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Evaluation of the potential migration of acaricides from stamped beeswax to honey simulating beehive conditions: A pilot study

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ABSTRACT

There is an issue concerning the migration of pesticides from stamped beeswax to other bee products, mainly, honey. This implies that honey and other beehive-based products could be contaminated, which would represent a potential risk to consumers and food safety. To determine the degree to which acaricides can be transferred from the stamped beeswax to the honey, a pilot experiment was designed consisting of placing beeswax that contained acaricides (endogenous content, τ-fluvalinate, chlorfenvinphos, and coumaphos; spiked samples, coumaphos) in contact with multifloral honey in an incubator. Temperature and shaking conditions were settled simulating beehive conditions. The isolation of the analytes from beeswax involved the use of a QuEChERS (quick, easy, cheap, effective, rugged & safe) method, and honey was analyzed through a solvent extraction procedure. Acaricides were determined in the resulting extracts by gas chromatography with mass spectrometry detection. Coumaphos was the only acaricide transferred into honey in beeswaxes with endogenous acaricide content; meanwhile, results of the experiments with spiked beeswax samples showed that the initial concentration present in the contaminated beeswax significantly influences the transfer to honey, as it was observed that in the spiked beeswax sheets with high levels of coumaphos, the migration from beeswax to honey arose.

1. Introduction

Honeybees and other insects are the key to effective and healthy pollination, sustainable agriculture, and natural ecosystems. However, there are studies showing that the number of honeybee colonies has declined significantly over the last hundred years especially in areas of Europe and the United States (Staveley et al., 2014). The reduction in the number of hives has been related to a wide range of phenomena such as: i) weakening of the insects due to mites and parasites; ii) exposure of bees to different agrochemicals used for the protection and care of plantations and pesticides used by beekeepers; iii) reduction of natural habitats, malnutrition and degradation of the natural habitat; iv) inadequate management of the hives; v) environmental pollution (mainly soil, water and atmosphere). Specifically, one of the critical health problems in the beekeeping field is varroosis, a parasitic disease affecting bees caused by the *Varroa destructor* mite. Control of this mite is based on the use of acaricides such as τ-fluvalinate or coumaphos (Adamczyk et al., 2010). These acaricides are mostly non-polar, fat--soluble, and their active principles are stable and do not degrade easily. Studies in Spain have shown concentration levels between 13 and 13900 μg/kg in hive matrices (Lozano et al., 2019). This highly variable range is because acaricide treatments carried out inside the hive cause the accumulation of residues in the beeswax due to the lack of elimination of these compounds. One of the most relevant applications of beeswax is in conventional beekeeping. It is used as a base for honeycombs to reduce the effort required by the bees to build new cells. The wax that goes into the honeycombs is usually obtained from old ones that have been subjected to various cleaning processes. However, it has been demonstrated that these treatments are not able to completely eliminate the residues of xenobiotics (Calatayud-Vernich et al., 2017), and in several studies carried out by our research group (Jiménez et al., 2005; Nozal et al. 2021; Yáñez et al. 2013), it has already been found that most of the acaricides frequently found on beeswax sheets tend to concentrate and do not disappear with cleaning treatments. This could be explained as those compounds are lipophilic, and they have a high degree of stability being able to resist the melting temperature of wax cleaning (Zhu et al., 2014). Acaricides can be retained in the wax without degrading so that it can take years for their complete

Abbreviations: **dSPE**, dispersive solid-phase extraction; **EMR-lipid**, enhanced matrix removal-lipid; **GC-MS**, gas chromatography-mass spectrometry; **IS**, internal standard; **LOQ**, limit of quantification; **MRLs**, maximum residue limits; **QuEChERS**, Quick, Easy, Cheap, Effective, Rugged & Safe; **SIM**, selected ion monitoring.

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disappearance (Medici et al., 2012; Serra-Bonvehí and Orantes-Bermejo, 2010). These residues accumulate in the wax (Serra-Bonvehí and Orantes-Bermejo, 2010) due to its repeated use, and its recycling, which can produce a potential risk for the beehive, for the ecosystem and even for consumers if transfer to other apiary matrices occurs. In addition, the level of acaricides in the beeswax and other beehive products is intensified by using them much more frequently, and sometimes in greater quantities than allowed by the European Union Regulation (García et al., 2017; Lozano et al., 2019).

