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Multi-residue method for the determination of pesticides and pesticide metabolites in honeybees by liquid and gas chromatography coupled with tandem mass spectrometry—Honeybee poisoning incidents

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ABSTRACT

A method for the determination of 200 pesticides and pesticide metabolites in honeybee samples has been developed and validated. Almost 98% of compounds included in this method are approved to use within European Union, as active substances of plant protection products or veterinary medicinal products used by beekeepers to control mites Varroa destructor in hives. Many significant metabolites, like metabolites of imidacloprid, thiacloprid, fipronil, methiocarb and amitraz, are also possible to detect. The sample preparation was based on the buffered QuEChERS method. Samples of bees were extracted with acetonitrile containing 1% acetic acid and then subjected to clean-up by dispersive solid phase extraction (dSPE) using a new Z-Sep+ sorbent and PSA. The majority of pesticides, including neonicotionoids and their metabolites, were analyzed by liquid chromatography tandem mass spectrometry (LC–MS/MS) but some of pesticides, especially pyrethroid insecticides, were analyzed by gas chromatography tandem mass spectrometry (GC–MS/MS). The procedure was validated according to the Guidance document SANCO/12571/2013 at four concentration levels: 1, 5, 10 and 100 ng/g bees and verified in the international proficiency test. The analysis of bee samples spiked at the limit of quantification (LOQ) showed about 98% mean recovery value (trueness) and 97% of analytes showed recovery in the required range of 70–120% and RSDr (precision) below 20%. Linearity and matrix effects were also established. The LOQs of pesticides were in the range of 1–100 ng/g. The developed method allows determination of insecticides at concentrations of 10 ng/g or less, except abamectin and tebufenozide. LOQ values are lower than the median lethal doses LD_{50} for bees. The method was used to investigate more than 70 honeybee poisoning incidents. Data about detected pesticides and their metabolites are included.

1. Introduction

Pesticides are widely used as plant protection products (PPPs) in agriculture. In Poland, there are more than 1500 PPPs authorized to use, which contains at least one of 200 different pesticides, very diverse in terms of chemical structure and toxic effects on bees. Those pesticides belong to many different categories such as insecticides, fungicides, herbicides, growth regulators, acaricides etc., but in terms of chemical properties they belong to much more different classes, e.g., insecticides included in this study belong to 16 different chemical classes.

Within last years due to global decline in honeybee population the bee health is a matter of public concern. Since 2003 in North America and Europe the phenomenon named Colony Collapse Disorder (CCD) occurs. The European data from EPILOBEE project showed that yearly colony mortality rates reported between 2012 and 2014 reached up to 36% [1]. The data from the United States showed that annual colony losses reported by beekeepers reached up to 45% [2]. The same data showed that commercial beekeepers in the United States reports greater loose of bee colonies in the summer than in the winter. Summer loses are usually connected with poisoning incidents. The mechanism of CCD remains unknown, but there is an agreement between scientists that there are several

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factors that could interact possible causes of colony losses. The scientists should expand the knowledge and understanding the role of pesticides, as one of the main factors that affect bee health, by development of new very sensitive and reliable methods detecting as much as possible pesticides, that even at very low levels at environmental doses and by interaction could weaken bees defence systems allowing parasites or viruses to kill the colony.

Till now a limited number of methods for the determination of pesticide residues in honeybees have been published. There are only few papers describing methods that can analyse more than 100 pesticides in this difficult matrix. and indicating method validation data. The proper selection of pesticides, by looking for those compounds which are currently approved or used as PPPs or as veterinary medicinal products (VMPs) by beekeepers, is of the same importance as the quantity of analysed pesticides. This could help to establish the most likely pesticide related risk for honeybee health. The effectiveness of the method used for the investigation of honeybee poisoning incidents is limited by the number of currently approved and used substances and not by the number of compounds banned to use many years ago.

By one of existing multiresidue methods honeybee samples can be analysed for 153 pesticides with gas chromatography system with dual selective detectors for electron capture and nitrogen-phosphorous (GC-NPD/ECD) and confirmatory analysis with different polarity column [3]. Roughly 150 pesticides can be analysed by method employing GC–MS/MS [4]. The drawbacks of this method are that gas chromatography is a technique that is unable to detect a lot of modern pesticides actually permitted to use in agriculture. Many of presently used pesticides could be analysed only with the methods involving liquid chromatography. Application of only LC–MS/MS determination of pesticides in honeybee are focused mainly on neonicotinoids [5–7] but there are also procedures that could provide valid data about the occurrence of 115 pesticides in honeybee colonies [8].

