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Determination of Pesticides in Dead Honeybees

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Pesticide residues were analyzed in dead honeybees by gas chromatography-tandem mass spectrometry (GC-MS/MS). Sample preparation using QuEChERS was followed by purification on a mixture of PSA and C18 sorbents. The extraction efficiency of > 80% was obtained for all analytes and pesticides were quantified in the range of 5-1000 ng/g. This method was used for investigating incidents of honeybees poisoning by pesticides. Fipronil was detected in all of the investigated samples originating from different regions of Russia. Additionally, τ-fluvalinate was detected in three samples.

Keywords: Dead honeybees, Pesticide residue analysis, QuEChERS, GC-MS/MS.

INTRODUCTION

Pesticides are chemicals such as insecticides, fungicides, herbicides, plant growth regulators, acaricides, *etc.* which are widely used in agriculture. In Russian Federation, over 350 insecticides and acaricides are allowed for use [1] and most of these chemicals are toxic for bees [2]. Recently, global decline of honeybees population caused concern worldwide.

Since 2003, a phenomenon known as colony collapse disorder (CCD) was observed in Europe and North America. The data obtained for Europe by the EPILOBEE project in 2012-2014 show that about 36% of bee colonies are lost annually [3]. In USA, the annual losses reached 45% [4]. The mechanism of CCD development remains unknown.

In Russia, significant losses of bee colonies are reported in spring and summer, which coincides with pesticide application to fields and orchards. In the summer of 2019, poisoning of honey bees was observed in over 20 regions of Russia, which is likely due to pesticides. The symptoms included large numbers of dead bees in the vicinity of beehives and decreased flight activity even in favourable weather. The main difficulty in analyzing pesticide residues is achieving reasonably high extraction efficiency (> 80%).

QuEChERS is an effective method for sample preparation in multi-residue pesticide detection in complex matrices. Few QuEChERS based methods for analyzing honey bees are already reported [5-7]. Another popular method for purifying honey bee extracts is matrix solid-phase dispersion (MSPD) [8,9].

Pesticide analysis in dead bees is complicated by the presence of widely disparate organic compounds, such as beeswax, chitin and proteins, which interfere with chromatographic separation. Both SPE and MSPD methods critically depend on the sorbents used. Those commonly used for bee extracts include primary secondary amine (PSA), octadecylsilane (C18), florisil and graphitized carbon (GCB). For matrices with high oil content, the use of some promising zirconium oxide-based sorbents (Z-Sep and Z-Sep+) was described [10]. Present goal was to analyze dead honeybees, which supposedly died from pesticide poisoning, using QuEChERS-based sample preparation followed by GC-MS/MS.

EXPERIMENTAL

All pesticide standards (amitraz, bifenthrin, carbaryl, fipronil, β-cyfluthrin, chlorpyrifosmethyl, coumaphos, esfenvalerate, malathion, fenvalerate, deltamethrin, cypermethrin, lambda-cyhalothrin, permethrin, propoxur, τ-fluvalinate, acetamiprid, thiacloprid, thiamethoxam) and the reagents were purchased from Sigma-Aldrich (USA).

Solid phase extraction was performed with 6 g of $MgSO_4$ and 1.5 g sodium acetate. Extracts were purified using 50 mg C-18, 50 mg PSA and 150 mg of $MgSO_4$ (Supel QuE acetate tube and Supel QuE PSA/C18 Tube, Sigma-Aldrich, USA).

TABLE-1
SORBENT COMBINATIONS USED FOR PURIFICATION OF EXTRACTS

A	B	C	C'	D	E	F	G
150 mg $MgSO_4$ 25 mg PSA	150 mg $MgSO_4$ 50 mg PSA 50 mg C18	150 mg $MgSO_4$ 35 mg Z-Sep+	150 mg $MgSO_4$ 50 mg Z-Sep+	150 mg $MgSO_4$ 35 mg Z-Sep	150 mg $MgSO_4$ 35 mg ZrO_2	100 mg $MgSO_4$ 40 mg PSA 90 mg C18 10 mg GCB	150 mg $MgSO_4$ 50 mg Z-Sep+ 50 mg PSA

Preparation of sample: Dried and homogenized bees (1 g) were placed in a 50 mL centrifuge tube with 10 mL of acetonitrile and shaken for 15 min on a Multi Reax vortex (Heidolph, Germany). A Supel QuE (6 g $MgSO_4$ and 1.5 g NaOAc) was added to the mixture, shaken for another 15 min and then centrifuged at 4000 rpm for 10 min. Supernatant liquid (1 mL) was transferred to a Supel QuE PSA/C18 tube, shaken for 15 min and centrifuged. The resulting supernatant was transferred to a 1.2 mL chromatographic vial, evaporated to dryness and redissolved in 0.1 mL of acetonitrile for GC-MS/MS analysis on TSQ8000 Evo (Thermo-Fisher Scientific). The chromatographic separation was achieved on a DB-5MS capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness, Agilent, USA). The following oven temperature program was used: initial temperature of 80 ºC hold for 1 min, increased to 180 ºC at 20 ºC/min, increased to 250 °C at 10 ºC/min, increased to 290 ºC at 4 ºC/min and hold for 5 min (post run). The injector and transfer line were operated at 270 and 280 ºC, respectively. The carrier gas was helium with constant flow rate of 1.1 mL/min. The injection volume was 1 µL in the splitless mode. The MS/MS system with electron ionization (EI) source in positive ionization was used for mass spectrometric analysis. The MS/MS settings were as follows: source energy: 70 eV, source temperature: 200 ºC.

