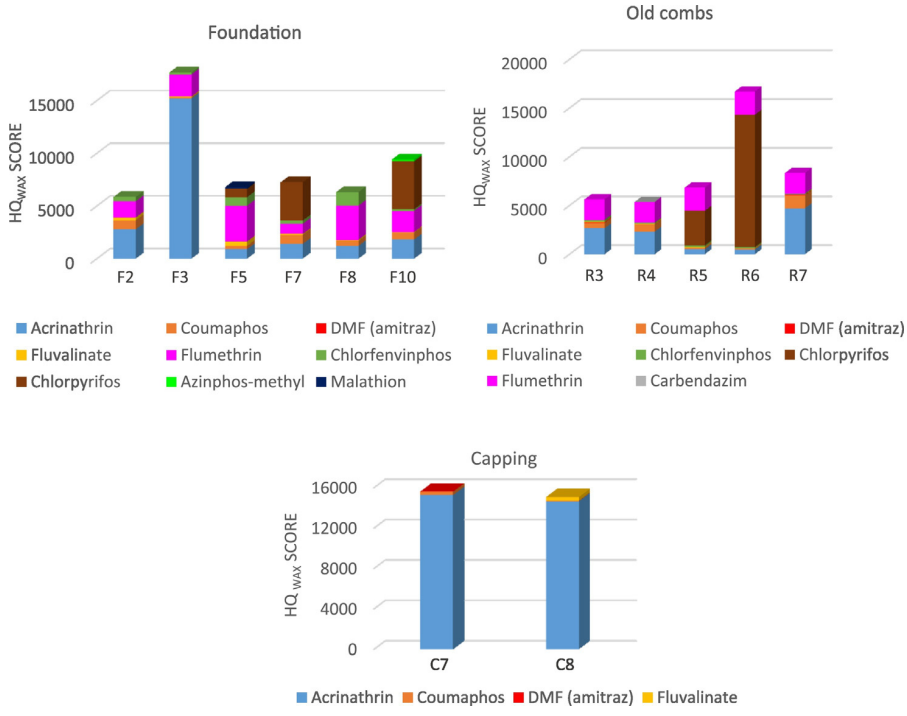

Fig. 4. Contribution of the detected pesticides to the HQ_{wax} scores in the samples (foundation, old combs and cappings). Beeswax samples with HQ_{WAX} > 5000 are illustrated.

(Supplementary material Tables S3 to S13). Each beekeeper realized a particular migratory route, so the differences in pesticides content among the groups were expected. Personal communication with particular beekeepers and the Apiarian Sanitary Defense Group stated that coumaphos (Checkmite®) and amitraz (Apivar®, Apitraz®) active substances were the principal veterinary treatments in the apiaries. The use of homemade preparations of different non-authorized products was not discarded. Conventional agriculture spraying of pesticides in the surroundings of the apiaries was confirmed by the sampling personal and proved in previous studies (Calatayud-Vernich et al., 2016a).

4.2.1. Beeswax and miticides

Since 2007, Checkmite® (with coumaphos active substance) has been one of the authorized products against varroa mite and its residues have been found at high levels in Spanish beeswax foundation: 340 $ng \cdot g^{-1}$ (Jimenez et al., 2005), 67.9 $ng \cdot g^{-1}$ (Serra-Bonvehi and Orantes-Bermejo, 2010), and 9486 $ng \cdot g^{-1}$ (present study, 2016). Coumaphos residues had a frequency of 100% and reached the highest mean concentration of all pesticides in R, F, C and B. Capping wax showed significant differences in coumaphos levels compared to foundation (F) and old combs (R) (Table 3). The slight difference between F (9486 $ng \cdot g^{-1}$) and R (11,431 $ng \cdot g^{-1}$) coumaphos content could be explained due to geographical reasons suggested above, otherwise indicated a general use of this product. Coumaphos contribution to the total pesticide load was over 50% for capping and exceeded the 70% for the rest of the beeswax sources. In American and Europe, numerous reports have exhibited very similar results (Lodesani et al., 2003; Chauzat and Faucon, 2007; Johnson et al., 2010; Mullin et al., 2010; Chauzat et al., 2011; Harriet et al., 2017).

Chlorfenvinphos was detected in 100% of the samples analyzed with a mean concentration of 1491, 429 for beeswax sources F and R respectively. However, was detected in 33% of wax capping samples (C) with a mean concentration of 354 $ng \cdot g^{-1}$, and significant differences were observed between capping and foundation beeswax (Table 3). As liphofilic residues are stable on beeswax matrix, previous treatments with this compound could be, in part, responsible of chlorfenvinphos incidence. However, levels detected in this work suggest an illegal use of this organophosphate acaricide against varroosis according to the current legislation (EU regulation 1107/2009). Previous works of Spanish and Italian beeswax have also supported results found in the present study where chlorfenvinphos was one of the most frequently detected pesticides, and the unauthorized use of this compound was proved (Jimenez et al., 2005; Lodesani et al., 2008; Orantes-Bermejo et al., 2010; Serra-Bonvehi and Orantes-Bermejo, 2010).

All foundation and recycled old combs beeswax samples contained fluvalinate (Apistan®) residues and it was also found in beeswax from virgin combs and cappings. As in the present study, fluvalinate residues have been found in beeswax matrix from many countries at high levels (Lodesani et al., 2003; Mullin et al., 2010; Chauzat et al., 2011; Serra-Bonvehi and Orantes-Bermejo, 2010; Adamczyk et al., 2010).

Although acrinathrin is an unauthorized compound as a veterinary treatment in beekeeping, their residues have appeared in high frequencies (>80%) in beeswax from foundation and recycled old combs. Residues were also found in wax cappings (58%) and its mean concentration reached 626 $ng \cdot g^{-1}$.Concentrations of this acaricide in the samples analyzed could indicate an irregular use of this pyrethroid against varroa mite. Previous works have also detected acrinathrin in Spanish beeswax, supporting our results (Jimenez et al., 2005; Serra-Bonvehi and Orantes-Bermejo, 2010).

Flumethrin (Bayvarol®) is other acaricide also detected in >80% of the samples in R and F beeswax. No differences were observed in levels found in both groups and concentrations were considered residual as literature also indicates (Serra-Bonvehi and Orantes-Bermejo, 2010).

Amitraz (Apivar®, Apitraz® and Amicel®) is the only acaricide unstable in beeswax ($t_{1/2}$ = 6.3 h) and is almost completely degraded within one day in this matrix (Korta et al., 2001). DMF is the principal breakdown product left in beeswax and has been used in the present work to trace amitraz applications in beehive. Of 35 beeswax samples analyzed, DMF was detected in 29. Foundation (F) and virgin wax were slightly contaminated, whereas beeswax from wax cappings (C) and recycled old combs (R) showed concentrations 7 to 43 times higher, respectively. Thereby, significant differences were detected between foundation and old combs wax (Table 3). High concentrations in wax cappings could indicate a recent use of amitraz in the apiaries against varroosis. This accused difference in DMF content between C, R and F group could also be explained if we consider methods used for rendering the beeswax. Hidrophilicity of DMF (Log P = −1.1) (TOXNET, 2017) would cause its wash off from beeswax matrix when in contact with liquid phase during long periods of time as occurs in foundation manufacturing steps (Serra-Bonvehi and Orantes-Bermejo, 2010).

4.2.2. Beeswax and insecticides

In despite of its little size, honeybees can patrol extensive areas when foraging in the search of nectar and pollen. Besides, pesticide residues from agriculture treatments are susceptible of being collected by forager honey bees during the flight due to their hairy body and pollen-collecting apparatus. Such reasons make honey bees a good sentinel of environmental contamination (Ghini et al., 2004; Mullin et al., 2010; Chauzat et al., 2011; Lambert et al., 2013). Compounds retained in the body of honey bee are transported to the hive where can be actively distributed throughout different apicultural matrices as beeswax (Tremolada et al., 2004). Samples in this study have revealed the presence of the pesticides dichlofenthion, ethion, carbendazim and azinphos-methyl, not approved in the EU through Regulation (EC) 1107/2009. Except for organophosphate dichlofenthion, the pesticides azinphos-methyl, ethion and carbendazim frequencies and concentrations were considered residual and could indicate a past use of these illegal compounds.

Dichlofenthion illegal insecticide together with chlorpyrifos and malathion higher frequencies and concentrations found in beeswax could indicate a widely use of these insecticides in the surrounding areas of honey bee colonies where beeswax came from. Several assessments of beeswax made in Europe and North America were in line with pesticide residues presented in the present work (Mullin et al., 2010; Chauzat et al., 2011).

4.3. Contaminated beeswax as a transference center of pesticides

Pesticide residues found in virgin and capping beeswax (C and B) evidenced a transfer of pesticide residues from areas of contaminated combs (F and R) to newly synthesized and uncontaminated beeswax. Pesticide mode of distribution in the hive ecosystem has been studied and supports our finding of white virgin wax progressive contamination (Tremolada et al., 2004; Harriet et al., 2017). Lipophilic beeswax can act as a trap of non-polar pesticides from which retained analytes can be transferred and actively distributed to other hive products (propolis, royal jelly, pollen, honey) by honey bees (Kochansky et al., 2001; Tremolada et al., 2004; Wu et al., 2011). To prevent transference of contaminants and guarantee beeswax quality, a maximum limit of pesticides (MRLs) should be stablished.

Most of pesticides found in beeswax are very stable once absorbed in this matrix. Many of the pesticides resist the process of comb recycling and some are concentrated by these treatments (e.g. coumaphos content do not decrease after 2 h at 140 °C) (Bogdanov et al., 1998; Martel et al., 2007). High half-life times (e.g. coumaphos, $t_{1/2}$ = 115–346 days) (Martel et al., 2007), and elevated partition coefficients (Log K_{ow}), between 5 and 7.6 for some compounds (EFSA, 2010; PubChem Project, 2017), are the main factors involved in their stability in beeswax. Such persistence in this matrix lead to long-term simultaneous accumulation of many pesticides, as shown in the present study. Long term accumulation of miticides in beeswax creates a propitious

environment to the appearance of acaricide resistant varroa (Bogdanov et al., 1998; Gonzalez-Cabrera et al., 2016).

4.4. Beeswax and toxicity

Pyrethroids acrinathrin and flumethrin, together with organophosphate chlorpyrifos, were the main contributors to the HQ_{wax} scores due to their great toxicity through contact for honey bees and significant concentrations in the samples (Fig. 4). Despite coumaphos, chlorfenvinphos and fluvalinate higher concentrations, contributions to HQ_{wax} were mostly residuals due to their very low toxicity for honey bees. Adverse implications for honey bee health may be occurring when $HQ_{wax} > 5000$ were detected. In this way, worker honey bee development, longevity and hive performance are adversely affected when developing in a pesticide contaminated brood comb at sublethal levels (Bevk et al., 2012; Wu et al., 2011). Additionally, synergistic adverse effects of miticides applied in-hive as fluvalinate and coumaphos have been described (Johnson et al., 2009). Queens and drones exposed to fluvalinate and coumaphos treatments were smaller and sexual vigor was impaired (Rinderer et al., 1999; Haarmann et al., 2002; Collins et al., 2004).

4.5. Beeswax in beekeeping

Cappings and recycled old combs are the only 2 beeswax sources used by manufacturers to elaborate foundation sheets used as templates for comb construction. In view of pesticide levels in old combs (14,421 $ng \cdot g^{-1}$), foundation (12,765 $ng \cdot g^{-1}$) and cappings (2726 $ng \cdot g^{-1}$) found in the present study, it was evidenced that wax manufacturers mainly utilize wax from old combs to elaborate foundation sheets. Consequently, same beeswax origin create a closed beeswax market where pesticide residues are maintained and incoming beeswax contaminated. The use of greater amounts of less contaminated beeswax, as capping beeswax, in foundation manufacturing processes is highly encouraged to dilute pesticide residues in this matrix.

5. Conclusions

Adequate results regarding accuracy, precision, sensitivity and robustness indicate that the methodology used is appropriate to assess levels of the selected pesticides in wax. Results pointed out that virgin and cappings wax were substantially less contaminated than foundation and old combs beeswax. Samples analyzed have revealed high levels of miticides applied in-hive and some insecticides and fungicides used to control pest of the crops. This widespread occurrence of pesticides in beeswax can result in pesticide residues transfer to other beehive matrices (e.g. honey). It is necessary to introduce Maximum Residue Limits (MRLs), as occurs in honey, to control the presence of pesticides in beeswax, preventing eventual transference to honey and guaranteeing beeswax quality, regardless of its subsequent use. Furthermore, given the concentrations detected, toxic effects as deterioration of honey bee health are not discarded. The use of greater amounts of capping beeswax in foundation manufacturing processes is necessary to dilute pesticide residues in this matrix. Furthermore, the right application of authorized veterinary treatments as well as the implementation of new and sustainable management practices are recommended to reduce miticide levels in beeswax.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.06.174.

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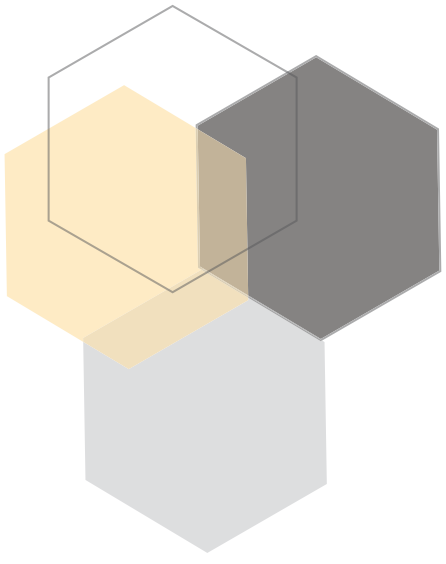
ARTICLE 05

SUPPLEMENTARY MATERIAL:

OCURRENCE OF PESTICIDE RESIDUES IN SPANISH BEESWAX.



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B

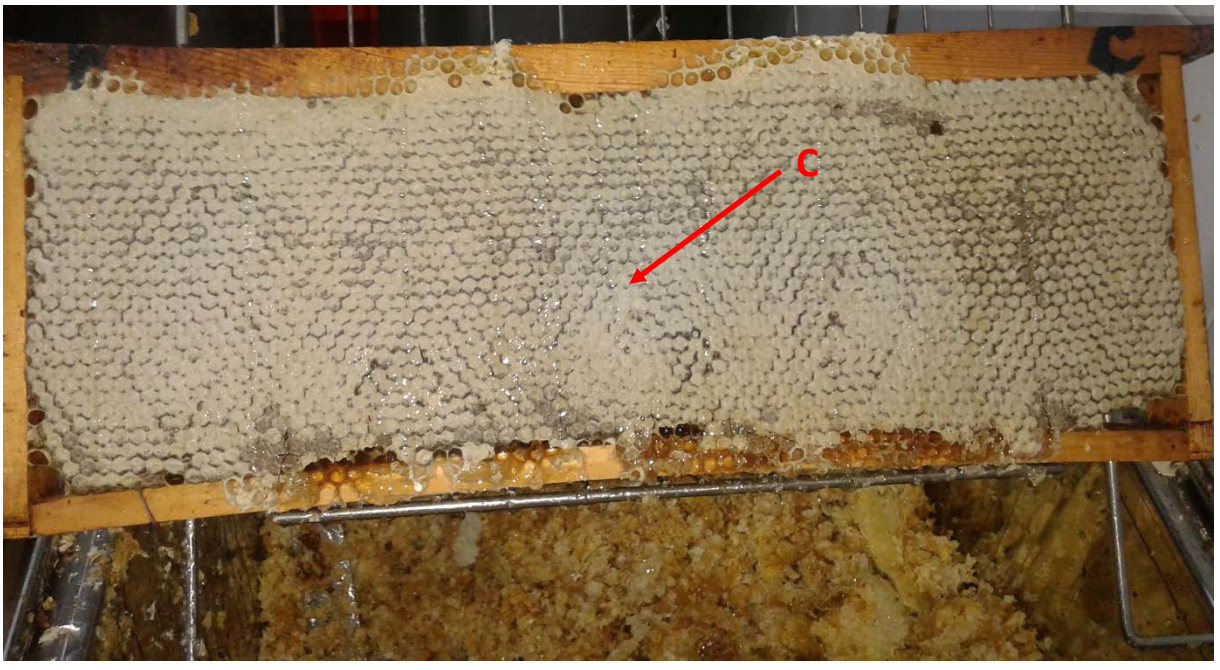
C

Figure S1. Beeswax types used in the present study: virgin combs (B), capping wax (C), recycled old combs (R) and fundation (F)

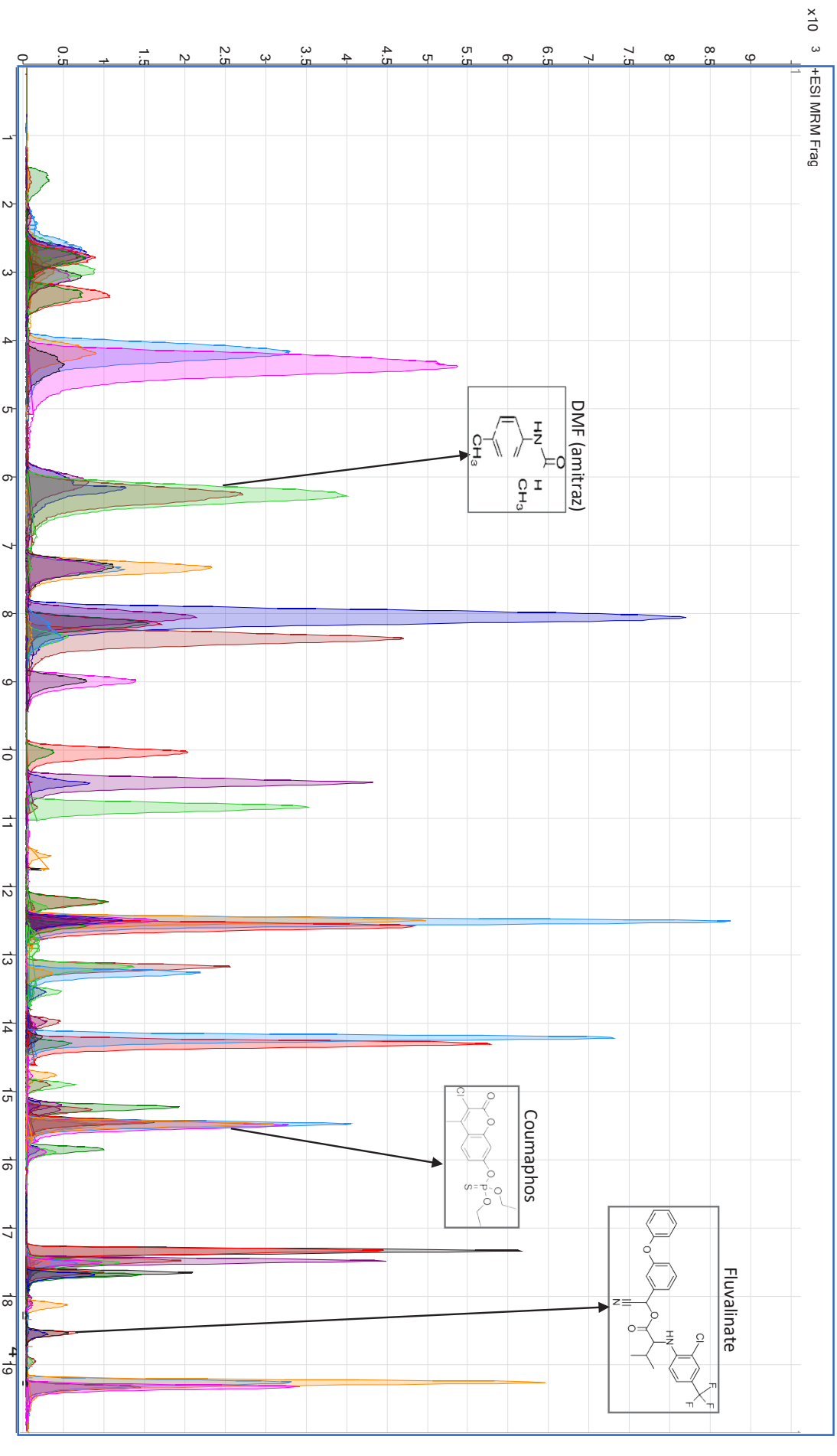

Figure S2. The peaks of the most frequent detected pesticides are indicated.

Table S1. Dynamic MRM conditions used for LC-MS/MS determination of pesticide residues.