Since in the beehive all cells are connected and beeswax is in contact with honey and other bee-related products, there is evidence that contaminated beeswax can contaminate honey in contact with it (Albero et al., 2023). Unfortunately, there are very few studies on this subject in the literature. This is probably due because it is really complicated for researchers to know the inner workings of the hive, simulate the bee-hive conditions and habitat, and establish whether the distribution of the acaricide is homogeneous or accumulates in a particular region. Moreover, the tendency of pesticides to distribute and accumulate in the hive products is conditioned by physicochemical properties of pesticides such as water solubility, vapor pressure, molecular structure, pKa values and lipophilicity (Goss and Schwarzenbach, 2001; Shimshoni et al., 2019). One of the most commented studies belongs to Kochansky et al. (2001) who reaffirms the results obtained by Wallner (1999) on the transfer of coumaphos from wax to honey. This situation was also investigated by Bogdanov et al. (2004) by analyzing a set of Swedish wax samples. In the study, the presence of p-dichlorobenzene residues was detected in all waxes and honeys, which gave an estimation of a possible wax-honey migration. Another example is that of Reybroeck et al. (2010) who evaluated the migration of sulfamethazine or experiences done by Mitrowska and Antczak (2017) about the transfer of nitroimidazoles. More recent studies (Baša Česnik et al., 2019; Kast et al., 2021; Murcia-Morales et al., 2020; Shimshoni et al., 2019) have attempted to evaluate this migration and experimentally determine the distribution and half-life time of pesticides between wax and honey and extrapolate the results to hives of different origins. It should be mentioned that the most frequently occurring acaricides have been found to be τ-fluvalinate, coumaphos, bromopropylate, and chlorfenvinphos (Benito-Murcia et al., 2021; Chauzat and Faucon, 2007; Nozal et al., 2021; Ostiguy and Eitzer, 2014; Ravoet et al., 2015). Consequently, it is not surprising that maximum residue limits (MRLs) have been established by the European regulation (European Union Pesticide Database, 2023) for honey, while this regulation specifies that no MRLs are applicable to other apiculture products until individual products have been identified and listed within this group. When these beeswax sheets are used in hives, a source of contamination is being introduced for the bees and the derived products. Therefore, it is essential to control the quality of the wax sheets by analyzing their pesticide content and at the same time to carry out migration studies to quantify their transfer from the stamped beeswax to honey. Consequently, this pilot study compromises an approximation to acaricide migration.

The main goal of the present study was to investigate the potential migration/transfer of acaricides from beeswax sheets to honey. The transfer study was carried out by putting in contact in an incubator beeswax sheets (endogenous content, τ-fluvalinate, chlorfenvinphos, and coumaphos; spiked samples, coumaphos) with multifloral honey, in which temperature and shaking conditions were selected to simulate beehive conditions. To our knowledge, this study has not been previously conducted with the selected acaricides and conditions. It should be mentioned that two different sample preparation methods, which were previously developed and validated by our research group (QuEChERS for beeswax samples; Nozal et al., 2021; solvent extraction for honey; Fuente-Ballesteros et al., 2023), were employed in combination with gas chromatography-mass spectrometry (GC-MS) to determine the acaricide residues in the different samples generated for this study.

2. Materials and methods

2.1. Reagents and materials

Analytical-grade standard of the studied acaricides (chlorfenvinphos 98.9%; coumaphos 99.5%; τ-fluvalinate 99.6%; chlorfenvinphos-d_{10} 99.1%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Chlorfenvinphos-d_{10} was chosen as internal isotope-labeled standard (IS) since it exhibits the same physical and chemical properties as the unlabeled analytes. Solvents of Pestinorm grade (acetonitrile, ethyl acetate, acetic acid, and cyclohexane) were obtained from VWR Prolabo Chemicals (Fontenay-sous-Bois, France). Solid reagents were obtained of analytical grade from Sigma Aldrich (Saint Louis, MO, USA), and QuEChERS (quick, easy, cheap, effective, rugged & safe) reagents were purchased from HPC standards GmbH (Cunnersdorf, Germany). QuEChERS dSPE EMR-Lipid (dispersive solid phase extraction enhanced matrix removal lipid) and polish (sodium chloride/magnesium sulfate) tubes were supplied by Agilent Technologies (Folsom, CA, USA).