RESULTS AND DISCUSSION

Pesticides were determined in dead bees following sample preparation by QuEChERS. To control pests, farmers often use compounds with either an acidic or basic nature, which, based on the pH of the extract, can exist in either a neutral or ionized state. Therefore, during QuEChERS extraction some analytes may remain in the more polar aqueous phase. To increase the extraction efficiency, the pH should be modified by buffering. The use of acetate and citrate buffers for this purpose was previously described [11,12]. The QuEChERS with citrate buffers was applied to the uncomplicated matrices without additional purification stages.

We are concerned that the use of a citrate buffer will result in the loss of acidic triazole fungicides because we intend to perform additional purification of the primary secondary amine (PSA) preparations. The PSA with acetate buffer is quite efficient in this case because of high amount of proteins with lower pK_a present in dead bees. Present experiments demonstrated that acetate buffering preserved constant pH values during the extraction and purification stages, keeping acidic-like analytes in their non-ionized forms and thus increasing the extraction efficiency on PSA.

Purification of extracts using various combinations of PSA with other sorbents (C18, Z-Sep, Z-Sep+, ZrO_2, GCB) have already been described in the literature [5,10,13,14]. The efficiency of PSA combinations with C18, Z-Sep, Z-Sep+, ZrO_2 and GCB were compared for the multi-residue pesticide extraction (Table-1).

Extraction efficiency was evaluated by weighing co-extracted material after evaporating 5 mL of acetate-buffered extract to dryness. The results are shown in Fig. 1. The combination of PSA with Z-Sep+ proved to be the most efficient (see G). However, further investigations showed that combining PSA with various Z-Sep sorbents decreases the extraction efficiency for some of the analytes. Therefore, a PSA-C18 mixture (see B) was chosen as a compromise between extraction and purification efficiency. This variant guaranteed acceptable degree of purification while maintaining the extraction efficiency at > 80% for all the analytes.

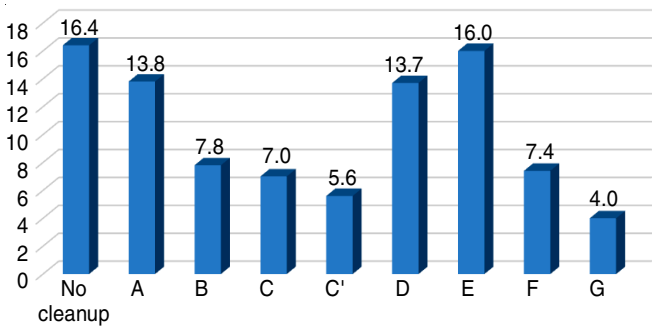

Fig. 1. Total weight of co-extractives after evaporating extracts obtained from 1 g of the matrix. For A-G (see Table-1)

Dead bees originating from Sverdlovsk area (four samples), Orel and Omsk areas (one sample each) were collected from the privately owned apiaries. In all the six samples, fipronil was detected at 8.0-111.8 ng/g. In addition, fluvalinate was detected in three of the Sverdlovsk samples at 8.0-48.7 ng/g (Table-2).

TABLE-2
PESTICIDE RESIDUE CONCENTRATIONS
DETECTED IN DEAD BEES

Site of sampling	Analyte detected	Conc. [ng/g (ng per bee)]
Omsk area, Lyubino-Malorossy	Fipronil	11.0 (2.2)
Sverdlovsk area, Sukhoi Log, Novopyshminskoe	Fipronil	111.8 (22.36)
Sverdlovsk area, Irbit, Zaikovo, Shkol'naya str.	Fipronil	42.7 (8.54)
	τ-Fluvalinate	48.7 (9.74)
Sverdlovsk area, Irbit, Zaikovo, Klubnaya str.	Fipronil	23.8 (4.76)
	τ-Fluvalinate	10.0 (2.0)
Sverdlovsk area, Irbit, Zaikovo, Krivaya str.	Fipronil	22.8 (4.56)
	τ-Fluvalinate	8.0 (1.6)
Orel area, Znamenka	Fipronil	31.0 (6.2)

Conclusion

According to the literature data, the LD_{50} of fipronil for bees is *ca.* 3-4 ng per bee. Given that an adult bee weighs *ca.* 0.2 g, whereas the fipronil concentrations estimated in the present studies correspond to 1.6-22.36 ng per bee, which could be lethal. Present findings underline the danger of fipronil use in the vicinity of apiaries. Furthermore, the QuEChERS was demonstrated to be a sensitive and accurate method for pesticide analysis in dead bees.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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