Target Pesticide	t_R[a] (min)	Δ t_R [b]	Precursor Ion	SRM_1[c]	Frag[d] (V)	CE[e] (V)	SMR_2[f]	Frag[d] (V)	CE[e] (V)	SMR_2/SRM_1 (%) (%RSD)[g]
Acetamiprid	3.21	2.97	223	126	111	22	56	111	14	37.4 (12)
Acetochlor	14	2	270	224	120	10	148	120	10	46.8 (22)
Acrinathrin (adduct) *	18.4	2	559.16	208.1	76	10	181.1	76	30	56.3 (3)
Alachlor	13.63	2	270	238	80	15	162	80	10	50.4 (13)
Atrazine	9.56	2.63	216	132	120	15	174	120	20	17.3 (14)
Atrazine-desethyl	4.06	2.5	188	146	120	15	104	121	24	29.1 (15)
Atrazine-desisopropyl	2.7	2.08	174	96	120	15	132	120	15	78.6 (13)
Azinphos-ethyl	13.8	1.71	346	97	80	20	137	80	32	83.5 (12)
Azinphos-methyl	11.3	1.24	318	125	80	8	132	80	12	85.4 (11)
Buprofezin	17.46	1.1	306	201	120	10	116	120	15	64.6 (13)
Carbendazim	4.16	4.74	192	160	95	17	132	95	25	11.4 (14)
Carbofuran	6.96	2.91	222	123	120	10	165	70	15	98.0 (9,3)
Carbofuran-3-hydroxy	3.87	2.48	255	163	70	5	220	70	15	90.8 (9)
Chlorfenvinphos	14.8	1.61	359	155	120	10	127	120	15	63.8 (11)
Chlorpyriphos	17.3	2.23	350	97	92	13	198	97	13	78.6 (14)
Coumaphos	15.4	2.15	363	335	134	10	307	134	10	24.8 (10)
Diazinon	14.86	1.89	305	169	128	17	153	128	21	66.3 (12)
Dichlofenthion	17.13	2	315	259	120	10	287	120	5	44 (11)
Dimethoate	3.22	2.59	230	199	80	10	171	80	5	45.3 (12)
Diuron	10.7	1.25	233	72	120	20	160	120	20	3.2 (13)
DMA (amitraz)	2.92	2.5	122	107	111	18	77	111	42	3.0 (17)
DMF (amitraz)	5.88	4.5	150	132	111	10	107	111	15	41.6 (16)
DMPF (amitraz)	2.88	4.12	163	122	111	15	107	111	15	0.1 (15)
Ethion	17.63	1.23	385	199	80	5	171	80	15	35.3 (11)
Fenitrothion	13.35	1.18	278	125	140	15	109	121	12	95.5 (12)

Target Pesticide	t_R[a] (min)	Δ t_R [b]	Precursor Ion	SRM_1[c]	Frag[d] (V)	CE[e] (V)	SMR_2[f]	Frag[d] (V)	CE[e] (V)	SMR_2/SRM_1 (%) (%RSD)[g]
Fenthion	14.63	1.83	279	247	114	5	169	114	13	76.6 (10)
Fenthion sulfone	8.7	2.3	311	125	146	21	109	146	17	66.7 (11)
Fenthion sulfoxide	7.65	2.68	295	109	136	33	280	136	13	98.1 (14)
Fipronil	14.6	2.9	437	368	150	15	290	150	25	21.8 (11)
Flumethrin (adduct) *	19	2	527.1	267	66	10	239	66	18	59.3 (35)
Fluvalinate	18.3	1.81	503	208	50	10	181	50	26	73.4 (10)
Hexythiazox	17.84	1.15	353	228	120	20	168	120	10	67.4 (9)
Imazalil	15.18	1.71	297	159	120	20	201	120	15	56 (14)
Imidacloprid	2.46	1.96	256	209	80	10	175	80	10	75 (11)
Isoproturon	10.3	2.37	207	72	120	20	165	120	10	16.8 (12)
Lambda-cyhalothrin (adduct) *	18.1	2	467.1	225	66	10	141	66	46	26.1 (32)
Malathion	12.5	1.96	331	99	80	10	127	80	5	98.5 (4)
Methiocarb	11.86	1.93	226	121	80	5	169	80	10	66.6 (11)
Metolachlor	13.67	2.04	284	252	120	15	176	120	10	10 (14)
Molinate	12.64	1.98	188	126	80	20	55	80	10	61.7 (11)
Omethoate	1.69	2.67	214	125	80	5	183	80	20	72.3 (12)
Parathion-ethyl	14.25	1.91	292	236	88	4	264	88	8	45.5 (13)
Parathion-methyl	12.06	1.5	264	125	120	20	232	110	5	34.5 (13)
Prochloraz	15.18	1.91	376	308	80	10	266	80	10	14.3 (9)
Propanil	11.9	2.01	218	162	120	20	127	120	15	92.4 (11)
Propazine	11.61	2	230	146	120	15	188	120	20	93.3 (14)
Pyriproxyfen	17.63	1.33	322	227	120	10	185	120	10	36.1 (12)
Simazine	7.04	1.76	202	124	120	20	132	120	20	93.8 (12)
Tebuconazole	14.6	2.87	308	125	95	25	70	95	21	6.6 (11)
Terbumeton	11.88	2.89	226	170	95	17	114	95	25	13.8 (14)
Terbumeton-desethyl	7.68	3.76	198	142	90	13	96	95	25	31.7 (12)
Terbuthylazine	11.97	3.01	230	174	95	13	96	95	25	16.4 (13)

Target Pesticide	t_R[a] (min)	Δ t_R [b]	Precursor Ion	SRM_1[c]	Frag[d] (V)	CE[e] (V)	SMR_2[f]	Frag[d] (V)	CE[e] (V)	SMR_2/SRM_1 (%) (%RSD)[g]
Terbuthylazine-2-hydroxy	7.91	3.28	212	156	95	13	86	95	25	28 (13)
Terbuthylazine-desethyl	8	2.81	202	146	95	13	79	95	25	13.2 (14)
Terbutryn	14.1	1.2	242	186	120	20	71	120	15	4.6 (14)
Thiabendazole	5.65	3.5	202	175	95	25	131	95	25	29.1 (18)
Thiamethoxam	3.09	2.58	292	211	78	10	132	78	10	21.3 (11)
Tolclofos-methyl	16.9	1.71	301	125	115	12	269	120	15	73.8 (19)

[a] t_R = retention time.

[b] Δ t_R = delta retention time, that is the centered retention time window.

[c] SRM_1 = selected product ion for quantification.

[d] Frag = Fragmentor.

[e] CE = Collision energy.

[f] SRM_2 = selected product ion for qualification.

[g] (%RSD) = relative standard deviation of the ratio SRM_2/SRM_1, calculated from mean values obtained from the matrix-matched calibration curves.

[*] = Aducts of target pesticides [Ion mass + NH_4^+]; non aduct target pesticides [Ion mass + H^+].

Table S2. Linearity of the analyzed pesticides prepared in beeswax extracts (concentration range from 10 to 500 ng·mL^{-1})

Pesticide	Linearity	R^2
Acetamiprid	y=125.565766x-1521.515225	0.998
Acetochlor	y=59.497900x+449.598041	0.991
Acrinathrin	y=3.465399x+6.203709	0.985
Alachlor	y=58.000749x-61.513515	0.993
Atrazine	y=531.921389x-5045.341432	0.993
Atrazine-desethyl	y=915.791068x-4698.040046	0.992
Atrazine-desisopropyl	y=201.103106x-789.863966	0.996
Azinphos-ethyl	y=68.171963x-282.185698	0.994
Azinphos-methyl	y=99.363847x+2661.258400	0.982
Buprofezin	y=757.573287x-2319.466022	0.992
Carbendazim	y=2769.516959x-17643.919428	0.994
Carbofuran	y=360.626139x-5604.769645	0.991
Carbofuran-3-hydroxy	y=201.442033x-2025.115866	0.992
Chlorfenvinphos	y=251.279016x-557.757571	0.990
Chlorpyrifos	y=241.017110x-437.267515	0.999
Coumaphos	y=456.422716x-3652.642940	0.990
Diazinon	y=666.767489x-5397.868789	0.994
Dichlofenthion	y=129.518877x-546.122557	0.996
Dimethoate	y=258.943629x-2225.059759	0.997
Diuron	y=465.326589x-132.041099	0.997
DMA	y=54.043642x+120.197315	0.993
DMF	y=680.395007x+353.950419	0.991
DMPF	y=861.012871x-1420.370946	0.982
Ethion	y=515.455482x+1486.081054	0.996
Fenitrothion	y=75.742394x+1673.118734	0.990
Fenthion	y=248.999836x-1682.238779	0.998
Fenthion.-sulfone	y=257.613661x-2702.363565	0.994
Fenthion-sulfoxide	y=293.856332x-4021.420511	0.996
Fipronil	y=134.439938x+250.656849	0.993

Flumethrin	y=21.486599x-68.040037	0.990
Fluvalinate	y=55.458304x-73.578469	0.992
Hexythiazox	y=194.223900x-370.029344	0.999
Imazalil	y=228.309611x-1599.079563	0.995
Imidacloprid	y=159.408996x+303.874534	0.997
Isoproturon	y=569.114882x-1657.549534	0.999
Lambda-cyhalothrin	y=4.742533x+39.071205	0.991
Malathion	y=426.182479x-5179.054998	0.991
Methiocarb	y=651.994924x-3377.609824	0.991
Metolachlor	y=625.124419x-6188.802615	0.998
Molinate	y=302.189303x-2543.512889	0.996
Omethoate	y=89.017940x-87.232652	0.999
Parathion-ethyl	y=202.097518x-996.096906	0.999
Parathion-methyl	y=27.162488x-119.739178	0.998
Prochloraz	y=329.124012x-1119.806135	0.997
Propanil	y=164.350717-1858.151015	0.995
Propazine	y=390.626147x-1342.941695	0.990
Pyriproxifen	y=167.245295x-609.088181	0.996
Simazine	y=265.551096x-1136.997366	0.997
Tebuconazole	y=241.636930x+104.673270	0.992
Terbumeton	y=1362.237673x-13318.848243	0.994
Terbumeton-desethyl	y=2344.83212x-9139.646699	0.993
Terbuthylazine	y=1026.597205x+1283.072890	0.993
Terbuthylazine- desethyl	y=821.057687x-1773.181389	0.995
Terbuthylazine-2-hydroxy	y=1425.058309x+2228.022249	0.991
Terbutryn	y=1236.483447x-5115.668978	0.990
Thiabendazole	y=403.471437x-6737.165241	0.992
Thiamethoxam	y=17.546177x-196.548147	0.995
Tolclofos-methyl	y=135.552060-1434.087511	0.991

Table S3-S4. First and second determination of pesticides residues in foundation beeswax (F).

PESTICIDES	F1 (ng·g $^{-1}$)	F2 (ng·g $^{-1}$)	F3 (ng·g $^{-1}$)	F4 (ng·g $^{-1}$)	F5 (ng·g $^{-1}$)	F6 (ng·g $^{-1}$)	F7 (ng·g $^{-1}$)	F8 (ng·g $^{-1}$)	F9 (ng·g $^{-1}$)	F10 (ng·g $^{-1}$)	F11 (ng·g $^{-1}$)
Acrinathrin	105.2	521.5	2801.8	0	166.8	0	256	213.9	503.8	330	175
Azinphos methyl	0	0	0	0	0	0	0	0	0	89.3	0
Carbendazim	0	0	0	0	0	0	0	0	0	0	0
Chlorfenvinphos	503.4	1888.8	787.9	304.2	3688.1	626.9	1362.3	5322.4	821.8	701.5	1471.1
Chlorpyrifos	79	0	0	0	57.2	24.3	258.3	0	0	36	27.3
Coumaphos	11752.7	17981.4	2136.2	42.5	6790.4	3893	18202.2	9610.5	12898.7	13935.2	13356.1
Dichlofenthion	162	0	86.8	0	75.9	0	0	0	110.3	79.4	50
DMF	38.9	61.3	0	105	24.1	16.5	57.5	34.4	21.05	120.9	0
Ethion	0	0	0	0	0	0	0	0	0	0	0
Fenthion-Sulfoxide	0	0	0	0	0	44.4	0	0	0	0	0
Flumethrin	0	96.9	104.7	0	171.3	108.6	96	158.5	101.5	95.8	110.9
Fluvalinate	374.8	2312.6	695.8	1204.9	3752.5	678.3	718.9	968.9	614.8	501.2	456
Imazalil	0	0	0	0	0	0	0	0	0	0	0
Malathion	229.8	0	0	209.1	69.3	0	0	0	0	0	0

PESTICIDES	F'1 (ng·g $^{-1}$)	F'2 (ng·g $^{-1}$)	F'3 (ng·g $^{-1}$)	F'4 (ng·g $^{-1}$)	F'5 (ng·g $^{-1}$)	F'6 (ng·g $^{-1}$)	F'7 (ng·g $^{-1}$)	F'8 (ng·g $^{-1}$)	F'9 (ng·g $^{-1}$)	F'10 (ng·g $^{-1}$)	F'11 (ng·g $^{-1}$)
Acrinathrin	87.4	428	2368	0	139.4	0	219.4	197.7	166.2	286.1	159.6
Azinphos methyl	0	0	0	0	0	0	0	0	0	61	0
Carbendazim	0	0	0	0	0	0	0	0	0	0	0
Chlorfenvinphos	364.5	1530	610.3	228.8	2956	533.5	1194.7	5247.3	704	592.7	1352.1
Chlorpyrifos	73.5	0	0	0	56.7	14.5	263.3	0	0	618.5	25.8
Coumaphos	10213	16019.5	1919.9	7.4	5657.9	3494.5	16539.2	9156.5	10912.3	12194.5	11982.4
Dichlofenthion	25	0	43.9	0	24	0	0	57.8	82.2	27.9	23.7
DMF	26.5	49.5	0	95.1	19	18.3	43.9	39.7	10.8	116.8	0
Ethion	0	0	0	0	0	0	0	0	0	0	0
Fenthion-Sulfoxide	0	0	0	0	0	0	0	0	0	0	0
Flumethrin-aduct	0	56.3	104.8	0	168.9	120	0	165.7	111.4	105	115.3
Fluvalinate	374.9	2346.1	656	1027.9	3434.2	705.3	651.6	900.5	574.7	453.8	472.5
Imazalil	0	0	0	0	0	0	0	0	0	0	0
Malathion	149.6	0	0	151.1	65.7	0	0	0	0	0	0

Table S5-S6. First and second determination of pesticides residues in beeswax from recycled old combs (R).

PESTICIDES	R1 (ng·g $^{-1}$)	R2 (ng·g $^{-1}$)	R3 (ng·g $^{-1}$)	R4 (ng·g $^{-1}$)	R5 (ng·g $^{-1}$)	R6 (ng·g $^{-1}$)	R7 (ng·g $^{-1}$)	R8 (ng·g $^{-1}$)	R9 (ng·g $^{-1}$)	R10 (ng·g $^{-1}$)
Acrinathrin	44.2	0	447.4	380.9	75	80.8	784.6	36.3	660.6	79.7
Azinphos methyl	0	0	0	0	0	0	0	0	0	0
Carbendazim	0	0	0	113.6	0	0	0	0	0	0
Chlorfenvinphos	237.9	664.9	840.1	440.7	588	607.1	398.4	349.5	361	413.3
Chlorpyrifos	0	0	0	0	443.7	1905	0	0	100.9	0
Coumaphos	440	12208	10924	15829.9	3289.7	3192.9	25676.8	22797.3	13104.9	17934.8
Dichlofenthion	0	1845.2	93	0	0	0	0	0	0	87.2
DMF	0	16	3201.3	143.6	0	0	1525.6	6947.7	105.1	3389.4
Ethion	0	0	0	0	0	0	0	0	0	0
Fenthion-Sulfoxide	0	50.5	0	0	0	0	0	0	0	0
Flumethrin-aduct	0	103.9	94.8	101.4	106	100.6	99.8	112.3	0	112.6
Fluvalinate	436.3	516.3	389.3	469.4	501.9	451.3	429.9	508	435.6	804.1
Imazalil	0	48.3	0	0	0	0	0	0	0	0
Malathion	0	0	0	0	0	0	0	0	0	0

PESTICIDES	R'1 (ng·g $^{-1}$)	R'2 (ng·g $^{-1}$)	R'3 (ng·g $^{-1}$)	R'4 (ng·g $^{-1}$)	R'5 (ng·g $^{-1}$)	R'6 (ng·g $^{-1}$)	R'7 (ng·g $^{-1}$)	R'8 (ng·g $^{-1}$)	R'9 (ng·g $^{-1}$)	R'10 (ng·g $^{-1}$)
Acrinathrin	44.1	0	459.8	406.3	98.5	85.8	819.4	25	420.1	68.4
Azinphos methyl	0	0	0	0	0	0	0	0	0	0
Carbendazim	0	0	0	113.5	0	0	0	0	0	0
Chlorfenvinphos	200.4	616.1	753.1	397.5	612.1	146.1	315.4	224	254.9	156.1
Chlorpyrifos	0	0	0	0	68.3	51.5	0	0	0	13.7
Coumaphos	422.7	12208.3	11265.9	14912.8	3668.7	706	28039.2	16423	10607.4	4970.1
Dichlofenthion	0	80.7	25.5	0	0	0	0	0	0	40.2
DMF	0	15.5	3337.4	138.8	0	0	1598.6	6821.4	91.7	2542.6
Ethion	0	0	0	0	0	0	0	0	0	0
Fenthion-Sulfoxide	0	12.7	0	0	0	0	0	0	0	0
Flumethrin-aduct	49	136.2	115.7	110.8	133	138.5	110.6	0	0	120
Fluvalinate	444.9	602.1	448.4	583.4	633.3	371.7	436.3	71.2	232.9	688.2
Imazalil	0	53.4	0	0	0	0	0	0	0	0
Malathion	0	0	0	0	0	0	0	0	0	0

Table S7-S8. First and second determination of pesticides residues in beeswax cappings (C).

PESTICIDES	C1 (ng·g $^{-1}$)	C2 (ng·g $^{-1}$)	C3 (ng·g $^{-1}$)	C4 (ng·g $^{-1}$)	C5 (ng·g $^{-1}$)	C6 (ng·g $^{-1}$)	C7 (ng·g $^{-1}$)	C8 (ng·g $^{-1}$)	C9 (ng·g $^{-1}$)	C10 (ng·g $^{-1}$)	C11 (ng·g $^{-1}$)	C12 (ng·g $^{-1}$)
Acrinathrin	0	692	777	0	0	0	2630	2513	25	622	0	287
Chlorfenvinphos	0	0	5	0	0	0	0	0	0	39	32	5
Chlorpyrifos	0	6	5	0	0	0	0	0	0	16	239	0
Coumaphos	120	448	576	240	85	357	6800	1069	599	1430	4479	737
DMF	171	161	193	115	0	252	317	398	269	1049	61	375
Flumethrin	0	0	0	0	48	0	0	0	0	0	0	0
Fluvalinate	33	21	24	0	0	0	0	3110	696	200	169	40
Hexythiazox	0	0	0	0	0	0	0	0	0	0	60	0
Pyriproxyfen	0	0	0	0	0	0	0	0	0	0	13	0

PESTICIDES	C'1 (ng·g $^{-1}$)	C'2 (ng·g $^{-1}$)	C'3 (ng·g $^{-1}$)	C'4 (ng·g $^{-1}$)	C'5 (ng·g $^{-1}$)	C'6 (ng·g $^{-1}$)	C'7 (ng·g $^{-1}$)	C'8 (ng·g $^{-1}$)	C'9 (ng·g $^{-1}$)	C'10 (ng·g $^{-1}$)	C'11 (ng·g $^{-1}$)	C'12 (ng·g $^{-1}$)
Acrinathrin	0	678	793	0	0	0	2560	2467	25	648	0	323
Chlorfenvinphos	0	0	5	0	0	0	0	0	0	21	68	5
Chlorpyrifos	0	4	5	0	0	0	0	0	0	4	281	0
Coumaphos	90	462	594	200	95	333	6960	1131	671	1500	4321	783
DMF	159	139	207	85	0	228	373	452	291	1081	89	405
Flumethrin	0	0	0	0	22	0	0	0	0	0	0	0
Fluvalinate	37	29	26	0	0	0	0	3020	714	170	131	70
Hexythiazox	0	0	0	0	0	0	0	0	0	0	30	0
Pyriproxyfen	0	0	0	0	0	0	0	0	0	0	37	0

Table S9-S10. First and second determination of pesticides residues in white combs beeswax (B).

PESTICIDES	B1 (ng·g $^{-1}$)	B2 (ng·g $^{-1}$)	PESTICIDES	B1' (ng·g $^{-1}$)	B2' (ng·g $^{-1}$)
Acrinathrin	0	0	**Acrinathrin**	0	0
Azinphos methyl	0	0	**Azinphos methyl**	0	0
Carbendazim	0	0	**Carbendazim**	0	0
Chlorfenvinphos	56.6	47.7	**Chlorfenvinphos**	25.4	0
Chlorpyrifos	83.4	0	**Chlorpyrifos**	27	0
Coumaphos	728	338.5	**Coumaphos**	860.4	274.4
Dichlofenthion	0	0	**Dichlofenthion**	0	0
DMF	48.1	16.9	**DMF**	56.4	15.8
Ethion	0	0	**Ethion**	0	42.5
Fenthion-Sulfoxide	0	0	**Fenthion-Sulfoxide**	0	0
Flumethrin	0	0	**Flumethrin**	0	0
Fluvalinate	38.5	0	**Fluvalinate**	60.8	0
Imazalil	0	0	**Imazalil**	0	0
Malathion	0	0	**Malathion**	0	0

Table S11. Mean concentration of pesticide residues found in beewax from recycled old combs (R)

Pesticides	R1 (ng·g $^{-1}$)	R2 (ng·g $^{-1}$)	R3 (ng·g $^{-1}$)	R4 (ng·g $^{-1}$)	R5 (ng·g $^{-1}$)	R6 (ng·g $^{-1}$)	R7 (ng·g $^{-1}$)	R8 (ng·g $^{-1}$)	R9 (ng·g $^{-1}$)	R10 (ng·g $^{-1}$)
Acrinathrin	44.1	0.0	453.6	393.6	86.7	83.3	802	30.7	540.4	74.0
Carbendazim	0.0	0.0	0.0	113.6	0.0	0.0	0.0	0.0	0.0	0.0
Chlorfenvinphos	219.1	640.5	796.6	419.1	600.05	376.6	356.9	286.8	307.9	284.7
Chlorpyrifos	0.0	0.0	0.0	0.0	256	978.3	0.0	0.0	50.4	6.8
Coumaphos	431.4	12208.2	11095.0	15371.3	3479.2	1949.4	26858.0	19610.1	11856.1	11452.5
Dichlofenthion	0.0	963.0	59.3	0.0	0.0	0.0	0.0	0.0	0.0	63.7
DMF	0.0	15.8	3269.3	141.2	0.0	0.0	1562.1	6884.6	98.4	2966.0
Fenthion-Sulfoxide	0.0	31.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Flumethrin	24.5	120.1	105.2	106.1	119.5	119.5	105.175	56.1	0.0	116.3
Fluvalinate	440.6	559.2	418.8	526.4	567.6	411.5	433.05	289.6	334.2	746.2
Imazalil	0.0	50.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table S12. Mean concentration of pesticide residues found in foundation beeswax (F).

Pesticides	F1 (ng·g $^{-1}$)	F2 (ng·g $^{-1}$)	F3 (ng·g $^{-1}$)	F4 (ng·g $^{-1}$)	F5 (ng·g $^{-1}$)	F6 (ng·g $^{-1}$)	F7 (ng·g $^{-1}$)	F8 (ng·g $^{-1}$)	F9 (ng·g $^{-1}$)	F10 (ng·g $^{-1}$)	F11 (ng·g $^{-1}$)
Acrinathrin	96.3	474.8	2584.9	0.0	153.1	0.0	237.7	205.8	335.0	308.0	167.3
Azinphos-methyl	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	75.1	0.0
Chlorfenvinphos	433.9	1709.4	699.1	266.5	3322	580.2	1278.5	5284.8	762.9	647.1	1411.6
Chlorpyrifos	76.2	0.0	0.0	0.0	56.9	19.4	260.8	0.0	0.0	327.2	26.5
Coumaphos	10982.9	17000.4	2028	25	6224.1	3693.8	17370.7	9383.5	11905.5	13064.8	12669.2
Dichlofenthion	93.5	0.0	65.3	0.0	50	0.0	0.0	28.9	96.2	53.6	36.8
DMF	32.7	55.4	0.0	100	21.6	17.4	50.7	37.0	15.9	118.9	0.0
Fenthion-Sulfoxide	0.0	0.0	0.0	0.0	0.0	22.2	0.0	0.0	0.0	0.0	0.0
Flumethrin	0.0	76.6	104.7	0.0	170.1	114.3	48	162.1	106.4	100.4	113.1
Fluvalinate	374.9	2329.3	675.9	1116.4	3593.3	691.8	685.3	934.7	594.7	477.5	464.2
Malathion	189.7	0.0	0.0	180.1	67.5	0.0	0.0	0.0	0.0	0.0	0.0

Table S13. Mean concentration of pesticide residues found in beeswax cappins (C).

