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Development and comparison of two multi-residue methods for the analysis of select pesticides in honey bees, pollen, and wax by gas chromatography–quadrupole mass spectrometry

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ABSTRACT

One of the hypotheses that may help explain the loss of honey bee colonies worldwide is the increasing potential for exposure of honey bees to complex mixtures of pesticides. To better understand this phenomenon, two multi-residue methods based on different extraction and cleanup procedures have been developed, and compared for the determination of 11 relevant pesticides in honey bees, pollen, and wax by gas chromatography–quadrupole mass spectrometry. Sample preparatory methods included solvent extraction followed by gel permeation chromatography (GPC) cleanup and cleanup using a dispersive solid-phase extraction with zirconium-based sorbents (Z-Sep). Matrix effects, method detection limits, recoveries, and reproducibility were evaluated and compared. Method detection limits (MDL) of the pesticides for the GPC method in honey bees, pollen, and wax ranged from 0.65 to 5.92 ng/g dw, 0.56 to 6.61 ng/g dw, and 0.40 to 8.30 ng/g dw, respectively, while MDLs for the Z-Sep method were from 0.33 to 4.47 ng/g dw, 0.42 to 5.37 ng/g dw, and 0.51 to 5.34 ng/g dw, respectively. The mean recoveries in all matrices and at three spiking concentrations ranged from 64.4% to 149.5% and 71.9% to 126.2% for the GPC and Z-Sep methods, with relative standard deviation between 1.5–25.3% and 1.3–15.9%, respectively. The results showed that the Z-Sep method was more suitable for the determination of the target pesticides, especially chlorothalonil, in bee hive samples. The Z-Sep method was then validated using a series of field-collected bee hive samples taken from honey bee colonies in Virginia.

1. Introduction

The honey bee, *Apis mellifera*, plays a critical role in agriculture and the global ecosystem by pollinating plants, while at the same time producing bee products with high economic value [1,2]. Globally, this value has been estimated to be approximately $210 billion, thus honey bees are an essential target for conservation [3]. However, in recent years, honey bee populations have been in a worldwide decline, which has been referred to as colony collapse disorder (CCD) and colony weakening [4,5]. Multiple causes of colony losses have been proposed, such as exposure to pesticides, pathogens, parasites, and natural habitat degradation [6,7]. Among these factors, pesticides are suspected by the scientific and beekeeping communities to have a strong impact on honey bee mortality and colony weakening [8,9]. In modern farming systems, honey bees are readily exposed to pesticides when they gather nectar and pollen from blooming crops, which are routinely treated with pesticides [10,11]. For example, researchers have demonstrated that low levels of pesticides, such as pyrethroid and neonicotinoid insecticides, may induce adverse sublethal effects in honey bees [8,12–15]. Honey bees are also exposed to miticides, like coumaphos and *tau*-fluvalinate, which are intentionally introduced to the hives to control the parasitic mite, *Varroa destructor* [16]. However, the relative contribution that pesticides have in colony losses remains unknown. Thus, to better understand the potential involvement that pesticides may have in colony losses, it is essential to develop reliable and sensitive analytical methods for the quantitation of pesticides in honey bees as well as in bee products, including pollen and wax.

In the past few years, several methods have been developed for the detection of pesticides in bee products like honey, pollen, wax, and honey bees [3,17–25]. However, most of the reported methods have focused on one or two matrices. To date, there have been very few multi-residue methods described in the literature for the simultaneous analysis of pesticide residues in honey bees, pollen, and wax. Since honey bees are most likely exposed to pesticides in both pollen and wax, it is important to be able to simultaneously quantify pesticide residues from these relevant matrices in one

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Table 1
Optimized gas chromatography–mass spectrometry method parameters.

Compounds	M.W.[a]	t_R (min)[b]	Target ion (*m/z*)	Qualifier ions (*m/z*)
DBOFB (NCI)[c]	455.9	7.049	454	456
Chlorothalonil (NCI)	265.9	7.73	266	264
Chlorpyrifos d10 (NCI)	360.6	8.565	323	322
Chlorpyrifos (NCI)	350.6	8.62	313	315
Bifenthrin (NCI)	422.9	14.50	386	387
PCB 204 (NCI)	429.8	14.594	430	432
Lambda-cyhalothrin (NCI)	449.9	17.10, 17.80	241	205
Coralox (NCI)	346.7	18.74	346	348
Permethrin (NCI)	391.2	19.91, 20.33	207	209
Coumaphos d10 (NCI)	372.8	20.299	372	374
Coumaphos (NCI)	362.8	20.40	362	364
Cyfluthrin (NCI)	434.3	21.62, 21.91, 22.08, 22.25	207	209
Cypermethrin (NCI)	416.3	22.42, 22.71, 22.85, 23.06	207	209
Flucynthrinate (NCI)	451.46	23.172	243.1	244
DCBP (NCI)	498.7	23.418	498	500
Fluvalinate (NCI)	502.9	25.40	294	296
Atrazine d5 (EI)[d]	220.7	9.697	205	207
Atrazine (EI)	215.7	9.744	202	215

[a] Molecular weight.
[b] Retention time.
[c] NCI=negative chemical ionization.
[d] EI=electron impact.

