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Determination of selected environmental contaminants in foraging honeybees

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ABSTRACT

Colony losses of honeybees have been of great concern in the last years. To explain these losses, several studies have been reported, and various factors, such as pathogens and pesticides, have been considered as possible causes. Nevertheless, organic contaminants, rather than pesticides, are continuously released to the environment, and can be intercepted by honeybees during foraging with the possible consequent damage. Azoles and organophosphorus esters have been selected in this work as environmental contaminants to be monitored in honeybees. A fast and robust method has been developed to determine these organic pollutants in honeybees. It is based on matrix solid phase dispersion (MSPD), which performs sample dispersion with extraction and clean up in the same step, followed by LC-ESI-MS/MS determination. Recoveries of the method varied between 73% and 119% and MQLs ranged from 0.8 to 4 ng g^{-1}. Honeybee samples from ten apiaries located in different regions were analyzed applying the developed method. Azole compounds were found at low levels, but not in all samples, while organophosphorus esters were found in most samples whatever location. Tris-(2-chloroisopropyl) phosphate, TCPP, and tributyl phosphate, TBP, were detected in all honeybees samples at levels higher than the rest of organophosphates analyzed.

1. Introduction

Honeybees (*Apis melifera* L.) are vital as pollinators of crops and they are bred commercially for their abilities to produce honey and pollinate crops. Nevertheless, annual losses of honeybee colonies averaged about 33 percent each year since the winter of 2006, which could threaten the economic viability of the bee pollination industry. Among the factors that could be the cause of honeybees disappearance, the pesticides, the infection by pathogens such as viruses, bacteria, fungi and parasites; poor nutrition or the global warming, are the most studied [1–5]. Furthermore, bee populations may also be vulnerable to contaminants in the environment, generated by the industrialization, housing development and agricultural practices (i.e. via sewage sludge amendments).

Organic environmental contaminants such as organophosphorus (OPs) and azole compounds have been selected for this study. OPs are widely used as flame retardants and plasticizers in different types of materials (building materials, electronic equipment, plastics….). They are used as additives and are not chemically bound in the material, therefore they can be released to the surrounding environment. OPs have been found in the environment, mainly in water and in air [6–10]. Triphenyl phosphate (TPhP) and tributyl phosphate (TBP) are suspected to be neurotoxic [11,12] while others such as tris-(2-chloroethyl) phosphate (TCEP), tris-(1,3-dichloro isopropyl) phosphate (TDCPP) or tris-(2-chloro- isopropyl) phosphate (TCPP) are carcinogenic [13,14].

Azoles are used as fungicides in agriculture, as biocides for commodities and material protection, and as antimycotic pharmaceuticals for animals and humans. These compounds are considered as a new group of endocrine-active agents in humans and animals, disturbing the biosynthesis of steroids by inhibition of enzymes like aromatase (CYP19) [15,16]. Azoles may enter the wastewater after use in households, agricultural fields and farms, and runoff into sewage. They can be found in water or in sewage sludge, which is other route of entrance into the environment [17–21].

OPs and azoles may be present in soil, water and air. Honeybees forage around their hive areas, collecting water, nectar and pollen from flowers: during this activity, honeybees can intercept environmental contaminants in their dense body hair, via inhalation or by intake of the pollutants present in pollen or water. Honeybees or even beehive products have been used as bioindicadors of environmental pollution, but in relation to presence of pesticide residues, heavy metals and radionuclides. To our knowledge, there are very few studies concerning environmental contaminants in

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honeybees, such as the determination of polycyclic aromatic hydrocarbons (PAHs) in honeybees [22,23] and the analysis of polychlorinated byphenyls (PCBs) [24] and brominated flame retardants (BFRs) [25,26] in honey samples.

In this work, a method to analyze OPs flame retardants and azole compounds in honeybees was developed to improve knowledge about potential exposure of honeybees to environmental contaminants. These compounds, which are widespread in the environment, were chosen. Azole compounds are endocrine-active agents inhibiting the synthesis of ergosterol that might affect honeybees. OP compounds have a chemical structure similar to that of OP insecticides and might affect the nervous system of honeybees by inhibiting the cholinesterase enzyme.

The difficulty for the extraction of those chemicals from honeybee samples is the content in wax, proteins and other substances that can interfere in the analytical determination. Reported methods that deal with the extraction of pesticide residues from honeybees are based on several modifications of the QuEChERS method, [27], followed by GC or LC determination [28–31]. Only few reported studies make use of MSPD (matrix solid phase dispersion) method to extract pesticides from honeybees [32–34]. MSPD has the advantage of using low amount of sample and solvent and, in addition, homogenization, extraction and purification are combined in the same step. Disruption of the honeybee structure is achieved without using freeze drying or blending with a disperser or mixer which requires a higher amount of sample. In order to provide a miniaturized method, we developed this procedure for the extraction of azoles and OPs in honeybees.

The aim of this work was to optimize a rapid and robust method for the simultaneous extraction of OPs and azole compounds from honeybees and subsequent quantitation by LC–MS/MS. MSPD based method was carried out for the extraction and purification. The method was applied for the determination of concentration levels of OPs and azoles in honeybees collected at various locations.