PESTICIDES	C1 (ng·g $^{-1}$)	C2 (ng·g $^{-1}$)	C3 (ng·g $^{-1}$)	C4 (ng·g $^{-1}$)	C5 (ng·g $^{-1}$)	C6 (ng·g $^{-1}$)	C7 (ng·g $^{-1}$)	C8 (ng·g $^{-1}$)	C9 (ng·g $^{-1}$)	C10 (ng·g $^{-1}$)	C11 (ng·g $^{-1}$)	C12 (ng·g $^{-1}$)
Acrinathrin	0	685	785	0	0	0	2595	2490	25	635	0	305
Chlorfenvinphos	0	0	5	0	0	0	0	0	0	30	50	5
Chlorpyrifos	0	5	5	0	0	0	0	0	0	10	260	0
Coumaphos	105	455	585	220	90	345	6880	1100	635	1465	4400	760
DMF	165	150	200	100	0	240	345	425	280	1065	75	390
Flumethrin	0	0	0	0	35	0	0	0	0	0	0	0
Fluvalinate	35	25	25	0	0	0	0	3065	705	185	150	55
Hexythiazox	0	0	0	0	0	0	0	0	0	0	45	0
Pyriproxyfen	0	0	0	0	0	0	0	0	0	0	25	0

Table S14. Hazard quotients (HQ_{wax})[1] values for the beeswax samples analyzed.

B1	**B2**										
823	23										
C1	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**
13	4127	4724	13	705	22	15616	15063	265	3997	3863	1847
F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	
2327	5861	17649	580	6771	2962	7241	6319	4949	9411	4645	
R1	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**R9**	**R10**		
875	3235	5635	5374	6841	16704	8332	2524	4587	3643		

[1](HQ = pesticide concentration in ppb ÷ pesticide topical LD_{50} as µg/bee).

Contact-LD_{50} (µg/bee)[2]						
Hexythiazox	Imazalil	Pyriproxifen	Azinphos-methyl	Ethion	Flumethrin	Carbendazim
200	39	100	0.42	11	0.05	50
Acrinathrin	Chlorpyrifos	Coumaphos	Chlorfenvinphos	Fluvalinate	Malathion	DMF
0.17	0.072	20	4.1	8.7	0.47	50

[2]LD_{50} values were from Sanchez-Bayo and Goka, (2014) and Hertfordshire, U. (2017). PPDB - Pesticides Properties DataBase.

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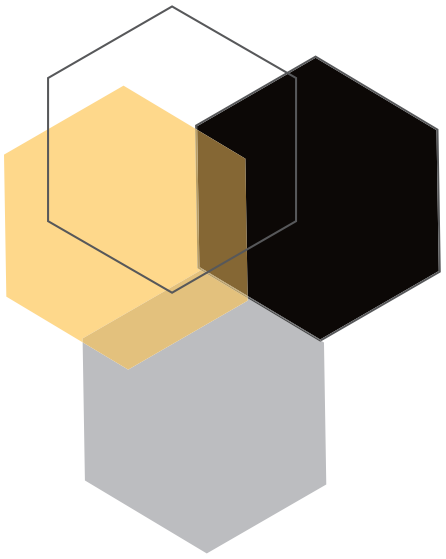

BEESWAX CLEANING BY SOLVENT EXTRACTION OF PESTICIDES

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Method Article

Beeswax cleaning by solvent extraction of pesticides

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A B S T R A C T

We set out to test if the methodology used to clean sheep wool wax (Lanolin) from pesticides could be used to clean beeswax as well. We first made an aggregate sample of brood comb wax from three different US beekeepers. Sub-samples of these aggregate wax samples were analyzed for pesticide contamination. The remaining wax, was then dissolved into hexane solution and run through four *N,N*-Dimethylformamide (DMF) washes. During these extractions, the pesticides partitioned into the DMF, and so were removed from the beeswax. Following the solvent extractions, the beeswax was tested again for pesticides. An average of 95% of the pesticide contamination was removed by the chemical wash procedure.

- Beeswax is the beekeeping matrix with the highest pesticide content.
- This study developed methodology for solvent-based removal of pesticides from beeswax (>95%).
- Of 24 pesticides detected in beeswax samples before to the solvent extraction, only 3 pesticides were detected after the extraction with DMF.

A R T I C L E I N F O
Method name: Beeswax cleaning by solvent extraction
Keywords: Beeswax, Solvent extraction, Pesticides removal, Hexane, *N, N*-Dimethylformamide (DMF)
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Specifications Table

Subject Area:	*Chemistry*
More specific subject area:	*Analytical chemistry*
Method name:	*Beeswax cleaning by solvent extraction*
Name and reference of original method:	*Jones, F. (1997). The removal of pesticide residues from wool wax by solvent extraction. J. Am. Oil Chem. Soc. 74, 1241-1245.*
Resource availability:	*Basic laboratory equipment like a fume hood, spatules, funnels, paper filters, pippetes, etc ...*

Method details

Material

- Beeswax from old brood combs.
- Hexane and *N*, *N*-Dimethylformamide (DMF).
- Beakers 50–500 mL.
- Analytical scale.
- Separating funnel (1 L).
- Flasks 250 mL.

Note: Availability of Standard laboratory equipment is assumed.

Purification of the beeswax samples

Prior to solvent removal of the pesticides from beeswax, three homogenous pools of beeswax from three different combs were prepared. When the brood comb beeswax source is old, as was the case for the combs collected from three different commercial beekeepers in this study, it is contaminated with many impurities such as bee silk, produced by pupating bees, propolis, pollen, and larvae excrement. These impurities are difficult to remove, and so, to obtain "clean beeswax" we used the follow procedure (Fig. 1). While beekeepers typically boil old combs (called slumgum), this process removes little wax, as the wax is absorbed by the bee silk cocoons that line the brood comb cells. Beekeepers eager to remove this wax, often use steam and pressure to separate impurities from the wax [1]. The separation of the beeswax from comb impurities using steam and pressure were not practical in our case, and so, we used the following chemical solvent method (Fig. 1):

1 Wax contained in the combs was obtained by dissolving the brood comb with the hexane solvent.
2 After the wax was dissolved in hexane, the solution was filtered to eliminate impurities.

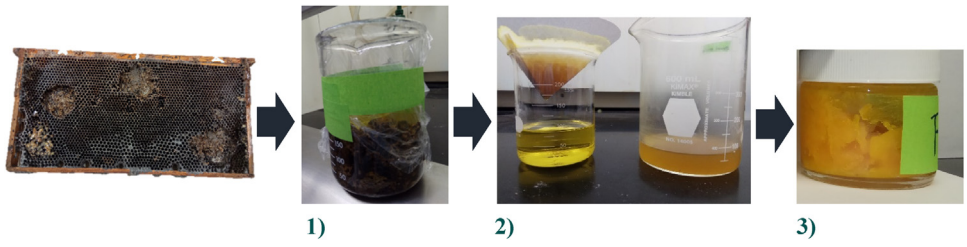

Fig. 1. Process to obtain the three batches of purified beeswax that were subjected to the solvent extraction of the pesticides. Wax was removed from old dark brood comb and dissolved into a hexane solution (1), which was then filtered (2), and had the hexane removed by evaporation under a stream of air. The resulting "pure" wax (3) was used to test for pesticides, and the pesticide wash study.

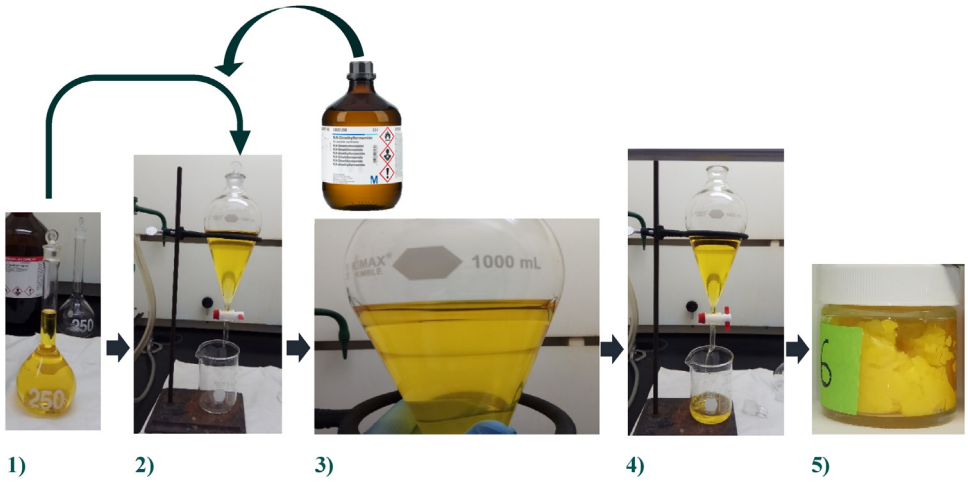

Fig. 2. Solvent extraction of the pesticides from beeswax. The wax-hexane solution (1) is placed into a separating funnel which also contains DMF (2). After vigorous agitation, the solution is let rest to separate the two phases (3), the DMF is then drained (4). The hexane is removed from the resulting solution by evaporation under an air steam resulting in a semi-solid beeswax (5).

3 Hexane was evaporated to obtain the three batches of beeswax. By weight, between a 15–19% of the old brood comb was recoverable beeswax. Of the 150 g of comb was collected per operation, 23 g of "pure" beeswax was recovered and used for further study.

Solvent extraction of pesticides from beeswax samples

Contaminant removal methods have successfully been used with lanolin (wool wax) [2], and for the current project, these methods were adapted and tested for their ability to remove pesticide contaminants of beeswax. The following procedure was repeated for each batch of beeswax removed from the three brood combs:

1) Prepare a solution of "pure" wax (see procedure above) in hexane 6% (250 mL, wt/vol).
2) Mix and agitate the 250 ml of wax solution added to 250 mL of DMF (extractant solvent) in a separating funnel (1 L).
3) Let the DMF and the hexane solution separate into two phases by letting the solution rest until the two physical layers are observed (hexane-wax solution, upper phase; DMF, lower phase) (Fig. 2).
4) Drain the DMF phase. Repeat the extraction process four times.
5) Keep the hexane-wax phase and evaporate hexane by evaporation under an air steam.

Method validation

Beeswax samples obtained before, and after the solvent extractions were sent to the USDA-AMS National Science Laboratory in Gastonia NC for multi-pesticide residue analysis to evaluate the effectiveness of the cleaning method. Wax was analyzed for 199 pesticides and associated degradates as described in Mullin et al. [3]. As can be observed in Tables 1–3, the solvent cleaning tested here is an effective method to decontaminate beeswax from pesticides. Pesticide incidence was reduced significantly in the three beeswax samples. Of 24 different pesticides detected before to the solvent extraction, only 3 pesticides were found after the decontamination procedure proposed was applied.

Table 1
Summary of pesticide detections in wax comb 1 before and after the solvent extraction of pesticides.

WAX COMB 1				
Pesticides and degradates	LOD ($ng \cdot g^{-1}$)	Detections before solvent extraction ($ng \cdot g^{-1}$)	Detections after solvent extraction ($ng \cdot g^{-1}$)	Removal (%)
2,4 Dimethylphenyl formamide (DMPF)	1.5	111	0	100
Azoxystrobin	1	2	0	100
Chlorthal-dimethyl (DCPA)	2	Trace	0	–
Coumaphos	4	1420	Trace	–
Coumaphos oxon	0.5	69	0	100
DEET	3	23	7	70
Diphenylamine	2	9	0	100
Fenpyroximate	3	264	0	100
Fluvalinate	25	1180	0	100
Iprodione	100	137	0	100
Metolachlor	25	Trace	0	–
Thymol	2	2750	0	100
Trifloxystrobin	1	1	0	100
			Removal Average	97

Table 2
Summary of pesticide detections in wax comb 2 before and after the solvent extraction of pesticides.

WAX COMB 2				
Pesticides and degradates	LOD ($ng \cdot g^{-1}$)	Detections before solvent extraction ($ng \cdot g^{-1}$)	Detections after solvent extraction ($ng \cdot g^{-1}$)	Removal (%)
2,4 Dimethylphenyl formamide (DMPF)	1.5	41	0	100
Azoxystrobin	1	8	0	100
Boscalid	5	25	0	100
Carbendazim	2	Trace	0	–
Chlorpyrifos	5	Trace	0	–
Chlorthal-dimethyl (DCPA)	2	3	0	100
Coumaphos	4	5270	Trace	–
Coumaphos oxon	0.5	157	0	100
Cyprodinil	2	121	0	100
DEET	3	5	5	0
Diphenylamine	2	7	0	100
Fenpyroximate	3	1330	0	100
Fluvalinate	25	2900	0	100
Iprodione	100	3330	0	100
Methoxyfenozide	1	4	0	100
Myclobutanil	7	41	0	100
Propiconazole	2	7	6	14
Pyraclostrobin	2	75	0	100
Pyridaben	2	9	0	100
Pyrimethanil	5	41	0	100
Thymol	2	12500	0	100
Trifloxystrobin	1	6	0	100
			Removal average	90

Table 3
Summary of pesticide detections in wax comb 3 before and after the solvent extraction of pesticides.

WAX COMB 3				
Pesticides and degradates	LOD ($ng \cdot g^{-1}$)	Detections before solvent extraction ($ng \cdot g^{-1}$)	Detections after solvent extraction ($ng \cdot g^{-1}$)	Removal (%)
2,4 Dimethylphenyl formamide (DMPF)	1.5	300	0	100
Boscalid	5	9	0	100
Coumaphos	4	1160	0	100
Coumaphos oxon	0.5	24	0	100
Cyprodinil	2	99	0	100
Fenpyroximate	3	610	0	100
Fluopyram	1	3	0	100
Fluvalinate	25	562	0	100
Iprodione	100	419	0	100
Methoxyfenozide	1	16	0	100
Myclobutanil	7	30	0	100
Penthiopyrad	1	57	0	100
Propiconazole	2	39	0	100
Pyraclostrobin	2	5	0	100
Thymol	2	766	0	100
Trifloxystrobin	1	6	0	100
			Removal average	100

Additional Information

Given the use of pesticides in beekeeping and plant protection, hive products exposure to pesticides is unavoidable. Acaricides, fungicides and insecticides have been found in beeswax from Europe [4,5] and America [6]. Wax is the most contaminated hive matrix, and its nature based on lipids and hydrocarbons, is in part, responsible of its high pesticide content. The most common wax contaminants are lipophilic, and do not degrade during the wax recycling. Moreover, some of the pesticides found in beeswax have not been used in years, suggesting its bioaccumulation in this matrix [7]. This makes it difficult for beekeepers to purchase uncontaminated foundation, and likely explains the persistence of contaminants in colonies even after comb replacement. Beeswax is also used by the cosmetic and pharmaceutical industries in numerous products like lipsticks, facial creams, pills coatings, chewing gum, and candles. Given that many of the pesticides detected in wax could pose endocrine disrupting effects, the development of methods to decontaminate wax will have a positive impact on human health as well. This study aims to improve managed honey bee colony health by developing methodology to decontaminate recycled wax and improve future work on wax decontamination.

Up to now, only few methods have been proposed to clean beeswax from pesticide residues. In this sense, methods developed propose the use of solid sorbents, like the patent US6586610B2 [8]. Serra Bonvehi and Jose Orantes-Bermejo [9], proved that activated charcoal is able to remove >95% of two organophosphorus —coumaphos and chlorfenvinphos—, widely detected in beeswax worldwide. However, this sorbent only removed fluvalinate pyrethroid a 35%. Our study demonstrated that organic solvent clean-up pose a wide scope, being able to eliminate pesticides belonging to many different families, and provides useful data of pesticide decontamination of beeswax by solvent extraction approach. Organophosphorus, but also carboxamide, pyrethroids and other pesticide families were removed from wax >95%. Pesticide content in the samples were reduced from $\mu g \cdot g^{-1}$ levels to less than 10 $ng \cdot g^{-1}$ in all cases. Although beeswax texture is softer after solvent extractions, reconstituted into a useable form for cosmetic and pharmaceutical industries.

The procedure here proposed is a preliminary study on the possibilities of solvent extraction, and could be an effective alternative to remove pesticides from beeswax. As a pilot study, this method is

feasible only on small scale because high amount of solvents are used during the extractions. A continuous solvent-solvent extractor design is needed to apply this methodology on a larger scale of wax production in order to minimize environmental harm and process cost. Hexane is easily evaporated, and its recovery during industrial processes would be of utmost importance to eliminate burdens to the environment. This would be the next mandatory step to fully implement this methodology within the beeswax sector, as an efficient, green and cheap method to get a proper clean-up of the beeswax from pesticide residues.

Acknowledgements

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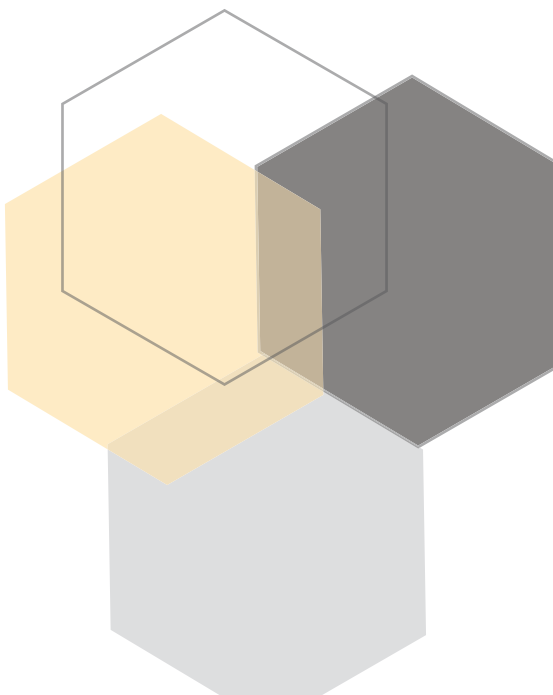
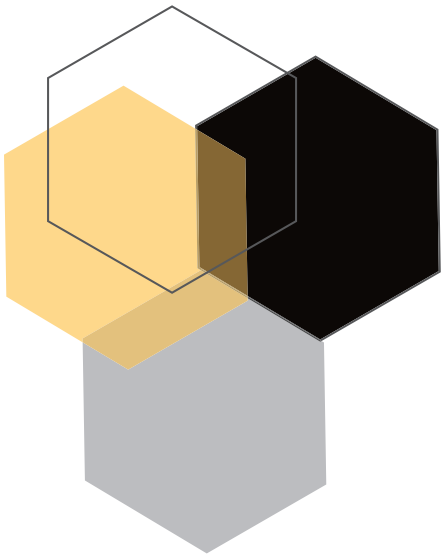


CHAPTER 6: SUMMARY

01

DEVELOPMENT OF THE ANALYTICAL METHODOLOGY



1.1 Extraction procedures

The procedures for the extraction of the pesticides from honey bees, pollen and beeswax were based on modified versions of the QuEChERS initially proposed by **Anastassiades et al. (2003)**, and adapted for each matrix (Figure 1). The varied nature of the hive products, like the hydrocarbons and lipids of the beeswax, proteins and lipids of the honey bees and a wide range of fat soluble carotenoids present in pollen, evidenced the versatility of the QuEChERS platform (**Niell et al., 2013; Niell et al., 2014; Barganska et al., 2014; Lozano et al., 2019**). Furthermore, QuEChERS is an economical and short procedure, and meets important components of green analytical chemistry due to the small amounts of solvent needed

1.1.1 Honey bees, pollen and beeswax

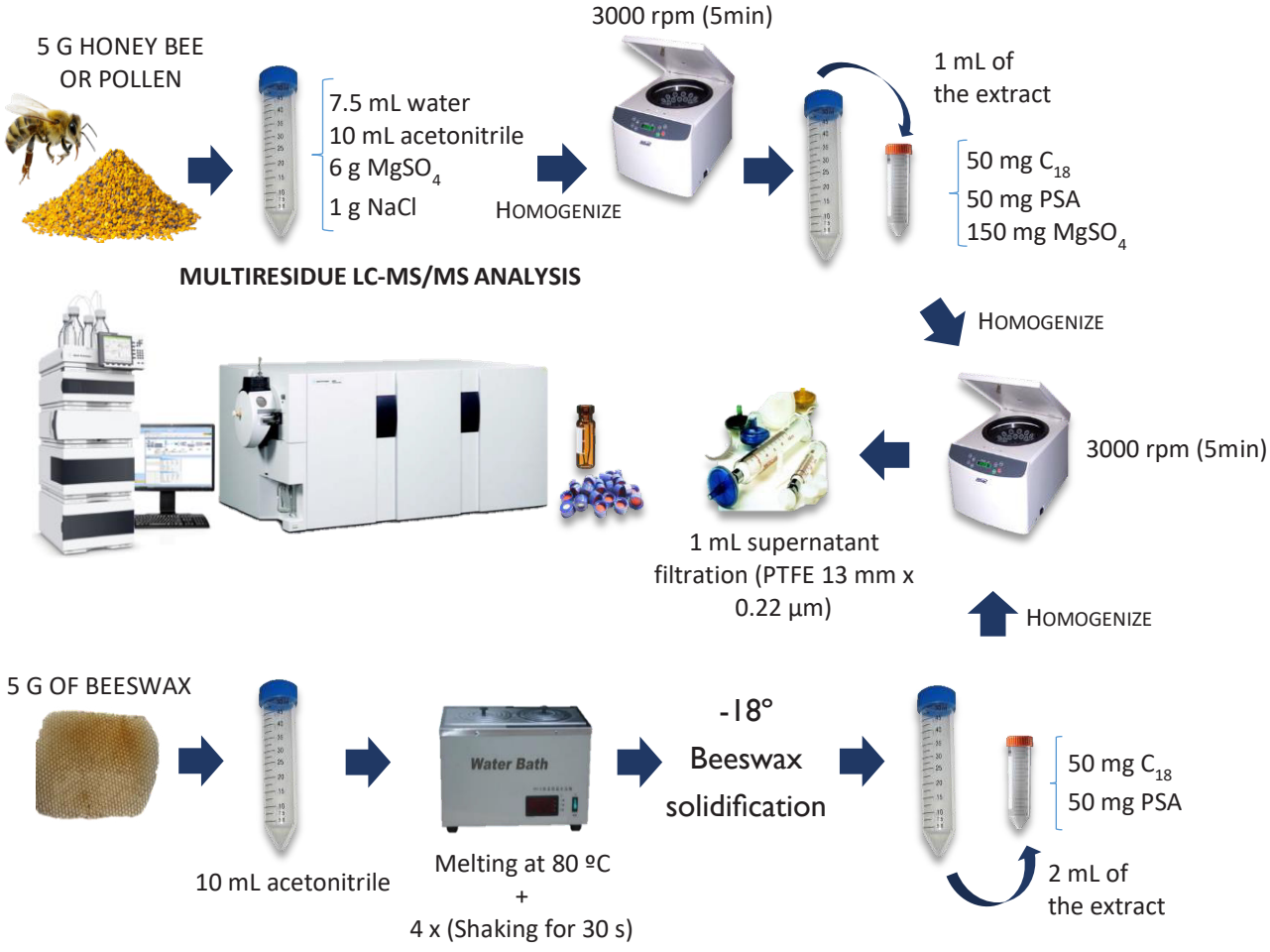


Figure 1. Procedures used for the extraction of the pesticides from honey bees, pollen and beeswax.

1.2 Determination method

The determination of the selected pesticides (Table 1) was performed by HPLC-QqQ-MS/MS. The chromatographic instrument was an HP1200 series LC equipped with an automatic injector, a degasser, a quaternary pump and a column oven-combined with an Agilent 6410 triple quadrupole mass spectrometer with an electrospray ionization (ESI) interface (Agilent Technologies, Waldbronn, Germany). Data were processed using a MassHunter Workstation Software for qualitative and quantitative analysis (A GL Sciences, Tokyo, Japan).