A vibromatic mechanical shaker (J.P. Selecta S.A., Barcelona, Spain), a thermostated ultrasound bath (J.P. Selecta S.A.), a drying oven (J.P. Selecta S.A.), a vortex mechanical mixer from Heidolph (Schwabach, Germany), a 5810 R refrigerated bench-top centrifuge from Eppendorf (Hamburg, Germany), a R-3 rotary evaporator from Buchi (Flawil, Switzerland), a M-20 grinder and an Ultra-Turrax® homogenizer T-18, both from IKA (Staufen, Germany), an analytical balance AE 240 from Mettler Toledo (Darmstadt, Germany), and an orbital shaker incubator ES-80 from Grant-bio (Cambridge, UK) were employed for sample treatments. Nylon syringe filters (17 mm, 0.45 μm) were from Nalgene (Rochester, NY, USA), and ultrapure water was obtained using Milipore Milli-RO plus and Milli-Q systems (Bedford, MA, USA).

2.2. Standards

Standard (matrix-free) stock solutions (~1000 mg/L) of each acaricide were prepared by dissolving different amounts of each accurately weighed compound of an ethyl acetate and cyclohexane mixture (20:80, v/v for beeswaxes and 50:50, v/v for honeys). These solutions were further diluted with the same solvent mixture to prepare the intermediate and calibration matrix-free standards. Previous work had determined that considering beeswax, coumaphos and τ-fluvalinate should be quantified with matrix-matched calibration curves as the matrix provoked a significant signal enhancement (Nozal et al., 2021); while for chlorfenvinphos and honey samples, standard in solvent calibration curves could be used due to the absence of a significant matrix effect (Fuente-Ballesteros et al., 2023; Nozal et al., 2021). To prepare the spiked beeswax samples with the acaricides (matrix-matched calibration curves, 5–5000 μg/kg; migration studies, coumaphos: 4, 40 and 400 mg/kg), it was followed the same procedure described by Nozal et al., (2021). Purified white beeswax (1.0 g), in which the absence of acaricide residues had been previously confirmed by GC-MS, was spiked with different amounts of the acaricides, and the IS (0.5 mg/kg). It was necessary to heat the beeswax at 70 °C when spiking with the acaricide to obtain homogenous samples. Stock solution was stored in glass containers in darkness at − 20 °C, and working and matrix-matched solutions were stored in glass containers and kept in the dark at 4 °C. All the solutions were stable for over two weeks (data not shown).

2.3. Sample procurement and treatment

2.3.1. Samples

Stamped beeswax sheets (acaricide-free and with endogenous content) were kindly donated by the Center for Agroenvironmetal and Apicultural Investigation (Marchamalo, Guadalajara, Spain). It must be specified that their appearance was slightly different. Thus, the tone of the beeswax sheets categorized as acaricide-free (decontaminated) was much whiter than that with acaricide content (also known as

commercial or raw) ones, which exhibited a yellowish color. This could be explained because the darker the color, the more times the beeswax sheet has been used. As mentioned above, beekeepers reuse beeswax sheets by subjecting them to various cleaning processes that are not fully effective (Bonvehi & Orantes-Bermejo, 2017; Navarro-Hortal et al., 2019). As the beeswax is bleached, it reaches a certain limit, and begins to darken. However, data on the decontamination procedure were not available. These were previously analyzed by GC-MS analysis to check for the absence of pesticides. Once it was confirmed that there were no residues of the acaricides studied, subsamples were used as blanks to prepare standards. All samples were stored at − 20 °C until analysis. Honey was purchased in local markets (Valladolid, Spain) and selected according to their botanical origin (light honey: multifloral). The absence of acaricides was previously confirmed by GC-MS. To homogenize the honey samples, it was individually stirred with a glass rod and subsequently stored in different tubes in darkness at 4 °C.

