5.5 Pesticide and Metabolites Residues in Honeybees: A 2014-2017 Greek Compendium

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In the period between 2014 mid-2017, more than 200 samples of honeybees were sent by authorities and individuals in Benaki Phytopathological Institute after incidents of unexpected deaths of bees in various parts of Greece. The samples were analyzed for pesticides and breakdown products, by two multi-residue methods based on an expanded HPLC-ESI-MS/MS and a newly developed GC-MS/MS method. Sample preparation was optimized and based on modified QuEChERS using for clean-up C18 and PSA.

Until mid-2017, 293 detections were registered in a total of 205 honeybee samples, resulting in a 76% percent of positive samples, to at least one active substance. Concentrations' range varied from 1 to 160000 ng/g $_{\text{bee body weight}}$. In some cases, these levels surpassed LD_{50} values indicating intoxication events.

Predominant substances were clothianidin, coumaphos, imidacloprid, acetamiprid and dimethoate. In less extent, other acaricides such as amitraz (mostly its breakdown products DMF, DMPF), tau-fluvalinate and certain pyrethroids exemplified by cyhalothrin, cypermethrin and deltamethrin were also recorded. In several samples, more than one active substance was detected.

Overall, this work aspires to provide valuable insight to pesticides and metabolites occurrence in honeybees in Greece between 2014-2017 and in parallel assist research community and apiculturists in this pivotal Mediterranean region that bee health and pollination services have prolific importance.

Introduction

Honeybee's death incidents are of great concern because declines in bee populations might have detrimental effect on agriculture and environment, affecting for some crops, pollination, and disrupting the stability of the agricultural ecosystems. The use of pesticides in agricultural cropping systems is often discussed as a factor influencing bee health (Johnson et al., 2010). Single events of poisonings by spray applications have been reported in many countries and by our group at the onset and middle of this decade (Kasiotis et al., 2014).

The presented study was pursued in the frames of the necessity to monitor pesticide residues in honeybess, after relatively constant incidents that have taken place in Greece since 2014 till the middle of 2017 and determine pesticide and metabolites residues in honeybees. The possibility of detecting several subsatnces in honeybee bodies and the possible synergsistic effects that they can elicit, intrigued us to expand the scope of the previously published LC-ESI-MS/MS method of our group to end up monitoring 150 active substances. In the same context, a complementary GC-MS/MS method was developed and validated, encompassing mainly pyrethroids to monitor 10 active substances. With regard to sample preparation approach, slight amendements to our previous work on the QuEChERS methodology were implemented.

Materials and Methods

Chemicals and solutions

Certified pesticide standards (purity >90%) were purchased from Sigma-Aldrich (Büchs, Switzerland), Dr. Ehrenstorfer GmbH (Augsburg, Germany), ChemService (Milan, Italy). Methanol, acetonitrile and water were purchased from Merck (Darmstadt, Germany) and were LC-MS grade. Magnesium sulphate anhydrous ($MgSO_4$) was purchased from Agilent Technologies, primary-secondary amine (PSA) from Interchim, Z-Sep from Supelco, endcapped C18 from Macherey-Nagel (Germany), while sodium acetate (NaOAc) from Panreac Quimica SAU (Barcelona, Spain).

Sample Collection-Regions with Incidents

Control honeybee samples were provided by Agricultural University of Athens, Greece experimental apiaries (Professor Harizanis), previously checked for interferences. The investigated samples were collected by individual beekeepers or veterinary authorities. Honeybee samples were collected very near or at the entrance of the hives. Special precaution was given to the transportation of the samples. The samples were immediately cooled at 0 °C with ice-packs or at -78 °C with dry ice (if available), packed and sent the same or early next day to the laboratory. After reaching laboratory, the samples were stored at -78 °C until analysis.

LC-MS/MS instrumentation, chromatographic and mass spectrometry conditions

An Agilent Technologies 6410 Triple Quad LC/MS system was used. The LC separation was achieved after injecting 10 µL of sample on a reversed phase column (ZORBAX Eclipse XDB-C_{18} Agilent, 2.1 x 150mm, 3.5µ) using a gradient system identical to the previously reported of our group. The mass spectrometer was operated in Multiple Reaction Monitoring (MRM) mode with positive and/or negative Electron Spray Ionization (ESI). Nitrogen was used as nebulizer and collision gas. For instrument control, Agilent Mass Hunter data acquisition Triple Quad B.01.04 and for data processing Agilent MassHunter Workstation Qualitative Analysis B.01.04. were used.

GC-MS/MS instrumentation, chromatographic and mass spectrometry conditions

The GC-MS/MS analysis was performed on a Chromtech Evolution MS/MS triple quadrupole mass spectrometer built on an Agilent 5975 B inert XL EI/CI MSD system. Samples were injected with a Gerstel MPS-2 autosampler using a 10 µL syringe. Separations were performed on a HP-5ms UI, length 30m, ID 0.25mm, film thick. 0.25 µm (J&W Folsom, USA). Helium was used as the carrier gas at a flow rate of 1.2 mL min^{-1}. The QqQ mass spectrometer was operated in EI-MS/MS mode in Multiple Reaction Monitoring (MRM) data acquisition mode. The transfer line, manifold and source of ionization temperatures were 300, 40 and 230°C. For the MS/MS experiments Argon 99.999% was used as a collision gas and the collision cell pressure was set at 1.7mTorr. The electron multiplier voltage was set at 2000 V. The total GC analysis time was 25 min.