The chromatographic column was a Luna C18 (15.0 cm × 0.21 cm) with a 3 µm particle size (Phenomenex, Torrance, USA). The column temperature was kept at 30 °C and the volume injected was 5 µL. A binary mobile phase at flow rate of 0.3 mL·min $^{-1}$ with a gradient elution was used. Solvent A was Milli-Q water with

10 mM ammonium formate and solvent B was methanol with 10 mM ammonium formate. The linear gradient was as follows: 0 min (50 % B), 10 min (83 % B), 12 min (83 % B), 12.5 min (98 % B), and 15.5 min (98 % B). Then, the mobile phase returns to the initial conditions with an equilibration time of 12 min.

Ionization and fragmentation settings were optimized by direct injection of pesticide standard solutions. MS/MS was performed in the selected reaction monitoring (SRM) mode using ESI in positive mode. For each compound, two characteristic product ions of the protonated molecule $[M+H]^+$ were monitored, the first and most abundant was used for quantification, while the second was used as a qualifier. Collision energy and cone voltage were optimized for each pesticide. Nitrogen was used as collision, nebulising and desolvation gas. The ESI conditions were: capillary voltage 4000 V, nebulizer 15 psi, source temperature 300 °C and gas flow 10 $L \cdot min^{-1}$. In order to maximize sensitivity, dynamic multiple reaction monitoring (MRM) was used, with MS1 and MS2 at unit resolution and cell acceleration voltage of 7 eV for all the compounds. The Dynamic MRM conditions used for the LC - MS/MS determination of the pesticides are listed in Table S1 from Article 1, 3, 4, 5.

Table 1. List of the selected pesticides included in the MRM of the present thesis.

Pesticide	Class	Use	LD_{50} µg · bee $^{-1}$ (*Apis mellifera*)	
			Contact	Oral
Acetamiprid	Neonicotinoid	Insecticide	7.9	14
Acetochlor	Chloroacetanilide	Herbicide	> 200	> 100
Acrinathrin	Synthetic pyrethroid	Insecticide/Acaricide	0.17	0.12
Alachlor	Chloroacetanilide	Herbicide	16	
Atrazine Atrazine-desethyl [M] Atrazine-desisopropyl [M]	Triazine	Herbicide	> 100	> 100
Azinphos-ethyl	Organophosphate	Insecticide	> 1.39	
Azinphos-methyl	Organophosphate	Insecticide	0.42	
Bifenthrin	Pyrethroid	Insecticide	0.015	0.2
Buprofezin	Unclassified	Insecticide	> 200	> 163.5
Carbendazim	Benzimidazole	Fungicide	> 50	> 756
Carbofuran Carbofuran-3-hydroxy [M]	Carbamate	Insecticide/Nematicide/Acaricide	0.036	0.05
Chlorfenvinphos	Organophosphate	Acaricide/Insecticide	4.1	0.55
Chlorpyrifos	Organophosphate	Insecticide	0.072	0.24
Clothianidin	Neonicotinoid	Insecticide	0.039	0.0035
Coumaphos	Organophosphate	Acaricide	20	4.6
Diazinon	Organophosphate	Insecticide/Acaricide	0.13	0.09
Diclofenthion	Organophosphate	Insecticide		
Dimethoate	Organophosphate	Insecticide	0.12	0.17
Diuron	Phenylamide	Herbicide	> 101.7	> 86.75
Amitraz [a] DMA DMF DMPF	Amidine	Acaricide/Insecticide	50	

Table 1. Cont

Pesticide	Class	Use	LD_{50} µg · bee $^{-1}$ (*Apis mellifera*)	
			Contact	Oral
Ethion	Organophosphate	Insecticide/Acaricide	11	
Etofenprox	Pyrethroid	Insecticide	0.015	0.024
Fenitrothion	Organophosphate	Insecticide	0.16	0.20
Fenthion Fenthion-sulfone [M] Fenthion-sulfoxide [M]	Organophosphate	Insecticide	0.22	
Fipronil	Phenylpyrazole	Insecticide	0.0059	0.0047
Flumethrin	Pyrethroid	Acaricide/Insecticide	0.05	
Fluvalinate	Synthetic pyrethroid	Acaricide/Insecticide	8.7	45
Hexythiazox	Carboxamide	Acaricide	> 200	> 112
Imazalil	Imidazole	Fungicide	39	37
Imidacloprid	Neonicotinoid	Insecticide	0.081	0.0037
Isoproturon	Urea	Herbicide	200	195
Lambda-cyhalothrin	Synthetic pyrethroid	Insecticide	0.038	0.91
Malathion	Organophosphate	Insecticide/Acaricide	0.16	0.40
Methiocarb	Carbamate	Insecticide/ Molluscicide	0.23	0.08
Metolachlor	Chloroacetamide	Herbicide	110	110
Molinate	Thiocarbamate	Herbicide		> 11
Omethoate	Organophosphate	Insecticide		0.05
Parathion-ethyl	Organophosphate	Insecticide/Acaricide		> 0.21
Parathion-methyl	Organophosphate	Insecticide/Acaricide	2.7	750
Prochloraz	Imidazole	Fungicide	141.3	101
Propanil	Anilide	Herbicide	> 100	> 94.3
Propazine	Triazine	Herbicide	16	
Pyriproxyfen	Unclassified	Insecticide	74	> 100
Simazine	Triazine	Herbicide	97	
Spinosad [b] Spynosyn A Spynosyn D	Micro-organism derived	Insecticide	0.003	0.057
Tebuconazole	Triazole	Fungicide	> 200	> 83.05
Terbumeton Terbumeton-desethyl [M]	Triazine	Herbicide		
Terbuthylazine Terbuthylazine-desethyl [M] Terbuthylazine-2 hydroxy [M]	Triazine	Herbicide	> 32	> 22.6
Terbutryn	Triazine	Herbicide	> 225	
Thiabendazole	Benzimidazole	Fungicide	> 34	> 4
Thiamethoxam	Neonicotinoid	Insecticide	0.024	0.005
Tolclofos-methyl	Chlorophenyl	Fungicide	> 100	

[M] Metabolite

[a] Amitraz is detected through its degradation products: DMA, DMF and DMPF.

[b] Spinosad is detected through its components spinosyn A and D.

LD_{50} were from **Sanchez-Bayo and Goka (2014)** and University of Hertfordshire Pesticide Properties Database (**Hertfordshire, 2019**).

1.3 Validation of the analytical methodology

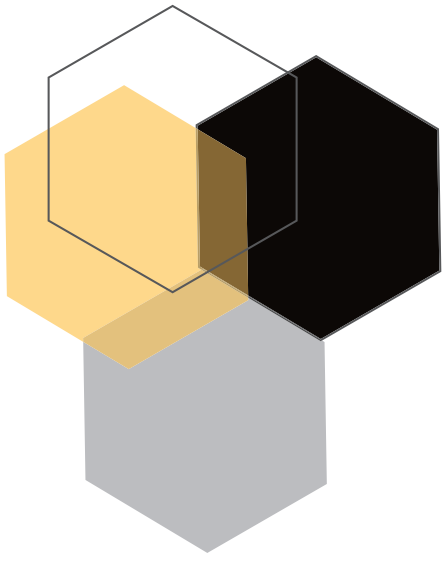
The multiresidue methods developed in the present thesis were evaluated regarding sensitivity, accuracy, precision and robustness according to European Union Guidelines on analytical quality control and validation procedures for pesticides (**SANCO/12571/2013; SANTE/11945/2015**). The validation of the method was carried out for each matrix and pesticide included in the analyses. The validation data can be found in Table 1 from Article 3 and 5, and Table S1 from Article 2.

In honey bees, recoveries ranged from 70 to 96 % and relative standard deviations (RSDs) were ≤ 20 % for most analytes, except for atrazine-desethyl, carbofuran, fenthion-sulfoxide, omethoate, parathion ethyl, and propazine. The limits of detection (LODs) were from 0.3 to 3 $ng \cdot g^{-1}$, whereas limits of quantification (LOQs) ranged from 1 to 10 $ng \cdot g^{-1}$. Matrix effects were in the range of - 60 to 20 % and were mostly suppressive, with the exception of carbofuran 3-hydroxy, carbofuran, fenthion sulfoxide and fenthion sulfone.

In pollen matrix, the average recoveries values at 10, 50 and 100 $ng \cdot g^{-1}$ spiked levels were 90, 86 and 91 %, respectively. Recovery values ranged from 70 to 116 %, and only 7 % of the compounds produced recoveries between 55 and 69 %. Precision, expressed as RSDs, was < 20 % in most of pesticides analyzed. LODs were lower than 2 $ng \cdot g^{-1}$ and LOQs were below 5 $ng \cdot g^{-1}$ for all pesticides. Matrix effects were mostly suppressive and ranged from - 54 to 50 %.

In beeswax, the recovery values ranged from 50 to 120 % with the exception of terbuthylazine-2-hydroxy. RSDs were < 20 % except for acetochlor, DMA, imazalil, fipronil, terbuthylazine-2-hydroxy and thiamethoxam at the 10 $ng \cdot g^{-1}$ spiked level. The 50 $ng \cdot g^{-1}$ spiked beeswax recoveries were from 50 to 112 % with DMPF and terbuthylazine-2-hydroxy exception. RSDs were < 20 % except for DMA. Recoveries at 100 $ng \cdot g^{-1}$ ranged from 52 to 108 % except for DMPF and terbuthylazine-2-hydroxy. RSDs were < 20 % for all pesticides. The LODs were from 0.3 to 4.2 $ng \cdot g^{-1}$, whereas LOQs ranged from 1 to 12.5 $ng \cdot g^{-1}$. The matrix effects were mostly suppressive and in the range of - 65 to 20 %.

02 DISTRIBUTION OF PESTICIDE RESIDUES IN HONEY BEES AND HIVE PRODUCTS



2.1 Honey bees

2.1.1 Live honey bees

Honey bees were collected from lateral combs, avoiding new emerging individuals from the brood nest, and were a pool of bees from five hives of each sampled apiary.

During June and July 2016-2017, 45 live honey bee samples were collected from 45 different Spanish apiaries (Article 2). Honey bees were contaminated with

7 different pesticides and 22 samples were free of any pesticide. The acaricides used in beekeeping coumaphos (33 %), fluvalinate (27 %) and amitraz (detected in the samples through its main degradate DMF) (16 %) were the most frequently detected at mean concentrations of 2.4, 7.2 and 3.5 $ng \cdot g^{-1}$, respectively. Chlorpyrifos, used in crop protection, was the insecticide most frequently detected (8.9 %) (Table 2, Article 2).

From June 2016 to June 2018, live honey bee samples (n= 38) were periodically collected within a program that monitored pesticides and mortality of honeybees from 3 experimental apiaries (Article 4). Ten samples (26 %) were free of pesticides. On the others, an average of 1 pesticide per sample was detected. Coumaphos and Amitraz (DMF) were detected in 55.3 and 42.1 % of samples, respectively (Table 3, Article 4). Organophosphates insecticides dimethoate (5.3 %) and chlorpyrifos (2.6 %) were detected in bees from apiaries located near agricultural settings.

Live bees are the less contaminated matrix in the hive and acaricides are the main source of contamination, whereas pesticides from plant protection are less frequent **(Mullin et al., 2010; Kiljanek et al., 2017; Fulton et al., 2019)**. While amitraz (compound currently used by beekeepers) detected in honey bees was a contamination from treatments against varroa in the apiaries, coumaphos (not used in the present) presence in honey bees likely came from residues trapped and accumulated in beeswax at high levels from past uses. Residues in bees are an indication that they are really exposed at least to the pesticides found in their bodies, but probably to many others. Guard bees that prevent the entry of poisoned bees with abnormal behaviors, the fast intervention of undertaker bees in removing poisoned dead bees from inside the hive and honey bees' detoxification mechanisms like biotransformation and rapid excretion could reduce pesticide load in their bodies.

2.1.2 Dead honey bees

All dead bees were collected through basket traps placed under the hive entrance (**Accorti et al., 1991; Porrini et al., 2003**) (Figure S1, Article 3).

From January to June 2014, 34 dead bee samples were collected during a pesticide and mortality monitoring program of 4 apiaries located in areas of intensive agriculture (Article 3). Residues of 8 pesticides were detected, 2 acaricides from beekeeping and 6 pesticides used in the surrounding crops. Coumaphos and fluvalinate were found in 94 and 9 % of samples, respectively. Chlorpyrifos (79 %), dimethoate (68 %) and omethoate (62 %) organophosphates were the most frequently detected insecticides. Imidacloprid (up to 223 $ng \cdot g^{-1}$) and acetamiprid (up to 44 $ng \cdot g^{-1}$) neonicotinoids were detected in 32 and 24 % of samples, respectively. Chlorpyrifos and dimethoate concentrations in dead bees were high and up to 751 $ng \cdot g^{-1}$, and were related to high mortality rates. Chlorpyrifos and dimethoate were detected together in 68 % of the cases and simultaneous detection of the three main agrochemicals implicated in honey bee mortality (chlorpyrifos, dimethoate, and imidacloprid) had a frequency of 29 %. Carbendazim was present in 32 % of

samples and concentrations ranged from 3 to 616 ng·g $^{-1}$ (Table 2, Article 3).

During the two-year monitoring of pesticide from June 2016 to June 2018, residues of 10 different pesticides were detected, 3 compounds used in beekeeping and 7 products used in plant protection (Article 4). The 17 samples of dead honey bees collected in mortality traps were mostly contaminated with dimethoate (76.5 %), its metabolite omethoate (52.9 %) and chlorpyrifos (41.2 %) (Table 3, Article 4). Chlorpyrifos (found up to 2700 ng·g $^{-1}$) and dimethoate (up to 338 ng·g $^{-1}$) were detected in dead honey bees with the highest mean concentrations, 232.9 and 89.9 ng·g $^{-1}$, respectively. Both organophosphates were involved in poisoning incidences in several occasions. Fluvalinate (35.3 %) was found at residual concentrations in most of the samples. Imidacloprid and acetamiprid neonicotinoids were found in two samples (11.8 %) with mean concentrations of 29.3 and 1.2 ng·g $^{-1}$, respectively. Hexythiazox (17.6 %) and pyriproxifen (11.8 %) concentrations ranged from 4 to 588 ng·g $^{-1}$. Coumaphos and amitraz (DMF) were found in one sample (5.9 %) and mean concentrations were < 3 ng·g $^{-1}$.

When exposed to sublethal doses of pesticides, forager bees often disorient and are unable to realize the homing-flight (**Schneider et al., 2012; Fischer et al., 2014**), so honey bees with considerable pesticide loads are lost in the fields and excluded from the analysis, underestimating and giving a biased vision of pesticide exposure in dead bees collected through traps. Furthermore, after days in the traps, certain quantity of each pesticide is lost by degradation and the concentration found in the samples is always lower than the original dose of the pesticide exposed to the honey bee. Even though, dead bees revealed the highest levels of insecticides detected in the apiaries, confirming the high exposure of bees to pesticides used in the surroundings crops (**Calatayud-Vernich et al., 2015**).

2.2 Pollen

Samples of recent stored pollen (bright colored pollen loads recently deposited by worker bees) and beebread (maturated pollen) were collected directly from combs. Since honey bees preferentially consume freshly stored pollen (**Carroll et al., 2017**), beebread was collected when recent stored pollen was not available or was too scarce. Calculations of hazard based on pesticide loads from this source of pollen are more accurate and realistic.

Pollen was the most contaminated hive product regarding the number of different pesticides found (**Porrini et al., 2016; Daniele et al., 2017**). Furthermore, the number of pesticides and concentrations detected in the samples were higher in apiaries from farmlands. Pollen balls transported from field to hive by foragers is contaminated with pesticides used in crops. Once the pollen is stored in honeycombs, can also be contaminated with other pesticides present in beeswax. The recent stored pollen and beebread analyzed in the present thesis have revealed the presence of compounds used in-hive against varroosis before sampling (amitraz), and not

applied in apiaries for years (coumaphos), thus indicating that beeswax can act as a source of contamination of incoming pollen.

In 2016 and 2017, 45 samples of pollen were collected from 45 apiaries located in different landscape contexts in Spain (Article 2). Analysis showed 14 different pesticides, with 8 pesticides derived from farmland use and 6 used against varroa. The most frequently detected pesticides were the authorized products in beekeeping coumaphos, fluvalinate and amitraz (DMF), found in 88.9, 46.7 and 37.8 % of samples, and which mean concentrations were 56.2, 10.9 and 17.6 $ng \cdot g^{-1}$ (Table 3 and 4, Article 2). The concentrations of chlorpyrifos (31.1 %) and acetamiprid (11.1 %) insecticides were significantly more elevated in pollen from apiaries located in intensive farming landscapes. Two non-authorized products against varroosis such as acrinathrin (20 %) and chlorfenvinphos (26.7 %) were detected. Although acrinathrin is also used in crops, high levels found in pollen (up to 458 $ng \cdot g^{-1}$) and wax could indicate an irregular use of this pyrethroid together with the organophosphate chlorfenvinphos (up to 194 $ng \cdot g^{-1}$) in some apiaries. The agricultural pesticides dimethoate, hexythiazox and pyriproxyfen were detected at frequencies ranging from 2 to 9 % of samples and concentrations reached 190 $ng \cdot g^{-1}$. Carbendazim, dichlofenthion and fenitrothion, not approved in the EU through Regulation (EC) 1107/ 2009, were detected in few samples (< 5 %), however the use of these non-authorized pesticides in the surrounding environment could not be discarded.

From June 2016 to June 2018, samples of beebread (n= 33) were collected periodically from the experimental apiaries located in wildlands and agricultural landscapes (Figure 1, Article 4). Residues of 6 pesticides from beekeeping and 11 from crop protection were detected. Amitraz and coumaphos were detected in most of the samples and both miticides had the highest mean concentrations, 71.2 and 31.6 $ng \cdot g^{-1}$, respectively. Miticides not used in the apiaries like fluvalinate, chlorfenvinphos and acrinathrin were detected with mean concentrations below 2 $ng \cdot g^{-1}$. Hexythiazox was detected in 24 % of samples with a mean concentration of 1 $ng \cdot g^{-1}$. So, while hexythiazox is used in fruit trees fields, and is likely to be transported to the hive through foraging activity, the main source of beebread contamination with miticides appears to be the wax matrix. Chlorpyrifos and dimethoate (organophosphates insecticides) were detected in 45 and 24 % of the samples, and mean concentrations were 16.2 and 3.4 $ng \cdot g^{-1}$ (Table 2, Article 4). Both compounds are the most used in citrus crops during bloom, and so, they were detected at high levels in pollen from apiaries located near agricultural settings. Imidacloprid and methiocarb (frequently product used in nectarine trees), were detected in 12 and 9 % of samples, respectively and their concentrations ranged from 1 to 28 $ng \cdot g^{-1}$. Acetamiprid and pyriproxyfen were detected in 27 and 12 % of samples, respectively, with mean concentrations below 2 $ng \cdot g^{-1}$. Buprofezin insecticide together with herbicide terbuthylazine were found in less than 10 % of samples and mean concentrations did not exceed 1.4 $ng \cdot g^{-1}$. Concentrations of carbendazim and tebuconazole were low (up to 29 $ng \cdot g^{-1}$).

2.3 Beeswax

Beeswax was collected during 2016 and 2017 from beekeepers operating in different regions of the country (honeycombs, melted wax from old combs and wax cappings) and diverse wax manufacturers (foundation) to give a representative profile of pesticide contaminants of Spanish beeswax.

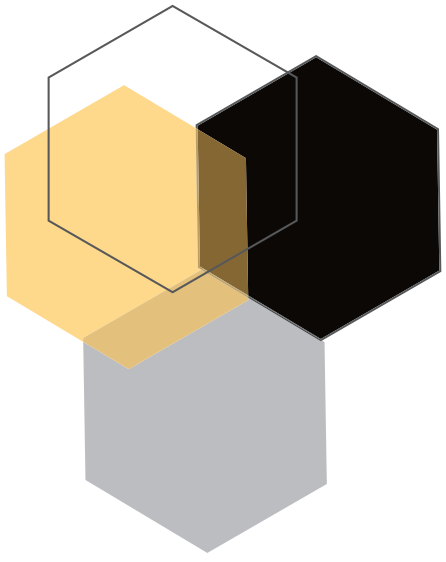
Analaysis of foundation (n = 11), honeycombs (n=43), melted wax from old combs (n= 10), cappings (n= 12) and virgin (n = 2) revealed that beeswax was uniformly contaminated with acaricides used in beekeeping, representing more than 95 % of total pesticide load, and to a much lesser extent with insecticides and fungicide residues (Figure 3, Article 5). Miticides used in beekeeping such as coumaphos, chlorfenvinphos, fluvalinate and acrinathrin were detected in more than 70 % of samples, and maximum concentrations reached were 53400, 5284, 6330 and 7500 $ng \cdot g^{-1}$, respectively (Table 5, Article 2; Table 3, Article 5). Previous works of Spanish and Italian beeswax support the results found in the present study. In these surveys, chlorfenvinphos and acrinathrin were also frequently detected, and the unauthorized use of both compounds was proved (**Jimenez et al., 2005; Lodesani et al., 2008; Orantes-Bermejo et al., 2010)**. Despite Amitraz was used in most of apiaries as the main miticide, the mean content of amitraz degradates in beeswax were significantly lower compared to other miticides detected. Amitraz (detected through DMF) was found in 46.5 % of honeycombs samples, 70 % of melted wax from recycled old combs, 81.8 % of foundation sheets and 91.7 % of cappings and concentrations ranged from 15.8 to 6884.6 $ng \cdot g^{-1}$. Amitraz is unstable in beeswax ($t_{1/2}$ = 6.3 h) and is almost completely degraded within one day in this matrix (**Korta et al., 2001**). In addition, the high polarity of its main degradate DMF ($\log K_{ow}$ = - 1.1) implies that this metabolite would be washed off during commercial recycling processes of wax. Flumethrin, a miticide used in beekeeping, ranged from 8 to 90 % among the differents sources of wax and concentrations were considered residuals as literature also indicates (**Serra-Bonvehi and Orantes-Bermejo, 2010**). Chlorpyrifos was the insecticide most frequently detected in beeswax (**Mullin et al., 2010**), ranging from 21.9 to 54.5 % of samples, with a maximum concentration of 978 $ng \cdot g^{-1}$. Compounds used in crop protection like dichlofenthion, malathion, fenthion-sulfoxide, azinphos-methyl, carbendazim, ethion, hexythiazox, imazalil and pyriproxyfen were less frequent and concentrations were mostly residual. Insecticides and fungicides residues provide evidence that beeswax receives pesticides applied in crops through forager bees activity.

Comparison of wax groups denoted an accused difference among beeswax nature. Virgin and cappings, with an average total pesticide loads of 680 and 2726 $ng \cdot g^{-1}$ were the less contaminated sources of beeswax. Foundation, honeycombs and recycled old combs exhibited similar average total pesticide loads: 12765, 8689 and 14421 $ng \cdot g^{-1}$, respectively. In view of these results, the use of greater amounts of less contaminated beeswax, as capping beeswax, in foundation manufacturing processes is highly encouraged to dilute pesticide residues in this matrix. Pesticide residues found in virgin (Table 2, Article 5) and capping wax evidenced a

transfer of pesticide residues from contaminated areas (foundation and honeycombs) to newly synthesized and uncontaminated beeswax (**Tremolada et al., 2004; Harriet et al., 2017**).