study. In view of these concerns, the aim of the current study was to develop a fast and reliable multi-residue analytical method for the trace analysis of relevant pesticides in honey bees, pollen, and wax. A total of 11 pesticides were selected for this study including the pyrethroid insecticides bifenthrin, *lambda*-cyhalothrin, permethrin, cyfluthrin, cypermethrin, and *tau*-fluvalinate, the organophosphate insecticides chlorpyrifos, coumaphos and coralox, the organochlorine fungicide chlorothalonil and the triazine herbicide atrazine (Table 1). These target analytes were chosen based on their potential toxicity to honey bees at low environmental concentrations and their widespread use in plant protection or in the bee hive directly. Two sample preparation methods, based on cleanup with gel permeation chromatography (GPC) and dispersive solid-phase extraction (d-SPE) with a new zirconium-based sorbent (Z-Sep) were compared with subsequent determination by gas chromatography coupled to a quadrupole mass spectrometry (GC–MS). Finally, the Z-Sep method was applied to bee hive samples collected in Virginia to validate this method as well as obtain preliminary data on the pesticides present in the hive.

2. Experimental

2.1. Chemicals and reagents

Pesticides analyzed in the current study were purchased from ChemService (West Chester, PA, USA), and their purities were $>97.0\%$ as certified by the manufacturer. Decachlorobiphenyl (DCBP) and 4,4′-dibromooctafluorobiphenyl (DBOFB) were used as surrogates and were purchased from Supelco (Bellefonte, PA, USA), and had purities $>99\%$. The internal standards, PCB 204, chlorpyrifos d10, coumaphos d10, flucynthrinate, and atrazine d5 (AccuStandard, New Haven, CT, USA) were added to the solutions before GC–MS analysis. The stock solution of each compound and surrogate was prepared at 1 μg/mL in hexane and stored in the freezer. Pesticide grade solvents including acetone, dichloromethane (DCM), ethyl acetate, and hexane along with Whatman GD/X polytetrafluoroethylene filters (13 mm) and acetic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA). The Z-Sep and Z-Sep+C_{18} were obtained from Supelco.

2.2. Sample collection and spiking procedures

The honey bees, pollen, and wax samples were randomly collected from apiaries located in Montgomery Co. and Frederick Co., Virginia, USA in 2014. From each hive, nurse honey bees (6–10 d) were collected from the brood frames. The pollen and wax samples were collected from the same brood frames with which the honey bees were collected. Each sample was collected in a sterile 50 mL conical tube, immediately placed on ice, and then stored at −80 °C for the multi-residue analysis.

Honey bees, pollen and wax samples that were found to have low or no detectable concentrations of the target pesticides were spiked with the 11 target pesticides and surrogates for method detection limit (MDL), recovery and quality assurance/quality control tests. The target pesticides and surrogates were spiked onto dry bee hive samples prior to adding solvents and homogenization. For detection of the target pesticides in field-collected honey bees, pollen and wax, only surrogates were added prior to extraction and internal standards added prior to analysis.

2.2.1. Procedure I-solvent extraction with GPC cleanup

2.2.1.1. Solvent extraction. For honey bees and pollen, 1 g of sample (~9 bees) was weighed and placed into a 50 mL polypropylene centrifuge tube. Next, 10 mL of a 1:1 DCM:hexane (v/v) solution was added, and the samples were then finely homogenized with a Power Gen 700 tissue homogenizer (Fisher Scientific) for approximately 2 min. The tube was capped and centrifuged for 10 min at 3000 rcf (relative centrifugal force) (Centrifuge 5702R, Eppendorf AG, Hamburg, Germany) at room temperature. A total of 6 mL of the supernatant was removed and used in the cleanup procedure.

For wax, 1 g of sample was weighed and placed into a 50 mL polypropylene centrifuge tube. Next, 10 mL of a 1:1 acetone:hexane solution was added, and the sample was capped and vigorously vortexed for 20 min using a Genie Z Vortex (Fisher Scientific), and then centrifuged for 10 min at 3000 rcf in a refrigerated Eppendorf 5702R centrifuge set at −3 °C. The lower temperature was necessary to aid in congealing the lipids, which were separated from the supernatant by centrifugation. A total of 6 mL of the supernatant was subjected to the cleanup procedure.

2.2.1.2. The GPC cleanup procedure. Prior to GPC cleanup, extracts were filtered through a 0.45 μm Whatman GD/X filter (13 mm diameter), and then concentrated to 1 mL using a Pierce Model 1878 Reactivap (Rockford, IL, USA). Next, 0.5 mL of the extract was injected into the GPC using a Rheodyne 7225 injector with a 0.5 mL sample loop (Cotati, CA, USA). The GPC procedure was performed on an Agilent 1100 high-pressure liquid chromatography (HPLC) system (Agilent Technologies, Palo Alto, CA, USA), and analytes were separated from interference using a 300 mm × 19 mm Envirogel GPC column equipped with a 5 mm × 19 mm guard column (Waters, Milford, MA, USA). A Foxy Jr. fraction collector (ISCO, Inc. Lincoln, NE, USA) was used to collect the fractions that eluted between 6.5 and 12.5 min, which contained the target compounds and surrogates. Dichloromethane was used as the mobile phase and the flow rate was set at 5 mL/min. The fractions were evaporated to near dryness and solvent exchanged to 0.5 mL of 0.1% acetic acid in hexane for subsequent GC–MS analysis. The acidification step was used to minimize isomerization of the pyrethroids [26].

2.2.2. Procedure II-solvent extraction with Z-Sep cleanup

For all three honey bee sample types, the extraction procedure started by weighing 1 g of material and then placing it into a