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Determination of Acaricide Residues in Saudi Arabian Honey and Beeswax Using Solid Phase Extraction and Gas Chromatography

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Determination of acaricide residues of flumethrin, *tau*-fluvalinate, coumaphos, and amitraz in honey and beeswax was carried out using a rapid extraction method utilizing C-18 SPE cartridges and an analytical method utilizing GC with ECD, NPD, and MSD detectors for the four acaricides. Recovery percentages from the extraction method ranged from 90–102%, while the minimum detection levels ranged from 0.01–0.05 mg/kg for the acaricides. Nine of the 21 analyzed samples were found to be contaminated with the acaricides *tau*-fluvalinate and coumaphos. Neither flumethrin nor amitraz was detected in any of the honey or wax samples. Coumaphos was found only in honey samples in which two samples exceeded the tolerance levels set by EPA and EC regulations. It has not been detected in beeswax. Five honey samples and eight beeswax samples were found to be contaminated with *tau*-fluvalinate. One of the wax samples was contaminated with a relatively high residue of *tau*-fluvalinate and contained above 10 mg/kg.

Key Words: Honey; Beeswax; Acaricides; Pesticide residues; GC/MS; *Varroa jacobsoni.*

INTRODUCTION

Some acaricides in Saudi Arabia are used in the form of formulated plastic strips to control the mite *Varroa jacobsoni* in beehives. Strips such as Apistan or

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Mavrik containing the pyrethroid insecticide *tau*-fluvalinate, Apiguard containing the pyrethroid flumethrin, Apilife, Var., Perizin containing the organophosphate coumaphos, and Apivar containing the formamidine amitraz are used in hives. The use of these acaricides is likely to increase in the future due to resistance developed by mites which requires increased treatments in the future.[1,2] Because of the high cost of these strips, some beekeepers misuse their application guidelines and reuse them. Strips are left inside the hive over the winter, or the used strips are sprayed over again with another formulation and are placed in the hives. Because most of the acaricide residues are lipophilic in nature, they accumulate in beeswax, while honey residues are relatively low and lie mostly below the allowed maximum residue limit (MRL).[3,4]

Various methods have been developed for the determination of acaricide residues in honey and beeswax samples. Problems for the determination of amitraz arose because this compound is extremely unstable in acidic media and it breaks down in honey,[5,6] therefore, determination of amitraz with its degradation products has to be considered. Amitraz has also not been detected in beeswax due to its breakdown and instability in wax. Other acaricides have also been reported to be unstable in honey such as coumaphos and fluvalinate.[7] On the other hand, *tau*-fluvalinate has been found to be stable in honey,[8] and flumethrin has been found stable upon storage up to three months.[9] This study describes a method of extraction and analysis of the four acaricides used by most beekeepers in Saudi Arabia utilizing C-18 solid phase extraction cartridges (SPE) and analysis by GC/NPD/ECD and GC/MS in the selected ion monitoring mode (SIM mode). Data from this study could be useful for governmental entities to provide guidelines on pesticide use to beekeepers under the Saudi conditions.

MATERIALS AND METHODS

The acaricide strips Apistan, Bayvarol, Perizin, and Apivar were purchased from the local market. Their active ingredients *tau*-fluvalinate (mixture of two isomers), flumethrin, coumaphos, and amitraz, respectively, as well as amitraz degradation products DMA, DMF, and DPMF were purchased from Chem Service, Pennsylvania, USA. All solvents and chemicals used were of HPLC or ACS certified grades. Solid phase extraction cartridges were Varian SPE C-18 (10 ml, 500 mg). The analytical instrument was an Agilent 6890 gas chromatograph with split/splitless inlet equipped with μ-ECD and NPD detectors. Another similar gas chromatograph was equipped with a 5973 mass selective detector. A HP-5MS capillary column (30 m $\times$ 0.32 mm, 0.5 μ) was attached to each detector using helium as carrier gas. The oven temperature was set to the initial temperature of 90°C and was held for 2 min, then programmed to 250°C at 20°C/min and held for 15 min. Splitless injections of 1 μl were used for both instruments with 250°C as the injector temperature. The MSD detector was

set to the selected ion monitoring (SIM) mode targeting the ions at m/z 293 for amitraz, m/z 502 and m/z 250 for *tau*-fluvalinate, m/z 216 for flumethrin, and m/z 362 for coumaphos at 100 msec dwell time. The interface temperature was 280°C for the GC/MS, the μECD detector temperature was 300°C, and the NPD detector temperature was 250°C.