Most of pesticides found in beeswax are very stable once absorbed in this matrix, many resist the process of comb recycling, and some are concentrated by these treatments (e.g. coumaphos content do not decrease after 2 h at 140 °C) (**Bogdanov et al., 1998**). High half-life times (e.g. coumaphos, $t_{1/2}$ = 115 - 346 days) (**Martel et al., 2007**) and elevated partition coefficients are the main factors involved in their stability in beeswax. As a result, beeswax act as a sink trap of lipophilic products from which retained pesticides can be transferred and actively distributed to other hive products by honey bees. Concentrations of pesticides in beeswax were remarkably higher compared to levels detected in pollen and honey bees. For example, compared to residues detected in live honey bees collected at the same time, levels of coumaphos in honeycombs were 1570 times higher.

03 EVALUATION OF PESTICIDE HAZARD IN THE APIARIES



In order to evaluate the hazard posed to bees by pesticide exposure in the studied beekeeping matrices, the hazard quotients (HQ= pesticide concentration ÷ pesticide topical/oral LD_{50}) proposed by **Stoner and Eitzer** (**2013**), were calculated. This is, the sum of all pesticide residue concentrations detected ($ng \cdot g^{-1}$) divided by their respective contact or oral LD50 in $\mu g \cdot bee^{-1}$, for each residue in a given sample. If we consider an individual pollen consumption of 100 mg by a nurse bee during the first 8-10 days of life (**Rortais et al., 2005**), then a nurse bee that consumed a pollen with a

HQ of 1000 would have consumed approximately 10 % of the LD_{50} of the pesticide during development stage. The HQ score provides an estimate based on percentages of LD_{50} equivalents present in pollen, wax and in honey bees themselves. Then, honey bees and pollen samples had a relevant HQ score when it was greater than 50, and the HQ score was considered as elevated when it was greater than 1000. In beeswax, pesticides are embedded in a lipophilic matrix and not all residues are in contact with honey bees, so HQ in beeswax samples was considered as relevant when it was greater than 250. Samples with HQ beeswax > 5000 were considered to have an elevate pesticide hazard (**Traynor et al., 2016**).

3.1 High mortality rates during pesticide poisoning episodes

Average pesticide hazard found in dead honey bees from both monitoring studies was considered elevated (Articles 3 and 4). Chlorpyrifos and dimethoate insecticides were the main contributors to the hazard quotients scores, and were related to pesticide poisoning episodes in apiaries located near agricultural settings.

In 2014, dead honey bees were collected periodically from 4 different apiaries during citrus and stone fruit trees blooming season to evaluate the potential impact of pesticides used in crops on honey bee death rate. The most relevant trait from this study were the mortality peaks between March and May in all apiaries (Figure 3-6, Article 3). During this period, the honey bees collected in the traps exceeded substantially the maximum natural death rate of 20 honey bees per day proposed by **Porrini et al**. (**2003**). Mortality peaks ranged between 50 and 300 bees/day. The increase of mortality took place during the citrus and nectarine flowering and could be related to the insecticides applied to crops, where farmers were frequently seen spraying in the surrounding of the experimental apiaries. There was a clear coincidence between elevated HQ scores and high death bee rates in apiaries and dimethoate and chlorpyrifos contributions to HQ were above 1000 points for each compound. On several occasions dimethoate, its metabolite omethoate and chlorpyrifos were detected simultaneously during high mortality peaks and HQs in those samples were above 3000 points. Imidacloprid neonicotinoid contributions to pesticide hazard was relevant in most of samples, and ranged from 197 to 1541 points. Residues of coumaphos were constant and its $HQ_{\text{dead bees}}$ contribution was very low (< 3 points) throughout the monitoring period. So, coumaphos was not a relevant cause of honey bee mortality. During May, at the end of citrus blooming season, honey bee mortality decreased beyond natural rate in all apiaries.

From June 2016 to June 2018, dead honey bees were collected and analyzed when acute mortality signs appeared in the apiaries, what means piles of dead or dying bees at the entrances of the colonies (Article 4). The apiary located in wildlands and with less agricultural pressure, was free of pesticide poisoning episodes and death rate followed a natural pattern throughout the monitoring period. Mortality was around 20 dead bees/day during periods of low activity, summer (July - August) and winter (December - January), and higher during periods of high activity like rosemary (February - March) blooming season (Figure 3, Article 4). During

flowering, hive population grows and honey bees intensify foraging flights, thus reducing their lifespan. As a result, there is a natural growth in mortality.

In the apiaries 1 and 2 located in farmlands (Article 4), elevated pesticide hazard appeared during and immediately after spraying and decreased after application periods, as also reported by **Beyer et al**. (**2018**).

In apiary 1, the highest mortality peaks were found in May 2017 (up to 256 dead bees/day) and May 2018 (up to 160 and 180 dead bees/ day) during citrus bloom, when dead bees were poisoned with chlorpyrifos and dimethoate organophosphate insecticides. Both compounds were also identified as responsible of poisoned honey bees from other European countries (**Porrini et al., 2014**; **Kiljanek et al., 2016b; Kiljanek et al., 2017**). $HQ_{dead\ bees}$ in May 2018 and 2017 exceeded from 3 to 37 times the threshold value considered as elevated hazard to honey bee health, respectively (Figure 4, Article 4).

In apiary 2, poisoning signs were observed during nectarine (February 2017) and citrus bloom (April-May 2017 and 2018). Dead honey bees collected in February 2017 were contaminated with imidacloprid, used in nectarine orchards near to the apiary. Sprayings of this neonicotinoid before and during bloom was banned in 2013, and since 2018, the use outdoors is completely prohibited by European Union (**EU regulation 2018/783**). Therefore, detections of this neonicotinoid suggest a violation of EU regulation. Levels detected of this compound and its high toxicity to honey bees were responsible of the rise in mortality (up to 95 dead bees/day). Contribution to $HQ_{dead\ bees}$ was elevated and exceeded 7000 points (Figure 4, Article 4). Death rate increased the second half of April, and in May 2017 mortality reached the highest value (>200 dead bees/ day). As occurred in apiary 1, chlorpyrifos, dimethoate and omethoate insecticides were sprayed in citrus orchards during blooming season, thus poisoning forager honey bees. Analysis of dead bees revealed that these compounds were responsible of the elevated pesticide hazard found in honey bee samples ($HQ_{dead\ bees}$ > 4700 points). In April 2018, mortality increased up to 95 dead bees/day, forager bees were poisoned with the compounds fraudulently applied during citrus bloom (chlorpyrifos, dimethoate and omethoate). Imidacloprid was also found in poisoned bees during this mortality peak and had a relevant contribution (360 points) to pesticide hazard.

In addition to acute mortality episodes, the bee colonies affected by poisoning events were debilitated, presenting a honey yield significantly lower and population of forager bees decreased. Considering the impact of pesticide exposure on managed bee colonies, it is necessary to take measures to reduce such stress and benefit wild pollinator health.

3.2 Pesticide hazard in-hive

3.2.1 Live bees

Acaricides used in beekeeping were the main contamination of live bees. Coumaphos and fluvalinate are low toxic to bees (Table 5, Chapter 1), and amitraz (the miticide applied in the apiaries during the studies) is safer for bees compared to other synthetic acaricides (**Gashout et al., 2018**). As a result, 95 % of live bees presented a low pesticide hazard (HQ < 50), and across all samples, only 4 of 83 samples had a relevant $HQ_{live\ bees}$. Chlorpyrifos and dimethoate insecticides were responsible of the relevant hazard found in such samples and contributed from 100 to 333 points to $HQ_{live\ bees}$ scores (Table 3, Article 4). As previously reported in the literature, pesticide hazard in live bees bodies are generally low (**Traynor et al., 2016**).

3.2.2 Pollen

Pollen from the 45 different Spanish apiaries exhibited an average HQ score of 222, 4 times higher than the lower threshold (50 points) established for relevant HQs (Article 2). Samples with relevant (49 %) and low hazard (49 %) were detected in the same frequency, and one sample (2 %) was considered to have an elevated pesticide hazard to honey bees. Despite most of HQ_{pollen} highest scores were calculated in samples from intensive agriculture environment, the main contribution to HQ was due to acrinathrin, pesticide likely misused against varroosis in some apiaries (Figure 3, Article 2). The samples where dimethoate and chlorpyrifos insecticides showed a relevant HQ_{pollen} contribution (> 100 points) came from apiaries located in an intensive agriculture environment.

During June 2016 to June 2018, apiaries located in areas with intensive agriculture surroundings exhibited average HQ_{pollen} between six and seven times higher than apiary located in wildlands and with less agricultural settings in the surroundings (Article 4). This apiary exhibited a low pesticide hazard in more than 90 % of samples. Pollen of the apiaries from agricultural contexts exhibited relevant pesticide hazard in more than 50 % of samples (Figure 2, Article 4). Therefore, apiaries surroundings influenced HQ_{pollen} scores (**Colwell et al., 2017**). Although amitraz and coumaphos were detected in most of the samples, contributions of both miticides to HQ_{pollen} were low and did not exceed 38 points (Table 2, Article 4). Contributions of miticides not used in the experimental apiaries like fluvalinate, chlorfenvinphos and acrinathrin to HQ_{pollen} were low (< 5 points) and did not pose substantial hazard to colonies health with the exception of acrinathrin, which showed low but also relevant contributions (>300 points) to hazard quotients. Chlorpyrifos was responsible of the highest contributions (up to 696 points) to pesticide hazard found in pollen. Chlorpyrifos is the most frequently detected insecticide in hive matrices worldwide, and levels in pollen and beebread have reached levels of concern for bee health (**Mullin et al., 2010; Tosi et al., 2017**). Dimethoate showed a relevant contribution to HQ_{pollen} (200 points) during nectar flow in 2018. Both insecticides had substantial contributions to pesticide

hazard during bloom in 2018. Imidacloprid and methiocarb were involved in relevant HQ_{pollen} scores (up to 350 points). Low levels of this neonicotinoid, as detected in this study, were proved to alter honey bee physiology and reduce foraging motivations in other pollinator species (**Lamsa et al., 2018; Cook, 2019**). Carbendazim and tebuconazole fungicides, detected in 30 and 6 % of pollen samples, contributed less than one point to HQ_{pollen} scores in positive samples for these compounds. In general, fungicides toxicity to honey bees is considered low, and in the HQ approach used in this study, indirect effect of fungicides on the colony are not contemplated. However, fungicides reduce the population of beneficial symbiotic fungi present in pollen that are crucial in the maturation of pollen into beebread. Therefore, nutritional value of beebread contaminated with fungicides is adversely affected and honey bee colony weakened (**Yoder et al., 2012; Steffan et al., 2017**).

3.2.3 Beeswax

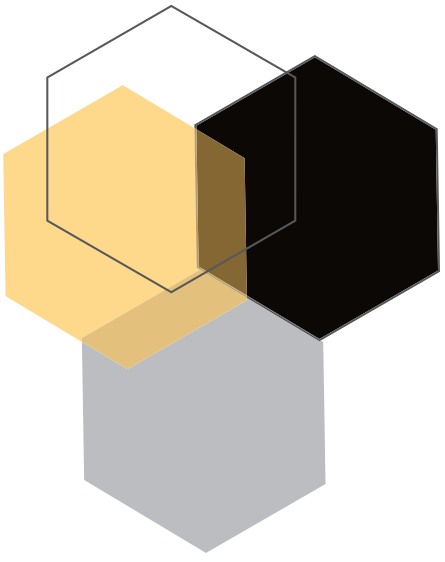
Beeswax is the most hazardous product in the hive (Article 2 and Article 5). The average HQ_{wax} was 30 times higher than the average HQ_{pollen}, and 300 times than the average $HQ_{live\ bees}$. However, in wax matrix only a fraction of the pesticide load become in contact with the bees (e.g. those on the surface), therefore the HQs calculated for beeswax overestimate the threat of pesticides detected.

Most of the samples exhibited an elevated pesticide hazard, and average HQs calculated in honeycombs (6948), foundation (6283) and recycled old combs (5775) were elevated. Pesticide hazard in cappings wax was moderately lower and average HQ was considered relevant (4188). Acrinathrin miticide was the main contributor to HQ_{wax} scores. In the highest HQ wax score (44544), acrinathrin contributed 44118 points. Flumethrin and chlorpyrifos contributed substantially to HQ_{wax} scores (Figure 4, Article 5; Figure 3, Article 2). Despite high concentrations of coumaphos, chlorfenvinphos, fluvalinate and amitraz (DMF), contributions to HQ_{wax} were mostly residuals.

Based on HQ model assumptions, a nurse bee that fed on pollen from the apiary with the highest HQ score (3829) (Table S5, Article 2), would be consuming 38 % of acrinathrin DL_{50}, 0.12 % of coumaphos DL_{50} and 0.005 % of fluvalinate DL_{50} (during her first 10 days of life). If we also consider the toxicity load of the wax from this colonies (HQ_{wax} = 44544), the honey bee health could be seriously compromised.

Most of acaricides and other pesticides detected in beeswax are not highly toxic to bees alone, but in combination there is potential for heightened toxicity due to interactive effects (**Johnson et al., 2013**). In this way, worker honey bee development, longevity and hive performance are adversely affected when developing in a pesticide contaminated brood comb at sublethal levels (**Bevk et al., 2012; Wu et al., 2011**). Additionally, synergistic adverse effects of fluvalinate and coumaphos miticides have been described (**Johnson et al., 2009; Zhu et al., 2014**). Queens and drones exposed to fluvalinate and coumaphos were smaller and sexual vigor was impaired (**Rinderer et al., 1999; Haarmann et al., 2002; Collins et al., 2004**). Harmful loads of pesticide in beeswax matrix also creates a propitious environment to the appearance of acaricide resistant varroa (**Bogdanov et al., 1998; Gonzalez-Cabrera et al., 2016**).

04 THE PESTICIDES IN BEESWAX; AN ONGOING ISSUE



Obtaining cleaned wax is of prime importance for beekeepers to reduce deleterious effects on bee colonies, avoid selective pressure of resistant mites and diminish the transfer of pesticides to other hive products. Currently, the only option to reduce comb pesticide levels in colonies is to replace old drawn and contaminated wax with foundation. However, the beeswax industry uses contaminated wax (mainly old combs) as raw material to produce wax foundation. This creates a market in which beeswax is reused and recirculated and pesticide residues are maintained. Beeswax is also used by food, cosmetic and pharmaceutical industries in numerous products like lipsticks, facial creams, pills coatings, chewing gums.

Given that many of the pesticides detected in wax could pose endocrine disrupting effects, the development of methods to decontaminate wax will have a positive impact on human health as well.

This important aspect was explored in a preliminary study about beeswax cleaning by solvent extraction of pesticides (Article 5, Chapter 5). This work was carried out during a three month research stay in the Departament of Entomology of University of Maryland (USA) under the supervision of Dr. Dennis VanEngelsdorp.

Up to now, only few methods have been proposed to clean beeswax from pesticide residues. In this sense, methods developed propose the use of solid sorbents, like the patent US6586610B2 (**Ulrich, 2019**). **Serra Bonvehi** and **Orantes-Bermejo**, (**2017**), proved that activated charcoal is able to remove > 95 % of two organophosphorus, coumaphos and chlorfenvinphos, widely detected in beeswax worldwide. However, this sorbent only removed fluvalinate pyrethroid a 35 %. Our study demonstrated that organic solvent clean-up pose a wide scope, being able to eliminate pesticides belonging to many different families. Organophosphorus, but also carboxamide, pyrethroids and other pesticide families were removed from wax > 95 %. Pesticide content in the samples were reduced from $\mu g \cdot g^{-1}$ levels to less than 10 $ng \cdot g^{-1}$ in all cases (Table 1-2-3, Article 5). Although beeswax texture is softer after solvent extractions, reconstituted into a useable form for cosmetic and pharmaceutical industries. However, this method is feasible only on small scale because high amounts of solvents are used during the extractions. A continuous solvent-solvent extractor design is needed to apply this methodology on a larger scale of wax production in order to save solvent and minimize environmental harm and cost.

05

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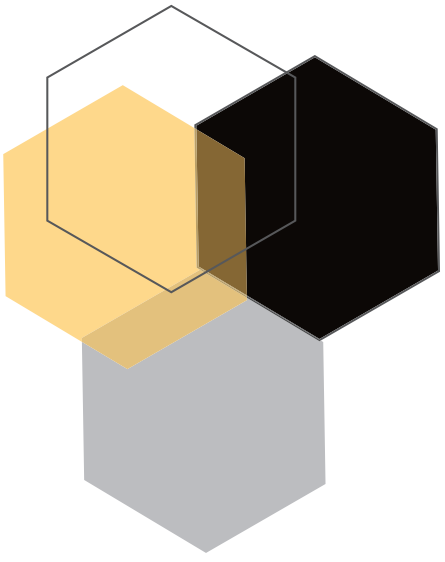

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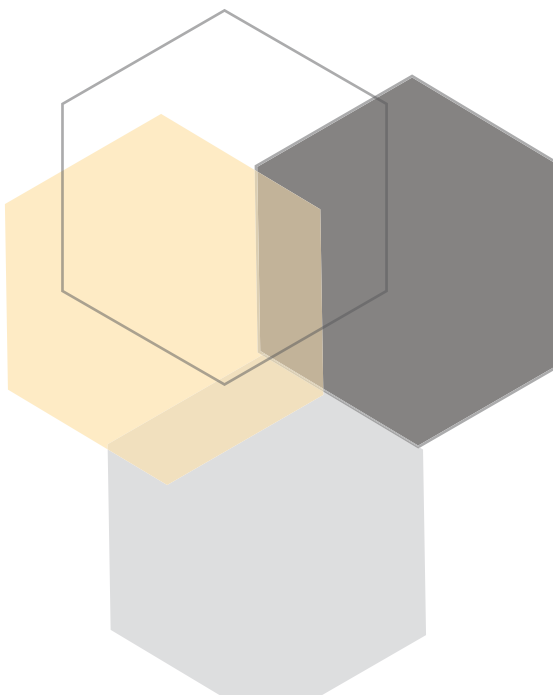


CONCLUSIONS

1. The QuEChERS procedure, slightly modified for each matrix, followed by high performance liquid chromatography tandem mass spectrometry methodology provided appropriate results in terms of accuracy, precision, sensitivity and quickness, and therefore was a suitable method for the determination of the selected pesticides in honey bees, pollen and beeswax.

2. Miticides used in beekeeping (i.e. coumaphos) were the most frequently detected pesticides in beeswax, pollen and live bees, whereas insecticides were the most frequent compounds found in dead honey bees.

3. Beeswax is the most contaminated hive compartment regarding levels of pesticides detected, whereas pollen revealed the highest number of different pesticide residues detected in the samples. Live honey bees were remarkably less contaminated in both quantities and number of pesticides detected.

4. Pesticide poisoning episodes took place only in apiaries located near agricultural settings, and dead honey bees revealed high levels of chlorpyrifos, dimethoate and imidacloprid insecticides, used in the surrounding crops.

5. Pollen from apiaries located in intensive farming landscapes showed concentrations of pesticides used in crops significantly higher than those pollen samples collected in rural, grassland or wildlands landscapes.

6. Beeswax was the beekeeping matrix with the highest pesticide hazard to honey bees and acrinathrin was the most important contributor to the HQ scores. However, the real pesticide exposure in this matrix is overestimated. The pesticide hazard of pollen was considered relevant for bees, and the main contributors to HQ scores were acrinathrin and chlorpyrifos. Pesticide hazard in live bees was considered low.

7. In view of high pesticide concentrations in honeycombs and foundations presented in this thesis, it was evidenced that wax manufacturers mainly utilize wax from old combs to elaborate foundation sheets. The use of less contaminated sources of beeswax, as capping beeswax, in foundation manufacturing processes is highly encouraged to dilute pesticides accumulated in this matrix and prevent future pesticide transferences from wax to honey bees and hive products.

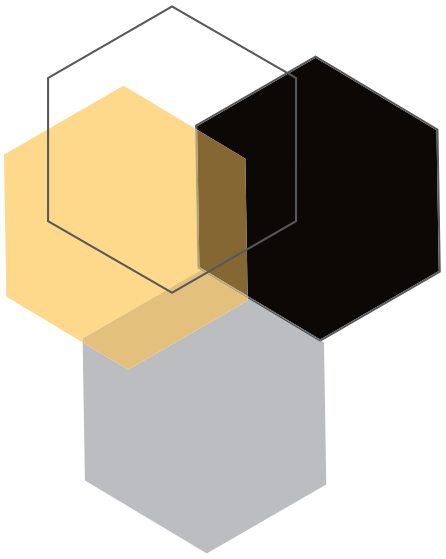
8. The use of solvent-based methodology is capable of extracting most of the pesticide content from the beeswax.

9. It is important to consider the landscape context of the apiaries to avoid honey bee poisoning events. It is also strongly recommended to reduce applications of persistent acaricides against varroosis in-hive to reduce pesticide exposure and improve bee health.

10. The results obtained showed the widespread occurrence of pesticides used in plant protection in pollen and dead bees samples, pointing out that the reliance on pesticides of modern agriculture should be reconsidered. A more sustainable management of the agro-environments would be developed since wild and managed pollinators are essential components in agroecosystems.

RESUM

01 DESENVOLUPAMENT DE LA METODOLOGIA ANALÍTICA



1.1 Procediments d'extracció

Els procediments d'extracció de plaguicides de les abelles, pol·len i cera estaven basats en versions modificades del procediment QuEChERS, inicialment proposat per **Anastassiades et al. (2003)**, i adaptats a cada matriu (Figura 1). Les diferències en la composició dels productes de la colmena, com els hidrocarburs i lípids de la cera, la mescla de proteïnes i grasses en les abelles i el gran contingut de carotenoides apolars presents al pol·len, van evidenciar la versatilitat del QuEChERS (**Niell et al., 2013; Niell et al., 2014; Barganska et al., 2014; Lozano et al., 2019**). A més, el *QuEChERS* és un protocol ràpid i econòmic, i el fet d'utilitzar xicotetes quantitats de dissolvent el converteixen en un mètode que compleix amb criteris importants de la química analítica verda.