Methods based on both gas and liquid chromatography have the potential to analyse the broadest spectrum of pesticides but it is a common issue that a share of actually approved pesticides is insufficient. There was described only one validated method involving both GC-ToF and LC–MS/MS determination of 80 environmental contaminants in honeybees [9]. This method was adopted to monitor presence of contaminants in France apiaries [10]. Simultaneous analysis both with GC–MS and LC–MS/MS, but without any validation data, was used to study the occurrence of 200 pesticides in different beehive matrices from North American apiaries [11]. The applicability of that method to European Union is limited because only about 50% of studied compounds is approved to use [12]. Very recently there was published second occurrence study that uses both GC–MS/MS and LC–MS/MS method to establish the exposure of native bees to pesticides [13], but similarly only about 60% from 136 examined pesticides is approved to use within EU [12].

Besides of detection system that is characterised by obvious abilities and limitations the second most important method related step is sample preparation. The QuEChERS method with dispersive solid phase extraction (dSPE) clean-up is a sample preparation technique that enable the multiresidue pesticide analysis of complex matrices. The QuEChERS is one of the most popular sample preparation approach in the area of pesticide residue analysis in food. There has been published methods of honeybee samples analysis with QuEChERS [4,9,11,14]. The matrix solid-phase dispersion (MSPD) technique is also popular approach for honeybee extract clean-up [3,15,16].

The analysis of pesticides in honeybees is challenging due to complexity of insect body and presence of all kind of natural compounds, like beeswax, chitin and proteins that in chromatographic analysis are difficult for clean-up impurities. Whatever dSPE or MSPD based method is used, a lot depends on the capabilities of sorbents used in clean-up technique. Sorbents commonly used to clean-up bee extracts are primary secondary amine (PSA), octadecylsilane (C18), Florisil and graphitized carbon black (GCB). GCB is useful for removal of pigments, but retains planar pesticides. The new promising sorbents, which have been applied to the determination of pesticide residues in avocado for a purification of high oil extracts, are zirconium dioxide coated silica sorbents Z-Sep and Z-Sep+ [17]. Due to presence of Lewis acid sites, Bronsted acid-base sites, and octadecylsilane group on the surface of these new sorbents they could be a good adsorbent of fatty acids and proteins.

The aim of this study was to develop and validate an analytical method for determination as much as possible pesticides currently approved to use within European Union and their metabolites in honeybee samples taking into account two sources of exposure: pesticides used in agriculture as PPPs, and pesticides intentionally introduced into hives by beekeepers as acaricides in order to control *Varroa destructor* mite. The development of this method is important because results of study with such a broad and most actual spectrum of pesticides analysed in honeybees, shown in Table 1, will help to assess the risk connected with the current used pesticides and their role in the bees decline.

2. Materials and methods

2.1. Selection of compounds

The selection of PPPs active substances to be included in this method was done after the verification of Polish database of plant protection products [18] and EU pesticide database [12]. Only carbendazim, nitenpyram and novaluron are not approved to use in EU as PPPs. Carbendazim and novaluron were included in this method because according to Regulation (EC) No 1107/2009 [19] these active substances are still temporary on the market. Nitenpyram is one of the neonicotinoid insecticide.

The number of VMPs for Varroa control authorised to use by European countries, besides ethereal oils and organic acids, is limited to a few pesticides like amitraz, coumaphos, tau-fluvalinate and flumethrin [20]. For the reason that resistance development of Varroa species against currently used VMPs is observed and there is a lack of new substances, other pesticides registered to use within non-EU countries (cymiazole) or formerly used (bromopropylate) were included in the method to check whether beekeepers uses them.

Among 200 pesticides and pesticide metabolites included in this method only five compounds, from both PPPs and VMPs groups, are not currently approved to use within EU.

2.2. Reagents

High purity pesticide analytical standards and internal standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany), Sigma–Aldrich (Seelze, Germany) and Toronto Research Chemicals (Toronto, Canada). Stock standard solutions (250–1500 μg/mL) were prepared in acetonitrile, acetone, methanol or dimethylformamide and stored in the dark at a temperature below −18°C. Individual standard solutions for optimization and mixed standard solutions for calibration and validation experiments were prepared by appropriate dilutions of stock standard solutions.

Ultra Resi-Analyzed purity acetonitrile used for the preparation of standards, LC–MS grade acetonitrile used as eluent in liquid chromatography, acetone and methanol were obtained from J.T. Baker brand of Avantor Performance Materials (Deventer, The Netherlands). Dimethylformamide, formic acid, ammonium formate, PSA, anhydrous magnesium sulphate, C18 sorbent—Discovery DSC-18, GCB sorbent—Supelclean ENVI-Carb