Sample Collection

Twenty-one samples were collected from beehives at the educational farm of King Saud University in Riyadh, Dirab, and Daraya. The hives were arranged at random into five groups, one group for each acaricide and a control group. Each group received two acaricide strips, except the control group, in the brood nest hung between two intermediate frames. Strips were kept in the hives for a period ranging from 60 days to six weeks, according to the instructions on each acaricide's label on the strip. Honey samples were collected directly from the hive about 100 g each from random locations near and far from the application site. Wax samples (about 50 g each) were randomly cut 2.5 cm^2 at different locations including the area of strip application. Three replicates of each sample were collected at the end of each experiment. Control samples were collected from untreated hives.

Extraction and Recovery Studies

Three levels of fortification (0.05, 0.5, and 1 μg/ml) were spiked into both honey and wax control samples. Extraction of the acaricides from honey samples was carried out following the procedure of Korta et al.,[10] with some modification as follows: One gram of spiked honey samples and the control were dissolved in 10 ml 0.1 M sodium phosphate buffer and sonicated until homogenous. The samples were passed through C-18 SPE cartridges after conditioning with 3 ml of tetrahydrofuran THF, acetonitrile, water, and finally with the buffer. The cartridge was then washed with 5 ml 100% acetonitrile, followed by 5 ml 10% THF, and were both discarded. The acaricides were eluted with 5 ml 100% THF, concentrated in a rotary evaporator, and the THF was replaced with methanol to a final volume of 1 ml and transferred to a GC vial.

Beeswax samples were extracted following the procedures of Bogdanov, Kilchenmann, and Imdorf[3] and Zimmermann, Gierschner, and Vorwohl[11] with some modification. Ten ml of 100% acetonitrile were added to 5 g wax sample and sonicated for 5 min. The extract was then frozen at −10°C for 15 min, centrifuged at 5000 RPM, and filtered. This step eliminates most of the high molecular weight hydrocarbons from the wax. The filtrate was frozen again at −10°C for 30 min, centrifuged at 8000 RPM, and filtered to purify the extract from other undesired hydrocarbons. The filtrate was then concentrated to 1 ml and transferred to a GC vial for injection.

Samples were then injected into the GC/ECD/NPD and GC/MS systems, and the amount of each analyte was calculated using Agilent Chemstation software. Acaricide reported amounts were calculated based on their GC/ECD/NPD signals and were confirmed by GC/MS.

RESULTS AND DISCUSSION

Determination of the four acaricide residues in honey and beeswax was carried out using a rapid extraction method utilizing C-18 SPE cartridges for honey samples and a freezing-centrifugation process for beeswax. The analytical method was carried out using GC with ECD, NPD, and MSD detectors. Recovery percentages from the extraction method ranged from 90–110%. Minimum detection levels of the acaricides ranged from 0.01–0.05 mg/kg for flumethrin (0.05 mg/kg), *tau*-fluvalinate (0.01 mg/kg), coumaphos (0.01 mg/kg), and amitraz and metabolites (0.03 mg/kg) using μ-ECD/NPD detectors, while they ranged from 0.05–0.1 mg/kg using MSD.

Figures 1 and 2 show the GC/MS ion chromatogram of the beeswax sample H1C1 containing 0.08 mg/kg *tau*-fluvalinate (retention times 32.16 and 32.74 min) and the GC/ECD chromatogram of the corresponding honey sample containing 0.05 mg/kg each of coumaphos (retention time 24.24 min) and *tau*-fluvalinate (retention times 27.43 and 27.64 min).

Nine of the 21 analyzed samples (Table 1) were found to be contaminated with the acaricides *tau*-fluvalinate and coumaphos. Neither flumethrin nor amitraz and its degradation products were detected in any of the honey or wax samples. Coumaphos was found only in honey samples and was not detected in beeswax. Two analyzed honey samples contained coumaphos residues

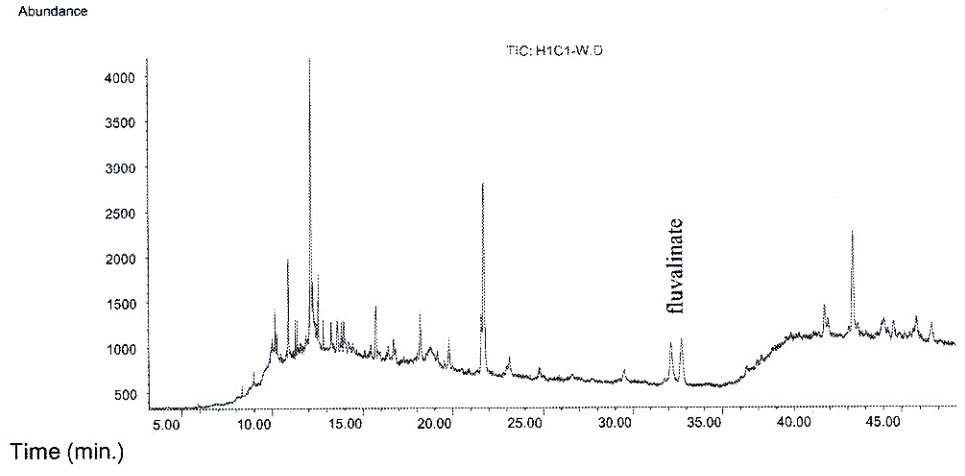

Figure 1: GC/MS ion chromatogram of the beeswax sample H1C1.