2. Experimental

2.1. Chemicals and standards

Tris-(2-butoxyethyl) phosphate (TBEP), tris- (2-chloroethyl) phosphate (TCEP), tributyl phosphate (TBP), triphenyl phosphate (TPhP), tris-(1,3-dichloro) isopropyl phosphate (TDCPP) and tris-(2-chloro)- isopropyl phosphate (TCPP) and fluconazole, clotrimazole, propiconazole and tebuconazole were provided by Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Trace analysis quality solvents, acetonitrile and n-hexane were supplied by AppliChem Panreac (Darmstadt, Germany) and Scharlab Chemie S.A. (Barcelona, Spain), respectively.

Florisil (60–100 mesh), aluminium oxide 90 standarized, silica (35–70 mesh), C18 (40–60 μm) and anhydrous sodium sulphate, were obtained from Acròs Organics (New Jersey, USA), Merck KGaA (Darmstandt, Germany), Panreac (Darmstandt, Germany), Scharlab Chemie S.A. (Barcelona, Spain) and Probus, S.A. (Badalona, Barcelona), respectively.

Individual stock standard solutions were prepared in acetonitrile (ACN) at 500 ng mL^{-1}. Working standard mixtures were prepared in ACN by dilution of stock standard solutions.

To avoid possible contamination of glass materials and solvents, due to the presence of OPs in indoor air, all glassware was previously cleaned with acetone and, samples and glassware were covered with aluminum foil.

2.2. Sample preparation

Foraging honeybees (*Apis melifera* L.) were sent to the laboratory thanks to the collaboration of beekeepers (Sample locations from different regions of Spain are shown in Fig. S1). Samples were stored at −80 °C until analysis. Approximately 0.5 g (5–8 insects) were mixed with 0.5 g of anhydrous sodium sulphate and dispersed with 1.5 g of Florisil in a glass mortar, with a pestle, until homogenization of sample was obtained. The mixture was transferred to a glass syringe barrel (glass column) closed with one way stopcock that contained two paper filters (Whatman Grade 1) and a layer of 1.0 g of co-sorbent (alumina). After the addition of the sample, another two paper filters were placed on top applying a slight compression with a syringe plunger. To eliminate the lipids and waxes, 3.5 mL of n-hexane were added to the sample located in the glass column and the eluate was discharged opening the stopcock. The stop valve was closed, 3 mL of ACN were added and the column was placed in an ultrasonic bath (Branson; Carouge, Switzerland) of 1 L capacity operating at 290 W, 40 kHz, at ambient temperature for 10 min. After extraction, the column was placed on a multiport vacuum manifold (Supelco/Sigma Aldrich-Chemie Gmb; Steinheim, USA) where the solvent was eluted from the column by opening the stopcock and collected in a glass graduated tube by gravity flow applying vacuum at the end of elution. This extraction procedure was repeated with 2 mL of ACN and the yielded extracts were combined and concentrated to 2 mL in a centrifugal vacuum concentrator (Genevac Limited, UK). Finally, the extracts were filtered through a 0.22 μm polypropylene syringe filter before LC–MS/MS analysis.

2.3. LC–MS/MS conditions

An Agilent 1200 (Waldbronn, Germany) liquid chromatograph equipped with an autosampler, a quaternary pump and a thermostatic column compartment was used. Separations were carried out using a Luna-C8 (150 mm × 4.6 mm i.d., 5 μ particle size) analytical column with a C8 security guard cartridge supplied by Phenomenex (Torrance, CA, USA). Eluent flow rate was set at 0.35 mL min^{-1} and the column was kept at 40 °C. The gradient elution program was performed with 0.15% formic acid in water as mobile phase A and ACN as mobile phase B. The gradient was as follows: 0 min, 90% A; 3 min, 50% A; 4 min, 40% A; 6 min, 20% A; 7 min, 5% A; 8 min, 5% A; 10 min 0% A, 13 min, 0% A; 16 min, 90% A. After recovering initial conditions, 9 min of equilibration time was included between runs resulting in a total analysis time of 25 min.

An Agilent 6410 triple quadrupole mass spectrometer equipped with an electrospray ionization interface (ESI), operating in positive ion (PI) mode was used. Drying and nebulizing gases for the ESI source were produced in situ by a nitrogen generator fed by compressed air at 7 bars. The optimized ESI parameters were: drying gas flow rate 9 L min^{-1}; drying gas temperature 350 °C; nebulizer gas pressure 40 psi and capillary voltage 4500 V.

Two different suitable transition pairs (precursor/product ion pair) were selected for each compound in multiple reaction monitoring (MRM) mode after direct injection to the interface. The optimized settings for fragmentor and collision energies were tested for each compound (Table 1).

3. Results and discussion

3.1. Optimization of MSPD conditions

The efficiency of MSPD method was based in the adequate selection of dispersant, co-sorbent and eluent solvent depending on the properties of target compounds and matrix complexity.