1.1.1 Abelles, pol·len i cera d'abella

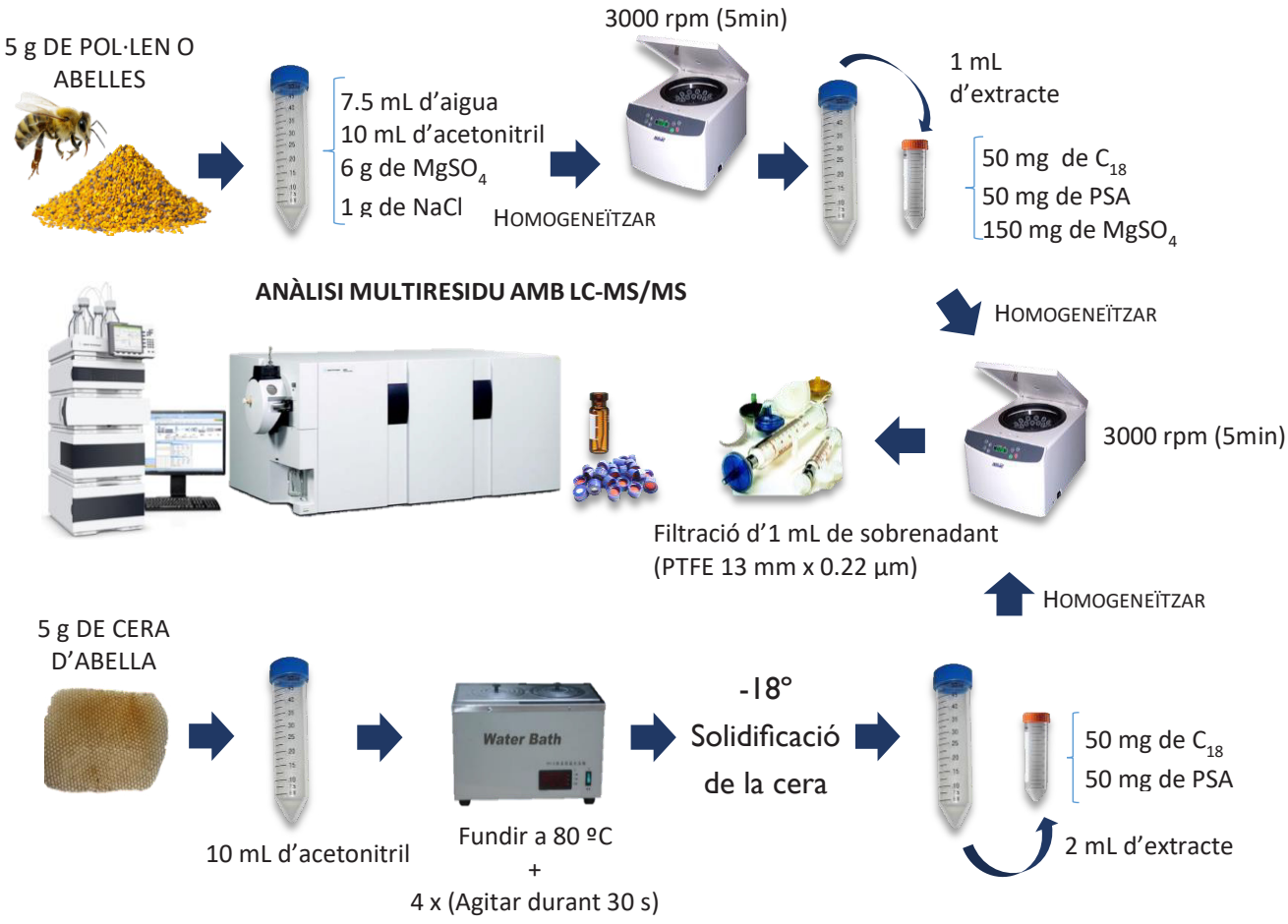


Figura 1. Procediments utilitzats per a l'extracció dels plaguicides de les abelles, pol·len i cera d'abella.

1.2 Mètode de determinació

La determinació dels plaguicides seleccionats (Taula 1) es va realitzar mitjançant cromatografia líquida d'alta eficàcia (HPLC) i espectrometria de masses en tàndem (MS/MS) amb triple quadrupol (QqQ). L'instrument cromatogràfic era un HP1200 equipat amb un injector automàtic, un desgasador, una bomba quaternària i una columna acoblada a un espectròmetre de masses de triple quadrupol Agilent 6410, amb una interfície de ionització electroesprai (ESI) (*Agilent Technologies*, Waldbronn, Alemanya). Les dades van ser processades i analitzades qualitativament i quantitativament amb *MassHunter Workstation Software* (*A GL Sciences*, Tòquio, Japó).

La columna cromatogràfica era una Luna C18 (15,0 cm × 0,21 cm) amb un tamany de partícula de 3 µm (*Phenomenex*, Torrance, EUA). La temperatura de la columna era de 30 °C i el volum d'injecció era de 5 µL.

Es va utilitzar una fase mòbil binària amb gradient d'elució amb un flux de 0,3 mL·min $^{-1}$. La fase A era aigua Milli-Q amb formiat amònic 10 mM i la fase B era metanol amb formiat amònic 10 mM. El gradient d'elució era el següent: 0 min (50 % B), 10 min (83 % B), 12 min (83 % B), 12,5 min (98 % B), i 15,5 min (98 % B). Tot seguit, la fase mòbil tornà a les condicions inicials amb un temps d'equilibrat de 12 minuts.

Els paràmetres de ionització i fragmentació van ser optimitzats mitjançant injeccions directes amb patrons comercials de cada plaguicida. MS/MS es va realitzar en mode *selected reaction monitoring* (SRM), utilitzant ESI en mode positiu. Per a cada compost, dos ions productes característics de la molècula protonada $[M+H]^+$ van ser monitoritzats, el primer i més abundant va ser utilitzat per a la quantificació i el segon va ser utilitzat per a l'anàlisi qualitativa. L'energia de col·lisió i el voltatge del con van ser optimitzats per a cada plaguicida. El nitrogen va ser utilitzat com a gas de col·lisió, nebulització i dessolvatació. Les condicions de l'ESI eren: voltatge del capil·lar 4.000 V, nebulitzador 15 psi, temperatura de la font 300 °C i el flux del gas 10 L·min $^{-1}$. Per tal d'optimitzar-ne la sensitivitat, es va utilitzar la *multiple reaction monitoring* (MRM) en dinàmic, amb una unitat de resolució en els dos espectròmetres (MS1 i MS2) i un voltatge en la cèl·lula d'acceleració de 7 eV. Les condicions del mètode multiresidu (MRM) utilitzades en la determinació dels plaguicides mitjançant LC - MS/MS apareixen en la Taula S1 dels articles 1, 3, 4 i 5.

Taula 1. Llista dels plaguicides seleccionats inclosos en el MRM de la present tesi.

Plaguicida	Classe	Ús	DL $_{50}$ µg · abella $^{-1}$ (*Apis mellifera*)	
			Contacte	Oral
Acetamiprid	Neonicotinoide	Insecticida	7,9	14
Acetoclor	Cloroacetanilida	Herbicida	> 200	> 100
Acrinatrín	Piretroide sintètic	Insecticida/Acaricida	0,17	0,12
Alaclor	Cloroacetanilida	Herbicida	16	
Atrazina *Atrazine-desethyl* ^{M} *Atrazine-desisopropyl* ^{M}	Triazina	Herbicida	> 100	> 100
Azinfòs d'etil	Organofosforat	Insecticida	> 1,39	
Azinfòs metil	Organofosforat	Insecticida	0,42	
Bifentrín	Piretroide	Insecticida	0,015	0,2
Buprofezina	No classificat	Insecticida	> 200	> 163,5
Carbendazim	Benzimidazol	Fungicida	> 50	> 756
Carbofuran *Carbofuran-3-hydroxy* ^{M}	Carbamat	Insecticida/Nematicida/Acaricida	0,036	0,05
Clorfenvinfòs	Organofosforat	Acaricida/Insecticida	4,1	0,55
Clorpirifòs	Organofosforat	Insecticida	0,072	0,24
Clotianidina	Neonicotinoide	Insecticida	0,039	0,0035
Cumafòs	Organofosforat	Acaricida	20	4,6
Diazinon	Organofosforat	Insecticida/Acaricida	0,13	0,09
Diclofention	Organofosforat	Insecticida		
Dimetoat	Organofosforat	Insecticida	0,12	0,17
Diuron	Fenilamida	Herbicida	> 101,7	> 86,75

Taula 1. Cont

Plaguicida	**Classe**	Ús	**DL $_{50}$ µg · abella $^{-1}$ (*Apis mellifera*)**	
			Contacte	**Oral**
Amitraz [a]	Amidina	Acaricida/Insecticida	50	
DMA				
DMF				
DMPF				
Etion	Organofosforat	Insecticida/Acaricida	11	
Etofenprox	Piretroide	Insecticida	0,015	0,024
Fenitrotion	Organofosforat	Insecticida	0,16	0,20
Fention	Organofosforat	Insecticida	0,22	
Fenthion-sulfone [M]				
Fenthion-sulfoxide [M]				
Fipronil	Fenilpirazol	Insecticida	0,0059	0,0047
Flumetrina	Piretroide	Acaricida/Insecticida	0,05	
Fluvalinat	Piretroide sintètic	Acaricida/Insecticida	8,7	45
Hexitiazox	Carboxamida	Acaricida	> 200	> 112
Imazalil	Imidazole	Fungicida	39	37
Imidacloprid	Neonicotinoide	Insecticida	0,081	0,0037
Isoproturon	Urea	Herbicida	200	195
Lambda-cihalotrín	Piretroide sintètic	Insecticida	0,038	0,91
Malatió	Organofosforat	Insecticida/Acaricida	0,16	0,40
Metiocarb	Carbamat	Insecticida/ Mol·lusquicida	0,23	0,08
Metolaclor	Cloroacetamida	Herbicida	110	110
Molinat	Tiocarbamat	Herbicida		> 11
Ometoat	Organofosforat	Insecticida		0,05
Paration d'etil	Organofosforat	Insecticida/Acaricida		> 0,21
Paration de metil	Organofosforat	Insecticida/Acaricida	2,7	750
Procloraz	Imidazole	Fungicida	141,3	101
Propanil	Anilida	Herbicida	> 100	> 94,3
Propazina	Triazina	Herbicida	16	
Piriproxifén	No classificat	Insecticida	74	> 100
Simazina	Triazina	Herbicida	97	
Spinosad [b]	Derivat de microorganisme	Insecticida	0,003	0,057
Spynosyn A				
Spynosyn D				
Tebuconazol	Triazol	Fungicida	> 200	> 83,05
Terbumeton	Triazina	Herbicida		
Terbumeton-desethyl [M]				
Terbutilazina	Triazina	Herbicida	> 32	> 22,6
Terbuthylazine-desethyl [M]				
Terbuthylazine-2 hydroxy [M]				
Terbutrín	Triazina	Herbicida	> 225	
Tiabendazole	Benzimidazol	Fungicida	> 34	> 4
Tiametoxam	Neonicotinoide	Insecticida	0,024	0,005

Taula 1. Cont

Plaguicida	Classe	Ús	DL_{50} µg · abella $^{-1}$ (*Apis mellifera*)	
			Contacte	Oral
Tolclofos-metil	Clorofenil	Fungicida	> 100	

[M] Metabòlit
[a] L'*Amitraz* és detectat mitjançant els seus productes de degradació: DMA, DMF i DMPF.
[b] L'*Spinosad* és detectat mitjançant els seus components: l'espinosina A i D.
Les DL_{50} eren de **Sanchez-Bayo and Goka (2014)** i de la base de dades de plaguicides de la *University of Hertfordshire* (**Hertfordshire, 2019**).

1.3 Validació de la metodologia analítica

La sensitivitat, l'exactitud, la precisió i la robustesa dels mètodes multiresidus desenvolupats en la present tesi van ser avaluats segons les directrius de control de qualitat i validació de mètodes multiparamètrics de plaguicides de la Unió Europea (**SANCO/12571/2013; SANTE/11945/2015**). La metodologia va ser validada per a cada matriu estudiada i plaguicida inclòs en les anàlisis. Les dades de validació dels diferents mètodes poden ser consultats a la Taula 1 de l'article 3 i 5, i a la Taula S1 de l'article 2.

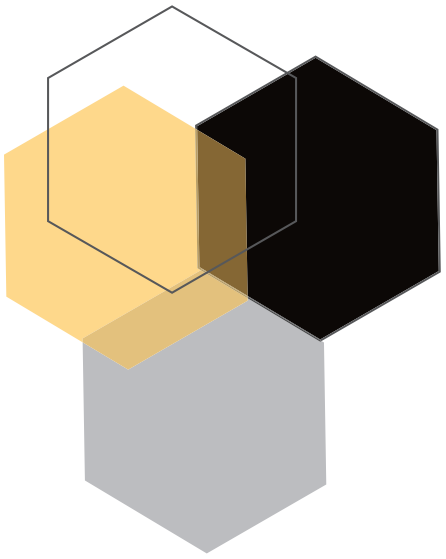
En les abelles, les recuperacions van variar entre el 70 i el 96 %, i les desviacions estàndard relatives (RSDs) van ser < 20 % per a la majoria d'anàlits, excepte per a *atrazine-desethyl, carbofuran, fenthion-sulfoxide, ometoat, paration d'etill,* i *propazina.* Els límits de detecció (LODs) van variar entre 0,3 i 3 ng·g $^{-1}$, mentre que els límits de quantificació (LOQs) estaven compresos entre 1 i 10 ng·g $^{-1}$. Els efectes matriu van ser principalment supressius i van variar entre el - 60 i el 20 %, amb l'excepció de *carbofuran, 3-hydroxy, carbofuran, fenthion-sulfoxide* i *fenthion-sulfone.*

En la matriu del pol·len, els valors de les recuperacions mitjanes realitzades als nivells de 10, 50 i 100 ng·g $^{-1}$ van ser 90, 86 i 91 %, respectivament. Els valors de les recuperacions van variar entre el 70 i el 116 %, i solament el 7 % dels compostos va mostrar recuperacions entre el 55 i el 69 %. La precisió, expressada com a RSD, va ser < 20 % en la majoria dels plaguicides validats. Els LODs van ser inferiors a 2 ng·g $^{-1}$ i els LOQs van ser inferiors a 5 ng·g $^{-1}$. Els efectes matriu van ser majoritàriament supressius i van variar entre el - 54 i el 50 %.

En la cera d'abella, les recuperacions realitzades a 10 ng·g $^{-1}$ van variar del 50 fins al 120 %, amb l'excepció de *terbuthylazine-2 hydroxy.* Les RSDs eren < 20 % excepte per a *acetoclor, DMA, imazalil, fipronil, terbuthylazine-2 hydroxy* i *tiametoxam.* Les recuperacions realitzades a 50 ng·g $^{-1}$ oscil·laren entre el 50 i el

112 %, amb les excepcions del *DMPF* i *terbuthylazine-2 hydroxy*. Les RSDs també van ser majoritàriament < 20 %. Les recuperacions realitzades a 100 $ng \cdot g^{-1}$ variaren entre el 52 i el 108 %, excepte per al *DMPF* i *terbuthylazine-2 hydroxy,* i les RSDs van ser < 20 %. Els LODs van variar entre 0,3 i 4,2 $ng \cdot g^{-1}$, mentre que els LOQs van oscil·lar entre 1 i 12,5 $ng \cdot g^{-1}$. Els efectes matriu van ser majoritàriament supressius i en el rang de - 65 fins al 20 %.

02 DISTRIBUCIÓ DELS RESIDUS DE PLAGUICIDES EN LES ABELLES I ELS PRODUCTES DE LA COLMENA



2.1 Abelles

2.1 Abelles vives

Les abelles es van agafar de les bresques laterals per tal d'evitar abelles recentment nascudes del niu de cria. Es van mostrejar 5 colmenes per apiari i cada mostra va tenir un nombre equivalent d'abelles.

Durant juny i juliol dels anys 2016 i 2017, es van agafar 45 mostres d'abelles vives procedents de 45 apiaris localitzats en diferents punts del territori espanyol (Article 2). Les abelles estaven contaminades amb 7 plaguicides diferents i 22 mostres no contenien residus de cap plaguicida. Els acaricides utilitzats en l'apicultura, com ara el *cumafòs* (33 %), el *fluvalinat* (27 %) i l'*amitraz*

(16 %) (detectat en les mostres a través del seu producte de degradació DMF), van ser els més freqüents i amb les concentracions mitjanes més elevades: 2,4, 7,2 i 3,5 ng·g $^{-1}$, respectivament. El *clorpirifòs*, utilitzat a l'agricultura, va ser l'insecticida més freqüent (8,9 %) (Taula 2, Article 2).

Des de juny del 2016 fins a juny del 2018, es van agafar periòdicament mostres d'abelles vives (n= 38) durant un programa de seguiment de la mortalitat i de residus de plaguicides en tres apiaris experimentals, l'un envoltat de vegetació silvestre i els altres dos en entorns agraris (Article 4). El 26 % de les mostres no contenia cap plaguicida i una mitjana d'1 plaguicida per mostra va ser calculada. El *cumafòs* i l'*amitraz* (DMF) van ser detectats en el 55,3 i el 42,1 % de les mostres, respectivament (Taula 3, Article 4). Els insecticides organofosforats *dimetoat* (5,3 %) i *clorpirifòs* (2,6 %) van ser detectats solament en les abelles d'apiaris localitzats en entorns agraris.

Les abelles vives són la matriu menys contaminada de la colmena i els acaricides són la principal font de contaminació d'aquestes, mentre que els plaguicides d'ús agrari són menys freqüents **(Mullin et al., 2010; Kiljanek et al., 2017; Fulton et al., 2019)**. La contaminació de les abelles vives amb *amitraz* és resultat dels tractaments veterinaris contra la varroosi en les colmenes dels apiaris, però la presència de *cumafòs* en les abelles probablement és resultat d'una transferència d'aquest compost des de la cera, on es troba acumulat en quantitats elevades degut al seu ús en anys previs a l'estudi. Els residus detectats en les abelles són un indicatiu que almenys estan exposades als residus trobats als seus cossos, encara que molt probablement n'estan exposades a molts altres que no arriben a ser detectats en les anàlisis. Les abelles guardianes que eviten l'entrada d'abelles intoxicades amb comportaments anormals, la ràpida actuació de les abelles enterradores que retiren les abelles mortes intoxicades de l'interior de la colmena i els mecanismes de destoxicació de les abelles com la biotransformació i l'excreció, podrien explicar la baixa concentració de plaguicides en les abelles vives.

21.2 Abelles mortes

Les abelles mortes van ser recollides mitjançant gàbies de mortalitat situades enfront i davall de l'entrada de les colmenes (**Accorti et al., 1991; Porrini et al., 2003**) (Figura S1, Article 3).

Des de gener fins a juny del 2014, es van agafar mostres d'abelles mortes (n= 34) durant un seguiment de mortalitat i de residus de plaguicides en 4 apiaris experimentals situats en entorns d'agricultura intensiva (Article 3). Es van detectar 8 plaguicides diferents, 2 acaricides utilitzats en l'apicultura i 6 fitosanitaris. *Cumafòs* i *fluvalinat*, utilitzats contra la varroa, van ser detectats en el 94 i el 9 % de les mostres, respectivament. Els organofosforats *clorpirifòs* (79 %), *dimetoat* (68 %) i *ometoat* (62 %) van ser-hi els insecticides més freqüents (Taula 2, Article 3). Els neonicotinoides *imidacloprid* (detectat fins als 223 ng·g $^{-1}$) i *acetamiprid* (detectat fins als 44 ng·g $^{-1}$) estaven presents al 32 i 24 % de les mostres, respectivament. Les concentracions de *clorpirifòs* i *dimetoat* en les abelles mortes eren altes (fins als 751 ng·g $^{-1}$) i van ser directament relacionades amb les

taxes de mortalitat elevades dels apiaris. Ambdós insecticides van ser detectats simultàniament en el 68 % de les mostres. La detecció d'aquests organofosforats juntament amb l'*imidacloprid*, també implicat en episodis de mortalitat, es va donar en el 29 % dels casos. El *carbendazim* estava present en el 32 % de les mostres i les seues concentracions oscil·laren entre 3 i 616 $ng \cdot g^{-1}$.

Durant el seguiment de plaguicides i mortalitat de juny del 2016 a juny del 2018, es van detectar 10 plaguicides diferents, 3 utilitzats contra la varroosi i 7 emprats en l'agricultura (Article 4). Les 17 mostres d'abelles mortes recollides en les trampes de mortalitat estaven contaminades principalment amb *dimetoat* (76,5 %), el seu metabòlit *ometoat* (52,9 %) i el *clorpirifòs* (41,2 %) (Taula 3, Article 4). El *clorpirifòs* (detectat fins als 2.700 $ng \cdot g^{-1}$) i el *dimetoat* (detectat fins als 338 $ng \cdot g^{-1}$) foren els plaguicides amb les concentracions mitjanes més altes, 232,9 i 89,9 $ng \cdot g^{-1}$, respectivament. Ambdós organofosforats també estaven implicats en episodis de mortalitat aguda en diverses ocasions. El *fluvalinat* (35,3 %) va ser detectat en les mostres amb concentracions residuals. L'*imidacloprid* i l'*acetamiprid* es van detectar en dos mostres (11,8 %) amb concentracions mitjanes de 29,3 i 1,2 $ng \cdot g^{-1}$, respectivament. Les concentracions de l'*hexitiazox* (17,6 %) i del *piriproxifén* (11,8 %) van variar entre els 4 i els 588 $ng \cdot g^{-1}$. El *cumafòs* i l'*amitraz* (DMF) foren detectats en una mostra (5,9 %) i les concentracions mitjanes foren residuals (< 3 $ng \cdot g^{-1}$).

Quan les abelles recol·lectores són exposades a dosis subletals de plaguicides poden patir desorientació i ser incapaces de realitzar el vol de tornada a la colmena (**Schneider et al., 2012; Fischer et al., 2014**), això provoca que abelles amb dosis considerables de plaguicides es perden al camp i són excloses de les anàlisis. Per tant, les abelles mortes recollides mitjançant trampes de mortalitat subestimen l'exposició real de les abelles als plaguicides. A més, els plaguicides es degraden després de dies a les trampes i la concentració mesurada en les mostres sempre és menor que la dosi original en contacte amb l'abella. Tot i això, les abelles mortes revelaren els nivells d'insecticides més alts dels apiaris, i confirmaren l'alta exposició de les abelles als plaguicides utilitzats durant la floració dels cultius del voltant (**Calatayud-Vernich et al., 2015**).

2.2 Pol·len

Les mostres de pol·len fresc (boles de pol·len brillants acabades de depositar a les bresques per abelles obreres) i de pa d'abella (pol·len madurat) van ser agafades directament de les bresques. El pa d'abella es va mostrejar quan no hi havia pol·len fresc o era molt escàs, degut al fet que les abelles consumeixen aquest últim preferiblement (**Carroll et al., 2017**). Per tant, els càlculs del perill per plaguicides basats en aquesta font de pol·len són més exactes i realistes.

El pol·len era el producte de la colmena més contaminat quant a nombre de residus de plaguicides diferents (**Porrini et al., 2016; Daniele et al., 2017**). El pol·len transportat des del camp fins a la colmena per les abelles recol·lectores està contaminat per plaguicides d'ús agrícola, i després de ser emmagatzemat en

les bresques es pot contaminar pels plaguicides presents en la cera. Les mostres de pol·len fresc i pa d'abella analitzades en la següent tesi han revelat la presència de compostos utilitzats a l'interior de la colmena per combatre la varroa (*amitraz*), i de productes no emprats en els apiaris experimentals en anys (*cumafòs*), i indiquen, així, que la cera pot actuar com a font de contaminació del pol·len que ve del camp.