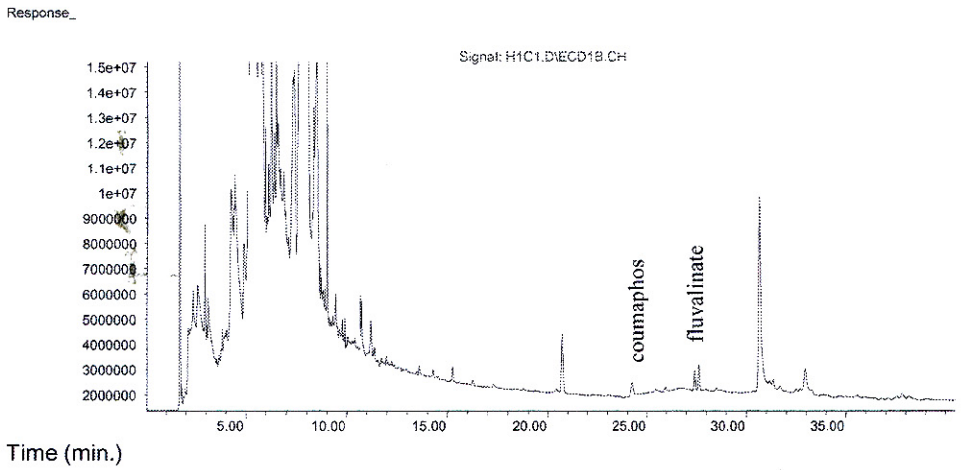

Figure 2: GC/ECD chromatogram of the honey sample H1C1.

exceeding the tolerance levels set by EPA at 0.1 ppm [12] and EC regulations set at 0.05 mg/kg.[13,14] Although fluvalinate was detected in five of the honey samples, however, they were all within the permissible limits for honey set by EPA at 0.05 ppm and by EC regulations at the same tolerance level. [14,15] Tolerance levels in beeswax were not set by either agency for fluvalinate; however, the tolerance level for coumaphos in beeswax was set by EPA at 100 ppm. [12] Eight beeswax samples contained residues of fluvalinate; one of the samples exceeded 10 mg/kg.

As expected, amitraz was not found in any of the honey or beeswax samples due to its instability in acidic media and in wax as previously found.[10] Its degradation products 2,4 dimethylaniline (DMA), 2,4 dimethylphenylformamide (DMF), and N-(2,4 dimethylphenyl)-N-methylformamidine (DPMF) were also not detected in any of the honey or beeswax samples.

Although flumethrin, *tau*-fluvalinate, and coumaphos are lipophilic compounds, they would be expected to be present in beeswax at higher

Table 1: Average amounts of acaricide residues found in honey and beeswax.

	tau-fluvalinate (mg/kg)		Coumaphos (mg/kg)	
Sample	**Honey**	**Beeswax**	**Honey**	**Beeswax**
H1C1	0.05	0.08	0.05	N.D.
H1C2	N.D.*	0.03	0.5	N.D.
H1C3	N.D.	N.D.	0.5	N.D.
H1CL	N.D.	0.02	N.D.	N.D.
H3C1	0.05	0.01	0.05	N.D.
H3C4	N.D.	0.17	N.D.	N.D.
H3C5	0.04	0.07	0.02	N.D.
H3C6	0.03	10.06	N.D.	N.D.
H3C7	0.02	0.02	0.02	N.D.

*N.D.: not detected.

concentrations than in honey. Our results show that only *tau*-fluvalinate was detected in beeswax at higher concentrations than in honey, while coumaphos was found only in honey and flumethrin was not detected in any honey or beeswax sample. Previous findings showed that the solubility of *tau*-fluvalinate in wax is very high, and it was detected in wax at concentrations reaching 8000 times as much as in honey and that these residues were stable for more than a year in wax.[3] These findings are in agreement with our results especially for the high residue of *tau*-fluvalinate found in one of the wax samples (H3C6) and was 335 times higher than its corresponding honey sample. We therefore conclude that the larger the number of applications by fluvalinate, the more it is accumulated in the wax, and not in the honey. Therefore, it is recommended that the number of applications per acaricide should be regulated and limited to a value beyond which its accumulated residue will not reach the maximum residue level (MRL) allowed. Also, the continuous reuse of wax in apiculture should be restricted.

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