2.3.2. Sample treatment for beeswax

Beeswax sheets used in this study were analyzed according to the methodology optimized by Nozal et al., (2021) with the only modification of the sample amount. Briefly, samples were crushed with dry ice in a mortar and homogenized. Next, 1.0 g of beeswax was mixed with 10 mL of 1% acetic acid in an acetonitrile mixture. The tube was then shaken in a vortex device and homogenized during 2.5 min in an Ultra-Turrax®. The extract was then centrifuged (7500 rpm, 5 °C, 5 min) after which 5 mL of supernatant was transferred to a QuEChERS dSPE EMR-lipid cartridge previously activated with 5 mL of ultrapure water. The mixture was shaken for 1 min in a vortex device, centrifuged again (7500 rpm, 5 °C, 5 min), and 5 mL of supernatant were transferred to the polish tube (sodium chloride/magnesium sulfate), shaken in a vortex device (1 min), and centrifuged using the above-mentioned conditions. Two milliliters of the supernatant were evaporated to dryness in a rotary evaporator (60 °C) and the dry extract was reconstituted with 1 mL of IS solution. The resulting solution was filtered (0.45 μm nylon), and finally a 1-μL aliquot was injected into the GC-MS system. It should be mentioned that the obtained limits of detection (LODs) and quantification (LOQ) were below the established MRLs for honey (European Union Pesticide Database, 2023) in all cases: **i**) chlorfenvinphos (LOD, 2 μg/kg; LOQ, 5 μg/kg; MRL, 10 μg/kg); **ii**) coumaphos (LOD, 2 μg/kg; LOQ, 5 μg/kg; MRL, 100 μg/kg); **iii**) τ-fluvalinate (LOD, 2 μg/kg; LOQ, 5 μg/kg; MRL, 50 μg/kg).

2.3.3. Sample treatment for honey

Honeys were treated following a solvent extraction protocol according to the method described by Fuente-Ballesteros et al., (2023). Briefly, 5.0 g of multifloral honey was mixed with 10 mL of ultrapure water and then shaken (vortex, 1 min) and ultrasonicated (3 min, 25 °C). Next, 10 mL of an ethyl acetate and cyclohexane (50:50, v/v) mixture was added, shaken (vibromatic, 10 min) and centrifuged (10000 rpm, 5 °C, 5 min). Then, 5 mL of the supernatant were collected and evaporated to dryness, reconstituted with 1 mL of IS solution, and filtered (0.45 μm nylon) prior to injection in the GC-MS system. The obtained LODs and LOQs were better than those previously reported for beeswax: **i**) chlorfenvinphos (LOD, 0.2 μg/kg; LOQ, 0.5 μg/kg; MRL, 10 μg/kg); **ii**) coumaphos (LOD, 0.2 μg/kg; LOQ, 0.6 μg/kg; MRL, 100 μg/kg); **iii**) τ-fluvalinate (LOD, 0.3 μg/kg; LOQ, 1.1 μg/kg; MRL, 50 μg/kg).