Al 2016 i 2017, es van recollir mostres de pol·len (n= 45) de 45 apiaris localitzats en diferents entorns del territori espanyol (Article 2). Les anàlisis detectaren 14 plaguicides diferents, 8 provinents de l'agricultura i 6 utilitzats a l'apicultura. Els productes més freqüents van ser els acaricides autoritzats contra la varroa, el *cumafòs*, el *fluvalinat* i l'*amitraz* (DMF), detectats en el 88,9, 46,7 i el 37,8 % de les mostres, amb unes concentracions mitjanes de 56,2, 10,9 i 17,6 ng·g $^{-1}$, respectivament (Taula 3 i 4, Article 2). Les concentracions dels insecticides *clorpirifòs* (31,1 %) i *acetamiprid* (11,1 %) van ser significativament més elevades en apiaris situats en entorns agraris. Es van detectar dos productes no autoritzats contra la varroosi, l'*acrinatrín* (20 %) i el *clorfenvinfòs* (26,7 %). Encara que l'*acrinatrín* també és utilitzat com a fitosanitari, els alts nivells detectats en el pol·len (fins a 458 ng·g $^{-1}$) i la cera podrien indicar un ús apícola irregular d'aquest compost juntament amb el *clorfenvinfòs* (fins a 194 ng·g $^{-1}$) en alguns apiaris. Els fitosanitaris *dimetoat*, *hexitiazox* i *piriproxifén* van ser detectats des del 2 fins al 9 % de les mostres, amb concentracions que arribaren als 190 ng·g $^{-1}$. Les analítiques de pol·len van revelar compostos no aprovats a la UE (**Regulació (EC) 1107/2009**) com el *carbendazim*, el *diclofention* i el *fenitrotion*, que van ser detectats en poques mostres (< 5 %). Per tant, l'ús d'aquests en els voltants dels apiaris no pot ser descartat.

Mostres de pol·len (n= 33) es van recollir periòdicament durant el seguiment de mortalitat i residus de plaguicides de 2016 a 2018 (Figura 1, Article 4). El pol·len recollit estava contaminat per 6 plaguicides d'ús apícola i 11 fitosanitaris. Els acaricides *amitraz* i *cumafòs* van ser-hi els productes més freqüents i amb les concentracions mitjanes més elevades: 71,2 i 31,6 ng·g $^{-1}$, respectivament. Altres acaricides no utilitzats en els apiaris experimentals com el *fluvalinat*, el *clorfenvinfòs* i l'*acrinatrín* van ser detectats en concentracions inferiors a 2 ng·g $^{-1}$. L'*hexitiazox* va aparéixer en el 24 % de les mostres amb una concentració mitjana d'1 ng·g $^{-1}$. Mentre que l'*hexitiazox* s'utilitza en els fruiters i és transportat fins a la colmena mitjançant l'activitat de les abelles recol·lectores, la principal font de contaminació del pol·len amb els acaricides sembla ser la matriu de la cera. El insecticides organofosforats *clorpirifòs* i *dimetoat* van ser detectats en el 45 i el 24 % de les mostres, amb concentracions mitjanes de 16,2 i 3,4 ng·g $^{-1}$ (Taula 2, Article 4). Ambdós compostos són molt utilitzats durant la floració dels cítrics i, en conseqüència, van ser detectats en nivells elevats en les mostres de pol·len d'apiaris situats en entorns agrícoles. L'*imidacloprid* i el *metiocarb*, utilitzats en els nectariners, van ser detectats en el 12 i el 9 % de les mostres i les seues concentracions van variar d'1 a 28 ng·g $^{-1}$. L'*acetamiprid* i el *piriproxifén* van aparéixer en el 27 i en el 12 % de les mostres, respectivament, i les seues concentracions mitjanes estaven per davall de 2 ng·g $^{-1}$. L'insecticida *buprofezina* juntament amb l'herbicida *terbutilazina* van ser detectats en menys del 10 % de les mostres i les concentracions mitjanes no van superar els 1,4 ng·g $^{-1}$. Les concentracions del *carbendazim* i *tebuconazol* foren baixes (fins a 29 ng·g $^{-1}$).

2.3 Cera d'abella

La cera analitzada durant els anys 2016 i 2017 provenia d'apicultors de diferents parts d'Espanya (cera de bresques, blocs de cera reciclada de bresques antigues i cera d'opercle) i de diferents productors de cera (làmines de cera). Amb els estudis sobre els contaminants de la cera, es pretenia mostrar un perfil representatiu dels plaguicides presents en aquesta matriu (Article 2 i Article 5).

Les analítiques de cera de làmines (n= 11), bresques (n= 43), cera reciclada de bresques antigues (n= 10), cera d'opercle (n= 12) i cera verge (n= 2) van revelar que la cera està contaminada uniformement amb acaricides d'ús apícola, que representen més del 95 % de la càrrega total de plaguicida d'aquesta matriu, i amb menor mesura amb insecticides i fungicides agrícoles (Figura 3, Article 5). Els acaricides utilitzats en l'apicultura, com ara el *cumafòs*, el *clorfenvinfòs*, el *fluvalinat* i l'*acrinatrín,* estaven presents en més del 70 % de les mostres, i se'n detectaren concentracions màximes de fins a 53.400, 5.284, 6.330 i 7.500 $ng \cdot g^{-1}$, respectivament (Taula 5, Article 2; Taula 3, Article 5). Estudis previs sobre el contingut de plaguicides en cera espanyola i italiana confirmen els nostres resultats sobre la presència de productes no autoritzats com el *clorfenvinfòs* i l'*acrinatrín,* i també suggereixen un ús fraudulent d'aquests productes als apiaris (**Jiménez et al., 2005; Lodesani et al., 2008; Orantes-Bermejo et al., 2010)**.

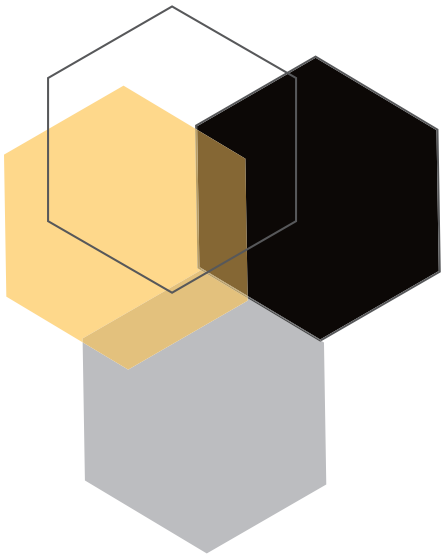
Malgrat que l'*amitraz* era utilitzat en la majoria dels apiaris com l'acaricida principal, el contingut mitjà d'aquest compost en les mostres era substancialment inferior a altres acaricides detectats i no emprats en els apiaris. L'*amitraz* va ser detectat al 46,5 % de les bresques, al 70 % de la cera reciclada de bresques antigues, al 81,8 % de les làmines de cera i al 91,7 % de la cera d'opercle, amb unes concentracions compreses entre 15,8 i 6.884,6 $ng \cdot g^{-1}$. L'*amitraz* és inestable en la cera ($t_{1/2}$ = 6,3 h) i es degrada quasi per complet després d'un dia en aquesta matriu (**Korta et al., 2001**). A més, l'alta polaritat del seu principal producte de degradació, DMF (log K_{ow}= - 1.1), podria provocar el rentatge d'aquest compost durant els processos de reciclat de la cera. Les deteccions de *flumetrina* van variar d'un 8 a un 90 % en les diferents mostres i fonts de cera, i les concentracions van ser considerades residuals com altres estudis també han corroborat, de forma que en reflectien un ús minoritari en el tractament de la varroa (**Serra-Bonvehí and Orantes-Bermejo, 2010**). L'insecticida *clorpirifòs* va ser el fitosanitari més freqüent en la cera (**Mullin et al., 2010**), present des del 21,9 fins al 54,5 % en les diferents fonts de cera i amb una concentració màxima de 978 $ng \cdot g^{-1}$. Els productes d'ús agrícola *diclofention*, *malatió*, *fenthion-sulfoxide*, *azinfòs metil*, *carbendazim*, *etion*, *hexitiazox*, *imazalil* i *piriproxifén* van ser menys freqüents i les concentracions detectades foren majoritàriament residuals. Els residus d'insecticides i fungicides detectats a les mostres de cera proven que aquesta matriu rep plaguicides aplicats al camp a través de l'activitat de les abelles recol·lectores.

La comparació del contingut mitjà de càrrega de plaguicides dels diferents grups de cera va mostrar una

acusada diferència. Les fonts de cera verge i d'opercle, amb una càrrega de plaguicida mitjana de 680 i 2.726 $ng \cdot g^{-1}$, van ser les menys contaminades. Les làmines, bresques i cera reciclada de bresques estaven més contaminades, amb una càrrega mitjana total de plaguicida similar: 12.765, 8.689 i 14.421 $ng \cdot g^{-1}$, respectivament. Aquests resultats suggereixen augmentar l'ús de cera d'opercle com a font principal de cera durant la producció de làmines, per tal de reduir la concentració de plaguicides en aquestes. Els residus de plaguicida detectats en la cera verge (Taula 2, Article 5) i d'opercle van demostrar la transferència de plaguicides d'àrees contaminades (làmines i bresques) a cera de nova síntesi i lliure de plaguicides (**Tremolada et al., 2004; Harriet et al., 2017**).

La majoria dels plaguicides adsorbits en la cera són estables, molts resisteixen el procés de reciclat d'aquesta matriu, i alguns són concentrats per aquests tractaments (p. ex., el contingut de *cumafòs* no disminueix després de 2 h a 140 °C) (**Bogdanov et al., 1998**). Els temps de vida mitjà (p. ex. *cumafòs*, $t_{1/2}$ = 115 – 346 dies) (**Martel et al., 2007**) i coeficients de partició elevats són els principals factors implicats en aquesta gran estabilitat. Com a resultat, la cera actua com un depòsit de productes lipòfils, des d'on els plaguicides retinguts poden ser activament distribuïts a altres parts de la colmena per les abelles. Així, les concentracions de plaguicides en la cera foren substancialment superiors als nivells detectats en el pol·len i les abelles. Per exemple, en comparació amb els residus detectats en mostres d'abelles vives mostrejades al mateix temps, els nivells de *cumafòs* en les bresques eren 1.570 vegades més elevats.

03 AVALUACIÓ DEL PERILL DELS PLAGUICIDES DETECTATS EN ELS APIARIS



Per a avaluar el perill que representa per a les abelles l'exposició als plaguicides de les diferents matrius estudiades, es va utilitzar el quocient de perillositat (HQ) proposat per **Stoner** i **Eitzer** (**2013**) (HQ = concentració de plaguicida ÷ DL_{50} del plaguicida oral/contacte). Açò és, la suma de les concentracions de tots els plaguicides detectats ($ng \cdot g^{-1}$) dividida per les seues respectives DL_{50} oral/contacte en $\mu g \cdot abella^{-1}$ per a cada plaguicida i mostra. Si considerem un consum de pol·len de 100 mg per abella nodrissa durant els seus 8 o 10 dies de vida (**Rortais et al., 2005**), aleshores una nodrissa que haja consumit pol·len amb un HQ de 1.000 hauria consumit aproximadament el 10 % de la DL_{50} d'un determinat plaguicida durant el seu desenvolupament. La puntuació de l'HQ proporciona una estimació d'equivalents de DL_{50} presents en el pol·len, cera o en les mateixes abelles. D'aquesta manera, els HQ de les abelles i el pol·len van ser considerats com a rellevants quan eren

majors de 50, i com a elevats quan eren més grans que 1.000. En la cera d'abella, com que els plaguicides es troben embeguts dins la matriu, no tots els plaguicides estan en contacte amb les abelles; per tant, els HQ d'aquesta matriu van ser considerats rellevants quan eren majors de 250. Les mostres de cera amb HQ > 5.000 van ser considerades com a exemples amb un perill per plaguicida elevat (**Traynor et al., 2016**).

3.1 Episodis de mortalitat elevada degut a intoxicacions per plaguicides

El perill per plaguicides calculat a les mostres d'abelles mortes dels dos estudis va ser considerat com a elevat (Articles 3 i 4). Els insecticides *clorpirifòs* i *dimetoat* van ser els principals contribuïdors a les puntuacions dels $HQ_{\text{abelles mortes}}$, i ambdós compostos van ser relacionats amb els episodis d'intoxicació que s'esdevingueren en els apiaris situats en entorns agraris.

A l'estudi del 2014, es van agafar periòdicament mostres d'abelles mortes de 4 apiaris diferents durant la floració de fruiters per a avaluar l'impacte dels fitosanitaris sobre la taxa de mortalitat de les abelles. Els trets més rellevants d'aquest estudi van ser els pics de mortalitat que ocorregueren durant març i maig en tots els apiaris (Figures 3-6, Article 3). Durant aquest període, les abelles mortes procedents de les gàbies de mortalitat superaven remarcablement el límit de mortalitat natural de 20 abelles per dia establert per **Porrini et al**. (**2003**). Els pics de mortalitat van variar entre les 50 i les 300 abelles mortes per dia. Aquest augment de la mortalitat va ocórrer durant la floració de cítrics i nectariners i estava relacionat amb els insecticides utilitzats en els camps, on els agricultors van ser freqüentment observats tractant els camps dels voltants dels apiaris. Hi havia una clara coincidència entre taxes de mortalitat altes i mostres amb HQ elevats, en les quals el *clorpirifòs* i el *dimetoat* aportaven individualment més de 1.000 punts. En diverses ocasions el *dimetoat*, el seu metabòlit *ometoat* i el *clorpirifòs* van ser detectats simultàniament en mostres recollides durant taxes de mortalitat altes, amb uns valors $HQ_{\text{abelles mortes}}$ per damunt dels 3.000 punts. Les contribucions del neonicotinoide *imidacloprid* als $HQ_{\text{abelles mortes}}$ va ser majoritàriament rellevant, i va variar entre els 197 i 1.541 punts. Els residus del cumafòs van ser constants durant tot l'estudi i les contribucions als HQ van ser molt baixes (< 3 punts). Per tant, aquest compost no pot tindre una implicació rellevant en els episodis de mortalitat. Durant el mes de maig i coincidint amb el final de la floració dels cítrics, la taxa de mortalitat dels apiaris va disminuir fins a valors considerats com a naturals.

En l'estudi de juny del 2016 fins a juny del 2018, es mostrejaren abelles mortes quan aparegueren signes de mortalitat aguda als apiaris, és a dir, piles d'abelles mortes o moribundes en les entrades de les colmenes (Article 4). L'apiari situat en un entorn de vegetació silvestre i amb poca pressió agrícola no va patir cap episodi d'intoxicació, i la taxa de mortalitat va seguir un patró natural durant tot l'estudi. La mortalitat era de 20 abelles per dia durant els períodes de baixa activitat, com l'estiu (juliol - agost) i l'hivern (desembre

- gener), i lleugerament superior en períodes de gran activitat com en la floració del romer (febrer - març) (Figura 3, Article 4). Durant la floració, la població de la colmena creix i les abelles recol·lectores intensifiquen els seus vols, es redueix la seua esperança de vida i augmenta de forma natural la mortalitat de la colmena, duplicant aproximadament aquesta.

En els apiaris 1 i 2, situats en entorns agrícoles, es van observar $HQ_{\text{abelles mortes}}$ elevats durant els tractaments agrícoles en floració i immediatament després, i $HQ_{\text{abelles mortes}}$ baixos després d'aquesta, com també han reportat **Beyer et al**. (**2018**).

En l'apiari 1, els pics de mortalitat més elevats van ocórrer al maig del 2017 (fins a 256 abelles mortes per dia) i al maig del 2018 (fins a 160 i 180 abelles mortes per dia), durant la floració dels cítrics. Les abelles mostrejades durant aquests episodis estaven intoxicades amb els insecticides *clorpirifòs* i *dimetoat*, com prèviament s'havia informat (**Calatayud-Vernich et al., 2015**). Ambdós compostos han sigut identificats com a responsables d'intoxicacions d'abelles en estudis de diferents països europeus (**Porrini et al., 2014**; **Kiljanek et al., 2016; Kiljanek et al., 2017**). Els $HQ_{\text{abelles mortes}}$ del maig del 2017 i 2018 van excedir des de 3 fins a 37 vegades el valor límit establert com a perill elevat per a la salut de les abelles.

En l'apiari 2, es van observar signes d'intoxicacions durant la floració dels nectariners (febrer del 2017) i dels cítrics (abril-maig del 2017 i 2018). Les abelles mortes mostrejades a febrer del 2017 estaven contaminades amb *imidacloprid*, utilitzat en els camps de nectariners propers. L'ús d'aquest neonicotinoide abans i durant la floració està prohibit des de 2013, i al 2018 es va prohibir completament el seu ús en exteriors (**EU regulation 2018/783**). Per tant, les deteccions d'aquest neonicotinoide suggereixen una violació de la regulació de la UE. Els nivells detectats a les mostres i la seua alta toxicitat el fan responsable de l'augment de la mortalitat durant aquest període (fins a 95 abelles mortes per dia). La seua contribució als $HQ_{\text{abelles mortes}}$ va ser elevada i va sobrepassar els 7.000 punts (Figura 4, Article 4). La taxa de mortalitat va augmentar la segona meitat d'abril, i a maig del 2017 es va arribar al punt més alt (> 200 abelles mortes per dia). Com va passar a l'apiari 1, els insecticides *clorpirifòs*, *dimetoat* i *ometoat* van ser utilitzats durant la floració i, en conseqüència, van intoxicar les abelles recol·lectores. Les mostres analitzades revelaren un perill elevat per a la salut de les abelles causat per aquests organofosforats ($HQ_{\text{abelles mortes}}$ > 4.700 punts). A l'abril del 2018, la mortalitat va augmentar fins a les 95 abelles mortes per dia. Les analítiques van revelar que les abelles estaven intoxicades amb els compostos *clorpirifòs*, *dimetoat* i *ometoat*, utilitzats irregularment durant la floració dels cítrics. L'*imidacloprid* va ser també detectat a les abelles durant aquest increment de la mortalitat i va tindre una contribució rellevant (360 punts) a l'HQ d'aquest episodi d'intoxicació.

La producció de mel i la població d'abella recol·lectora van disminuir substancialment a les colònies que patiren intoxicacions, i això provocà un greu afebliment de les colmenes, però sense arribar a produir-se'n el col·lapse. Tenint en compte l'impacte dels plaguicides sobre les colònies d'abelles mel·líferes, és necessari prendre mesures per a reduir els seus efectes i beneficiar la salut de tots els pol·linitzadors.

3.2 Perill dels plaguicides de l'interior de la colmena

3.2.1 Abelles vives

Els acaricides utilitzats en l'apicultura van ser la principal font de contaminació de les abelles vives analitzades en aquesta tesi. El *cumafòs* i el *fluvalinat* són poc tòxics per a les abelles (Taula 5, Capítol 1), i l'*amitraz* (el producte utilitzat en els apiaris durant els estudis) és més segur per a les abelles que la resta d'acaricides sintètics (**Gashout et al., 2018**). Com a resultat, el 95 % de les abelles presentaven un perill per plaguicides baix (HQ < 50), i sols 4 de les 83 mostres contenien un $HQ_{\text{abelles vives}}$ rellevant. Els insecticides *clorpirifòs* i *dimetoat* van ser responsables dels $HQ_{\text{abelles vives}}$ rellevants d'aquestes mostres, i les seues contribucions van variar entre els 100 i els 333 punts (Taula 4, Article 4). Aquests resultats estan en consonància amb les dades d'altres autors, on les abelles vives presentaven HQ generalment baixos (**Traynor et al., 2016**).

3.2.2 Pol·len

El pol·len procedent dels 45 apiaris espanyols tenia un HQ mitjà de 222, quatre voltes més gran que el límit inferior (50 punts) establert per als $HQ_{\text{pol·len}}$ rellevants (Article 2). Les mostres amb una càrrega de plaguicida que representava un perill baix i les mostres amb perill rellevant van tindre una freqüència del 49 %, mentre que les mostres amb un HQ elevat sols representaven el 2 % del total. Tot i que la majoria dels $HQ_{\text{pol·len}}$ més alts corresponien a mostres d'apiaris localitzats en entorns agrícoles, el principal contribuïdor als HQ va ser l'*acrinatrín*, un acaricida no autoritzat contra la varroosi i que probablement va ser transferit des de la cera fins al pol·len (Figura 3, Article 2). Les mostres on els insecticides *clorpirifòs* i *dimetoat* van contribuir de forma rellevant als HQ (> 100 punts) procedien d'apiaris situats prop d'ambients amb agricultura intensiva.

Des de juny del 2016 fins a juny del 2018, els apiaris experimentals situats en entorns agrícoles van mostrar uns valors mitjans d'$HQ_{\text{pol·len}}$ entre 6 i 7 vegades més alts que l'$HQ_{\text{pol·len}}$ de l'apiari situat en un ambient de vegetació silvestre i amb menor pressió agrícola (Article 4). Aquest apiari va mostrar un $HQ_{\text{pol·len}}$ baix en més del 90 % de les seues mostres. Per contra, el pol·len dels apiaris situats en contextos agrícoles va obtindre $HQ_{\text{pol·len}}$ considerats com a rellevants en més del 50 % de les mostres (Figura 2, Article 4). Per tant, els entorns dels apiaris van influenciar els $HQ_{\text{pol·len}}$ (**Colwell et al., 2017**). Encara que l'amitraz i el cumafòs van ser detectats en quasi totes les mostres, les contribucions d'ambdós acaricides als $HQ_{\text{pol·len}}$ va ser baixa i no va sobrepassar els 38 punts (Taula 2, Article 4). Les contribucions dels acaricides no emprats en els apiaris experimentals com el *fluvalinat*, el *clorfenvinfòs* i l'*acrinatrín* als HQ_{pollen} van ser molt baixes (< 5 punts) i no van suposar un perill substancial per a la salut de les colònies; amb l'excepció de l'*acrinatrín*, que va tindre contribucions baixes però també rellevants (> 300 punts) als $HQ_{\text{pol·len}}$. El *clorpirifòs* va ser responsable de les contribucions als $HQ_{\text{pol·len}}$ més elevades (fins als 696 punts). Aquest organofosforat és l'insecticida més freqüent als diferents productes de la colmena a tot el món, i els nivells detectats en el pol·len i el pa d'abella

representen una amenaça per a la salut de l'abella mel·lífera (**Mullin et al., 2010; Tosi et al., 2017**). El *dimetoat* va tindre contribucions rellevants als $HQ_{pol·len}$ (200 punts) durant el flux de nèctar de l'any 2018. Ambdós organofosforats (*clorpirifòs* i *dimetoat*) van mostrar contribucions importants als $HQ_{pol·len}$ dels apiaris experimentals situats en entorns agraris durant aquest mateix any. Les aportacions de l'*imidacloprid* i del *metiocarb* als $HQ_{pol·len}$ van ser rellevants i fins als 350 punts. Concentracions baixes d'aquest neonicotinoide, com les detectades en aquest estudi, són suficients per a provocar alteracions fisiològiques i reduir les motivacions de recol·lecció d'altres espècies de pol·linitzadors (**Lamsa et al., 2018; Cook, 2019**). Els fungicides *carbendazim* i *tebuconazol*, detectats al 30 i al 6 % de les mostres, van tindre contribucions insignificants als $HQ_{pol·len}$ (< 1 punt). En general, la toxicitat dels fungicides es considera baixa per a les abelles, i, en l'enfocament dels HQ plantejat en aquesta tesi, els efectes indirectes dels fungicides sobre les colònies no estan contemplats. No obstant això, els fungicides redueixen la població de fongs simbiòtics beneficiosos, que són crucials en la maduració del pol·len a pa d'abella. Per tant, és d'esperar que la qualitat nutricional del pa d'abella contaminat amb fungicides siga pitjor i que la salut de l'abella s'hi veja afectada negativament (**Yoder et al., 2012; Steffan et al., 2017**).