Sample preparation

1g of bees were placed in a beaker and extracted by means of acetonitrile (ACN 7mL), hexane (3 mL) and deionised water (3 mL) using an Ultra Turax homogenizer for 5 minutes. Afterwards, 0.5 g magnesium sulphate anhydrous ($MgSO_4$), 0.2 g of sodium acetate (NaOAc) and 0.2 g of primary-secondary amine (PSA) were added and the mixture was vortex shaken for 2 min at 2500 rows per min. Then samples were centrifuged for 5 min at 4000 rpm. The organic layer was transferred to a new falcon tube containing $MgSO_4$ (0.5 g), PSA (0.1g) and C18ec (0.05 g). The mixture was vortex-mixed for 1 min, the organic phase was decanted, and evaporated to dryness under a vacuum resulted. The dry concentrate was reconstituted in 1mL of a ACN/H_2O (3:2) solution. For the GC-MS/MS, reconstitution was carried out with ACN. Finally, the sample was filtered with a PTFE disk with 0.45 µm pore size (CHROMAFIL ®Xtra PTFE-45/25, Macherey-Nagel) into the respective vials.

Analytical Method Validation

The method was validated following in principal SANTE/11945/2015 guideline. Good recoveries were observed for the majority of analytes that varied between 70 and 120% with relative standard deviations of <20% in most cases. Limits of Quantitation (LOQs) varied from 1 to 10 ng/g depending on the analyte.

Results

In this work, the existing sample preparation of our group was optimized by testing several materials (Z-Sep, C18 ec..) involved in the clean-up ff the samples. The modified QuEChERS performed adequately when apart from PSA, endcapped C18 was introduced in the clean-up step in dispersive mode.

Table 1 Indicative results for pesticides and metabolites residues in honeybees in Greece (2014-2017)

Bees Sum Detections 2014-2017*	Active Substance	Percent	Concentration Range (ng/g $_{\text{bee body weight}}$)
59	Clothianidin	21.0	1.2-174.2
41	Coumaphos	14.6	3.4-60057
41	Imidacloprid	14.6	1-6906.7
16	Acetamiprid	5.7	1.1-698.4
15	Dimethoate	5.3	7.7-123400
8	Thiamethoxam	2.8	0.7-126
8	Thiacloprid	2.8	0.9-295.9
8	Carbendazim	2.8	1.7-10.7
7	Tau-fluvalinate	2.5	10.1-1316
6	Fipronil Sulfone	2.1	1.3-93.8
6	DMF	2.1	8.6-223.9
6	DMPF	2.1	10.3-230.7
5	Methomyl	1,8	2120-166555
4	Spinosad A	1,4	1.5-6.7
3	Cyhalotrhin	1,1	101-899
3	Cyprodinil	1,1	65.3-392.5
3	Cypermethrin	1,1	21.1-1503
3	Tebuconazole	1,1	5.9-522.2
3	Azimsulfuron	1,1	1,8-4,5
3	Etoxazole	1,1	3.4-9.7
2	Deltamethrin	0,7	513-822

*overall 293 detections were registered

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Predominant substances in terms of number of detections were clothianidin, coumaphos, imidacloprid, acetamiprid and dimethoate. In less extent, other acaricides such as amitraz (mostly its breakdown products DMF, DMPF), tau-fluvalinate and certain pyrethroids exemplified by cyhalothrin, cypermethrin and deltamethrin were also recorded. In several samples, more than one active substance was detected.

It is noteworthy that the majority of pyrethroids, amitraz and tau-fluvalinate were incorporated to the analytical portfolio the last 1.5 years. Hence, this is probably one of the reasons that their prevalence is lower than coumaphos or neonicotinoidss. However, retrospective analyses are currently underway (depending on matrix availability and analytes stability) to disclose additional results and harmonize the findings for all major classes of pesticides-acaricides. The latter is expected to augment detections of certain active substances and breakdown products.

With regard to particular neonicotinoids prevalence-fluctuations after the banning of seed treatment products no clear trend was observed. Last but not least, a high-resolution mass spectrometry untargeted approach is now finalized that for certain cases such as breakdown products is of significant value. Certain metabolites such as 5 hydroxy imidacloprid, imidacloprid olefin, imidacloprid urea, 6-chloro nicotinic acid, coumaphos oxon, and desmethyl-acetamirpid were identified using this approach.

References

Johnson RM, Ellis MD, Mullin CA, Frazier M. Pesticides and honey bee toxicity - USA. Apidologie 2010; 41: 312-331.

Kasiotis KM, Anagnostopoulos C, Anastasiadou P, Machera K. Pesticide residues in honeybees, honey and bee pollen by LC-MS/MS screening: reported death incidents in honeybees. Sci Total Environ 2014; 485-486: 633-642.