3.2.3 Cera d'abella

La cera d'abella és el producte de la colmena amb més perill per càrrega de plaguicida (Article 2 i Article 5). L'HQ_{cera} mitjà era 30 vegades més elevat que l'$HQ_{pol·len}$, i 300 vegades superior a l'$HQ_{abelles\ vives}$. Però, s'ha de tindre en compte que en la matriu de la cera sols una fracció de la càrrega de plaguicida detectada està en contacte amb les abelles (p. ex., les molècules de la superfície) i, per tant, els HQ calculats en aquesta matriu sobreestimen l'exposició real d'aquestes als plaguicides.

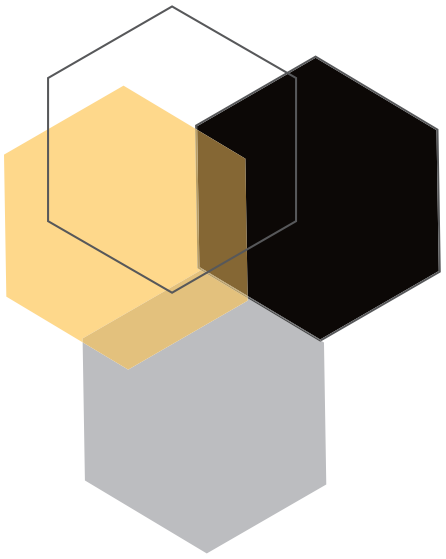
La mitjana del perill pel contingut de plaguicides de les diferents fonts de cera va ser elevada: bresques (HQ = 6.948), làmines (HQ = 6.283), bresques antigues (HQ = 5.775). L'HQ calculat a la cera d'opercle va ser considerat rellevant i de 4.188 punts. L'acaricida *acrinatrín* va ser el principal contribuïdor als HQ_{cera}. En la puntuació d'HQ_{cera} més elevada (44.544), l'*acrinatrín* era responsable de 44.118 punts. La *flumetrina* i el *clorpirifòs* van contribuir substancialment a les puntuacions dels HQ_{cera} (Figura 4, Article 5; Figura 3, Article 2). Tot i les concentracions elevades de *cumafòs, clorfenvinfòs, fluvalinat* i *amitraz (DMF)*, les seues aportacions als HQ_{cera} eren majoritàriament residuals.

Sobre la base de les assumpcions del model HQ, una abella nodrissa que s'alimentara del pol·len amb l'HQ més alt (3.829) (Taula S5, Article 2) estaria consumint el 38 % de la DL_{50} d'*acrinatrín*, el 0,12 % de la DL_{50} del *cumafòs* i el 0,005 % de la DL_{50} del *fluvalinat* (durant els 10 primers dies del seu desenvolupament). Si, a més, considerem la càrrega tòxica de la cera d'aquestes colònies (HQ_{cera} = 44544), la salut de les abelles podria estar seriosament compromesa.

La majoria dels acaricides i plaguicides trobats en la cera no són molt tòxics de forma aïllada per a les

abelles, però en combinació la seua toxicitat pot veure's augmentada sinèrgicament (**Johnson et al., 2013**). En aquest context, el desenvolupament i la longevitat de les obreres, així com el vigor de la colmena, es veuen afectats quan la colònia conté bresques amb nivells subletals de plaguicides (**Bevk et al., 2012; Wu et al., 2011**). A més, s'han descrit reaccions perjudicials i amb sinergia quan el *cumafòs* i el *fluvalinat* estan presents simultàniament a la colmena (**Johnson et al., 2009; Zhu et al., 2014**). Aquests dos compostos afecten el desenvolupament motor i sexual d'abellots i reines (**Rinderer et al., 1999; Haarmann et al., 2002; Collins et al., 2004**). Les càrregues altes d'acaricides, com les detectades en aquesta tesi, creen un ambient propici per a l'aparició de varroes resistents als acaricides (**Bogdanov et al., 1998; Gonzalez-Cabrera et al., 2016**).

04 ELS PLAGUICIDES EN LA CERA D'ABELLA: UN PROBLEMA D'ACTUALITAT



L'obtenció de cera amb la menor quantitat de plaguicides possible és de vital importància per a reduir l'impacte d'aquests sobre la salut de les abelles, evitar la pressió selectiva sobre àcars resistents i disminuir-ne la transferència a les abelles i resta de productes de la colmena. Actualment, si els apicultors volen reduir el contingut de plaguicides de les seues bresques, han de reemplaçar la cera vella i contaminada per làmines de cera menys contaminades. Malauradament, la indústria de la cera utilitza principalment cera de bresques velles (altament contaminada) per a la producció de "noves" làmines; açò crea un cercle viciós on els residus dels plaguicides es mantenen i la cera que entra nova es contamina amb aquests. La cera d'abella també s'utilitza en la indústria cosmètica, alimentària i farmacèutica, en nombrosos productes com ara pintallavis, cremes facials, recobriments de pastilles i xiclets. Degut al fet que molts dels plaguicides presents en la cera poden actuar com a

disruptors endocrins, el desenvolupament de mètodes de descontaminació de la cera no sols beneficiaria l'apicultura, sinó que també tindria un impacte positiu en la salut humana.

El treball presentat en aquesta tesi (Article 5, Capítol 5) és un estudi preliminar realitzat durant l'estada doctoral a la *University of Maryland* (EUA) sota la supervisió del Dr. Dennis VanEngelsdorp. Aquest article tracta sobre la neteja de la cera d'abella mitjançant l'extracció dels plaguicides amb dissolvents.

Fins ara, el nombre de mètodes proposats per a la descontaminació dels plaguicides de la cera ha sigut bastant escàs. Alguns mètodes proposen la utilització d'adsorbents sòlids, com la patent US6586610B2 (**Ulrich, 2019**). **Serra-Bonvehí** i **Orantes-Bermejo**, (**2017**) van provar que el carbó activat era capaç d'eliminar més del 95 % de dos organofosfats com el *cumafòs* i el *clorfenvinfòs*, àmpliament detectats a la cera. Però, aquest adsorbent sols va eliminar el 35 % d'un altre compost àmpliament detectat en la cera, el *fluvalinat*. El nostre estudi va demostrar que la neteja amb dissolvents orgànics és capaç d'eliminar plaguicides de diferents famílies. Plaguicides organofosfats, carboxamides, piretroides i d'altres famílies van ser extrets de la cera > 95 %. El contingut en plaguicides de les mostres va ser reduït de nivells de $\mu g \cdot g^{-1}$ fins a menys de 10 $ng \cdot g^{-1}$ en tots els casos (Taula 1-2-3, Article 5). Encara que la textura de la cera va canviar després de les extraccions, era vàlida per a ser utilitzada en la indústria farmacèutica i cosmètica. No obstant, s'ha de remarcar que aquest mètode és solament factible a xicoteta escala perquè consumeix grans quantitats de dissolvents durant les extraccions. Per poder aplicar aquesta metodologia a una escala més gran, s'hauria de dissenyar un extractor en continu per tal de reduir el volum de dissolvents emprats i minimitzar-ne, així, el cost mediambiental i econòmic.

05

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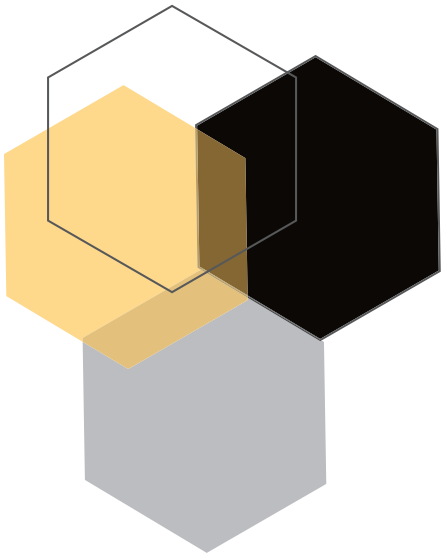

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CONCLUSIONS

1. La metodologia de QuEChERS, lleugerament modificada per a cada matriu, acoblada a la cromatografia líquida d'alta eficàcia i espectrometria de masses en tàndem va mostrar una exactitud, precisió, sensibilitat i rapidesa adequades per a la determinació dels plaguicides en les abelles, el pol·len i la cera.

2. Els acaricides utilitzats en l'apicultura (p.ex. coumaphos) van ser els plaguicides més freqüents en la cera, el pol·len i les abelles vives, mentre que els insecticides foren els compostos més detectats a les abelles mortes.

3. La cera d'abella és la matriu més contaminada de la colmena quant a les concentracions dels plaguicides detectades, mentre que el pol·len va revelar el major nombre de residus de plaguicides diferents. Les abelles vives van mostrar uns nivells de plaguicides substancialment menors.

4. Els episodis d'intoxicació van ocórrer solament als apiaris situats en entorns agrícoles, i les mostres d'abelles mortes van mostrar alts nivells dels insecticides utilitzats al camp; clorpirifòs, dimetoat i imidacloprid.

5. El pol·len procedent d'apiaris situats en entorns d'agricultura intensiva contenia nivells de plaguicides significativament superiors respecte al pol·len d'apiaris situats en zones rurals, deveses i entorns de vegetació silvestre.

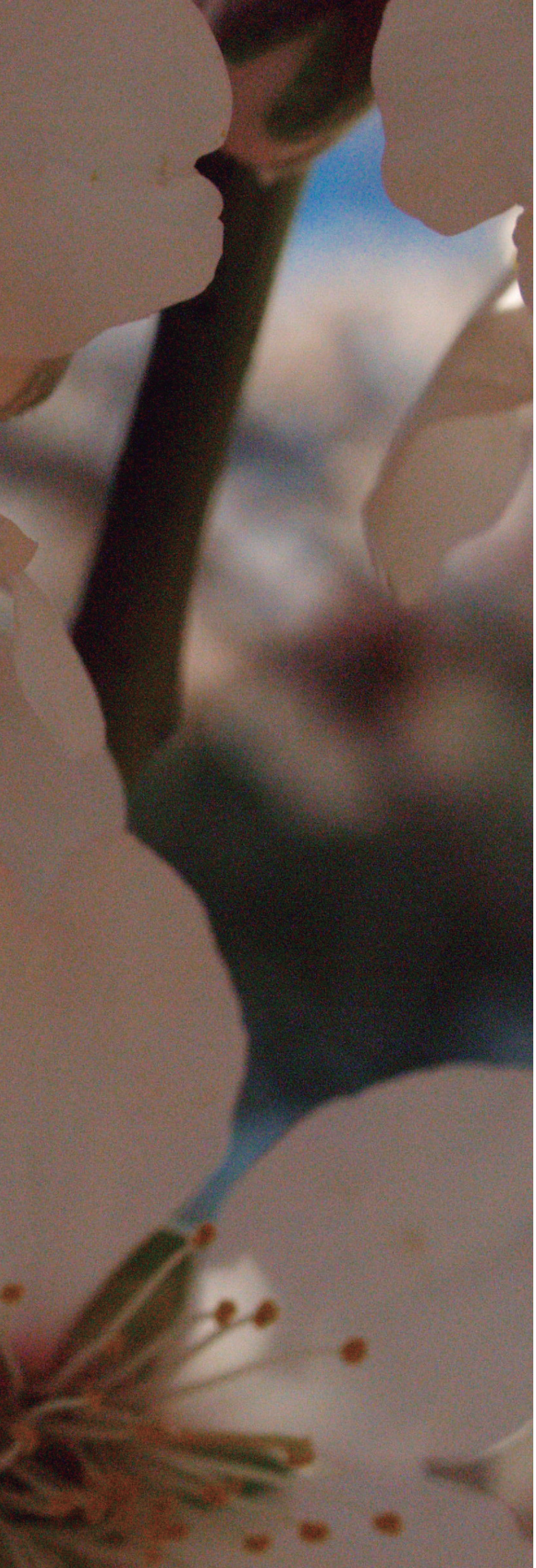
6. La cera d'abella va ser la matriu amb el major perill per plaguicides per a les abelles, i l'acrinathrin va ser el principal contribuïdor als HQ. No obstant això, l'exposició real als plaguicides en aquesta matriu està sobreestimada. El perill per plaguicides presents al pol·len va ser considerat com a rellevant per a la salut de les abelles, i els majors contribuïdors a les puntuacions dels HQ van ser el chlorpyrifos i l'acrinathrin. Els HQ de les abelles vives van ser considerats baixos.

7. Tenint en compte les elevades concentracions de plaguicides trobades a les bresques i les làmines, es va demostrar que els productors de cera utilitzen principalment cera de bresques velles per produir noves làmines. Es recomana encaridament l'ús de cera menys contaminada, com la cera d'opercle, durant la producció de noves làmines per tal de diluir els plaguicides acumulats en aquesta matriu i previndre futures transferències cap a les abelles o altres matrius apícoles.

8. L'ús de dissolvents orgànics és un metodologia capaç d'extraure els plaguicides presents en la cera d'abella.

9. És important considerar l'entorn dels apiaris per evitar episodis d'intoxicació d'abelles. A més, reduir l'ús d'acaricides persistents contra la varroosis a l'interior de les colmenes és altament recomanat per disminuir l'exposició a aquestos i millorar la salut de l'abella.

10. La gran quantitat de fitosanitaris trobats al pol·len i a les abelles mortes ha de fer reflexionar sobre l'ús sistemàtic d'aquests compostos a l'agricultura moderna. Les abelles mel·líferes i els pol·linitzadors natius deurien ser valorats com a components essencials en els agro-ecosistemes per tal de desenvolupar una forma més sostenible de gestionar les zones agrícoles.



ANNEX

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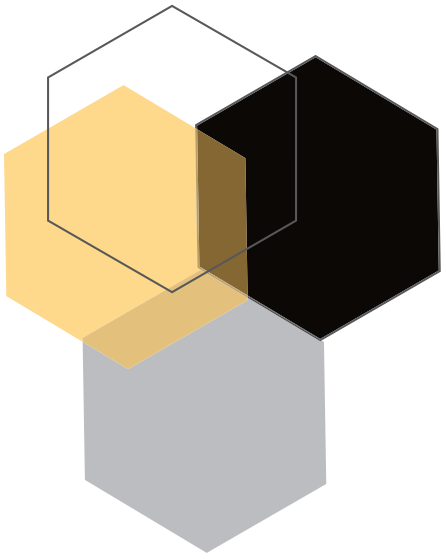


GLOSSARY OF BEEKEEPING TERMS

Apis mellifera

Species of an eusocial flying hymenopter from apidae family. Western honey bee originating in southern Asia, and now located around the world. They are known for the large size of their colonies and for their surplus production and storage of honey.

Apiary

A place in which a number of beehives are kept; also known as a bee yard.

Beebread

Pollen pellets that are packed in the combs by honey bees, with a small cover of honey and glandular secretions, bacteria and mold, resulting in a fermented mixture.

Brood

Immature stages of bees that not yet emerged from their cells. Brood can be in the form of eggs, larvae, or pupae of different ages.

Brood chamber

A part of the hive, usually in the bottom boxes. Box or boxes containing the combs of the brood nest and main food storage of the colony.

Brood nest

Combs containing the brood of the colony.

Cappings

A thin layer of beeswax used to cover the full cells of maturated honey. This layer of wax is sliced from the surface of a honey-filled comb during honey extraction.

Cocoon

The silky envelope spun by last stage larvae, serving as a protective covering while they are developing.

Comb

A structure of hexagonal prismatic wax cells built by honey bees in which brood is reared and honey and pollen are stored. A comb filled with honey is a honeycomb and a comb essentially filled with brood is a broodcomb.

Drone

A drone is a male honey bee. Unlike the female worker bee, drones do not have stings and gather neither nectar nor pollen. A drone's primary role is to fecundate the queen during mating flights.

Forager bee

Worker bees generally two to three weeks old that work to collect nectar, pollen, water and resins for the colony. Foraging is the last task in the life of a worker. Also known as field bees.

Foundation

An artificially and commercially made structure consisting of thin sheets of beeswax with the cell bases of worker cells embossed on both sides in the same manner as they are produced naturally by honey bees.

Frame

A piece of equipment made of either wood or plastic designed to hold a comb.

Hive

A shelter structure, generally a wooden box, constructed for housing a colony of honeybees.

Larva

The immature feeding stage of a bee hatched from the egg; with a white, legless, grub-like appearance.

Nurse bee

The young worker bees, five to ten days old, which feed and take care of developing brood.

Pollen

The male reproductive cell bodies produced by anthers of flowers. It is collected and used by honey bees as their only protein source.

Propolis

Resinous materials collected from trees or plants by bees with antimicrobial properties, playing an important role in the social immunity of the colony. It is also used to coat the interior hive walls, strengthen the comb, seal cracks and reduce openings; also called bee glue.

Pupa

The last immature stage in the development of the honey bee, during which it changes (in capped cells) from a larva to an adult bee.

Queen

A female bee with a fully developed reproductive system, larger than a worker bee and responsible for laying fertile eggs. Queens are developed from larvae selected by worker bees and specially fed in order to become sexually mature. Queens are raised in specially constructed queen cells.

Supers

Any hive body, or smaller box, used for the storage of surplus honey, which the beekeeper will harvest. Normally it is placed over the brood chamber.

Varroa destructor

An ectoparasitic mite that infest honey bee colonies and feeds on the honey bees' fat body. The varroa reproduces on pupae. *Varroa destructor* is the greatest single driver of the global honey bee colonies decline.

Worker bee

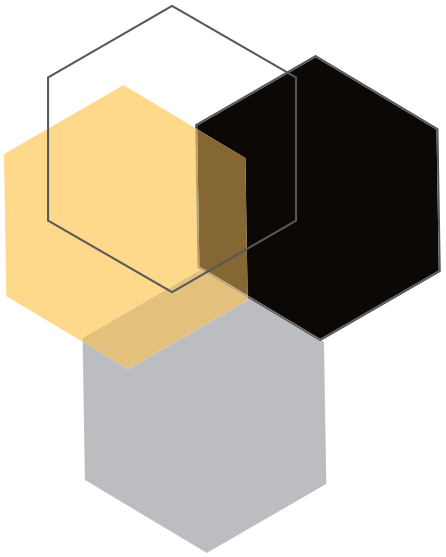
A female bee whose reproductive organs are undeveloped. The most numerous caste of individuals that undergoes all the tasks (nursing, cleaning, guarding, foraging…) of the colony.

Sources:

(Betterbee.com, 2019; Wordreference.com, 2019; Wikipedia.com, 2019)

02

GLOSSARI DE TERMES APÍCOLES



Abella nodrissa

Abella obrera jove, de cinc a deu dies de vida, que s'encarrega d'alimentar i de cuidar a la cria.

Abella obrera

Abella femella amb òrgans reproductius no desenvolupats. És la casta més nombrosa de la colònia i s'encarrega de totes les tasques (tenir cura de la cria, neteja, guàrdia, recol·lecció...).

Abella recol·lectora

Abella obrera que en complir aproximadament dos setmanes de vida comença a recol·lectar nèctar, pol·len, aigua i pròpolis per a la colònia. L'activitat recol·lectora és l'última tasca en la vida d'una abella obrera. També coneguda com abella de camp.

Abella reina

Abella femella amb el sistema reproductiu totalment desenvolupat, de major tamany que una abella obrera i responsable de posar ous fecundats. Les reines es desenvolupen a partir de larves seleccionades per abelles obreres i són alimentades de forma especial per poder desenvolupar òrgans sexuals madurs. Aquestes es desenvolupen en cel·les especials anomenades cel·les reals o reialeres.

Abellot

Abella mascle. A diferència de les obreres femelles, els abellots no tenen agulló i no recol·lecten nèctar ni pol·len. El rol principal d'un abellot és el de fecundar una reina durant els vols d'emparellament.

Alces

Cos de la colmena, normalment situat per damunt de la cambra de cria i utilitzat per a l'emmagatzematge extra de la mel, d'on l'apicultor extraurà la collita.

Apiari

Lloc on s'instal·len un nombre més o menys gran de colmenes. També anomenat colmenar.

Apis mellifera

Espècie d'himenòpter eusocial volador de la família *apidae*. Coneguda com abella de l'oest, és originària del sud-est asiàtic i és troba a tot el món. Han sigut tradicionalment utilitzades en l'apicultura per a extraure la mel i altres productes.

Bresca

Estructura de cera construïda per les abelles, composta de cel·les prismàtiques hexagonals i destinada a l'emmagatzematge de mel, pol·len i com a receptacle de la cria. Una bresca plena de mel s'anomena bresca de mel i una bresca amb molta cria s'anomena bresca de cria.

Cambra de cria

Part de la colmena, sovint a la part inferior, que conté les bresques de cria de la colònia.

Capoll

Coberta de seda secretada per les larves i que servix de protecció mentre aquestes es desenvolupen.

Cera de làmina

Estructura feta artificialment que consisteix en làmines de cera d'abella estampades amb cel·les hexagonals, d'uns mil·límetres de grossària i que servix com a base per a la construcció de la bresca per part de les abelles.

Cera d'opercle

Capa fina de cera d'abella que aquestes fan servir per a cobrir les cel·les plenes de mel madura. Aquesta capa de cera és eliminada de la superfície de les cel·les durant el procés d'extracció de la mel.

Colmena

Estructura, generalment de fusta, construïda per donar refugi a una colònia d'abelles.

Cria

Abelles immadures que no han emergit de les seves cel·les. La cria està composta per ous, larves i pupes en diferent estadis.

Larva

Estat d'abella immadur posterior a l'eclosió de l'ou, amb capacitat d'alimentar-se, blanca, sense potes i vermiforme.

Marc

Element de la colmena, fet de plàstic o de fusta, dissenyat per a la subjecció de les bresques.

Niu de cria

Totes les bresques que contenen cria en una colònia.

Pa d'abella

Pol·len empaquetat a les bresques per les abelles amb una coberta de mel i secrecions glandulars, que fermenta amb l'ajuda de bacteris i fongs.

Pol·len

En les plantes fanerògames, polsina formada en l'antera i constituïda per cèl·lules masculines. Aquest és recollit per les abelles i utilitzat com a única font de proteïna de la colònia.

Pròpolis

Substància resinosa que les abelles arrepleguen de les gemmes d'alguns arbres, amb activitat antimicrobiana i un paper molt important en la immunitat social de la colònia. També s'utilitza per revestir les parets de la colmena, per a tapar clevills, reduir la entrada de la colmena i per a reforçar les bresques.

Pupa

L'últim estat immadur en el desenvolupament d'una abella, durant el qual canvia de larva a abella adulta dins de cel·les operculades.

Varroa destructor

Àcar ectoparàsit que infesta les colònies i s'alimenta del cos gras de les abelles. La varroa es reprodueix en les pupes. *Varroa destructor* és considerat com el principal causant del declivi mundial de les colònies d'abelles mel·líferes.

Fonts:

(avl.gva.es, 2019)

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