2.4. GC-MS parameters

GC-MS parameters were based on previous studies by our research group (Fuente-Ballesteros et al., 2023). To sum up, an Agilent Technologies 7890 A gas chromatograph coupled to an Agilent Technologies 5975 C mass spectrometer was used with an Agilent DB-5MS column (30 m × 0.25 mm × 0.2 μm) and helium as carrier gas. The GC-MS parameters were selected according to previous works (Fuente-Ballesteros et al., 2023; Nozal et al., 2021). The GC was operated under programmed temperature conditions, which differed slightly depending on the matrix: A) beeswax, from 60 °C (1 min) to 170 °C (5 min), at 40 °C/min and then increased to 195 °C (10 min) at 9 °C/min. Then, temperature was increased to 310 °C (3 min) at 10 °C/min; B) honey, from 60 °C (1 min) to 170 °C (5 min), at 40 °C/min and then increased to 310 °C (3 min) at 8 °C/min. An injection volume of 1 μL was employed with the autosampler in pulsed splitless mode, the injector temperature set at 280 ºC, and helium (Carburos Metálicos, Barcelona, Spain) used as the carrier gas at a flow-rate of 1.2 mL/min. MS SCAN parameters included a mass range of 50–400 *m/z*, operating in electron ionization mode with an ionization energy of 70 eV. The ion source and quadrupole temperatures were 230 °C and 150 °C. Analyses were performed in selected ion monitoring (SIM) mode, with one target/quantification and two qualifier ions for each analyte: i) chlorfenvinphos (retention time 11.8 min; 267 target ion; 270 and 329 qualifier ions); ii) coumaphos (retention time 18.2 min; 362 target ion; 109 and 226 qualifier ions); iii) τ-fluvalinate (retention time 20.4 min; 250 target ion; 181 and 208 qualifier ions).

2.5. Design of the migration study

A working protocol has been designed and tested based on placing in contact beeswax with honey applying constant shaking and temperature that simulates bees flapping and the nature of the beehive. Acaricide residues in both matrices have been monitored over time. This workflow was applied to two types of beeswaxes from the same origin, but with some differences. Specifically, raw and beeswaxes sheets with endogenous acaricide content using a maximum incubation time of twenty four weeks (*test 1*), and decontaminated beeswax sheets spiked with coumaphos standard using maximum incubation times of four and twenty four weeks (*test 2*) were used as shown in Table 1. Regarding *test 2*, it must be specified that two maximum residence times in the incubator were selected depending on the level of fortification. Particularly, samples fortified at low concentrations with coumaphos (F: 40 mg/kg, and G: 4 mg/kg) remained in the incubator for up to twenty four weeks. By contrast, the sample fortified at high concentration (H: 400 mg/kg) was only kept for up to four weeks. This was done in this way since what

Table 1
Design of experiments for test 1 and test 2.

Experiment	Replica	Description	Max. incubation time (weeks)
test 1			
$W_0(1)$	$W_0(1)_1$; $W_0(1)_2$; $W_0(1)_3$;	No-fortified	0
A	A_1; A_2; A_3	No-fortified	4
B	B_1; B_2; B_3	No-fortified	8
C	C_1; C_2; C_3	No-fortified	16
D	D_1; D_2; D_3	No-fortified	20
E	E_1; E_2; E_3	No-fortified	24
$MH_0(1)$	-	-	24
test 2			
$W_0(2)$	$W_0(2)_1$; $W_0(2)_2$; $W_0(2)_3$;	No-fortified	0
F	F_1; F_2; F_3	Fortified beeswax (40 mg/kg)	24
G	G_1; G_2; G_3	Fortified beeswax (4 mg/kg)	24
H	H_1; H_2; H_3	Fortified beeswax (400 mg/kg)	4
$MH_0(2)$	-	-	24

A, B, C, D and E, non-fortified beeswax and honey tests; F, G and H, fortified beeswax with coumaphos and honey tests; $MH_0(1)$, blank (acaricide-free) and control multifloral honey in test 1; $MH_0(2)$, blank (acaricide-free) and control multifloral honey in test 2; $W_0(1)$, raw beeswax, which contained acaricides, without incubation; $W_0(2)$, decontaminated beeswax (acaricide-free) without incubation.

was intended was to verify the time at which residues of acaricides appeared in the honey, and in the case of beeswax spiked at high concentration, these were observed from the first week, with which it was considered sufficient the time of four weeks for this test. In both tests, beeswaxes were analyzed prior to incubation to determine the initial acaricide concentration. Coumaphos was selected to fortified beeswaxes in one of the studies not only because it is an acaricide frequently found in high concentrations in beehive products (El-Nahhal, 2020), including beeswax (Calatayud-Vernich et al., 2017; Nozal et al., 2021), but it was also the only of the three acaricides that was initially present in the stamped beeswax which was found in honey after performing *test 1* (**see** Table 2).

The steps for incubation study were as follows: i) 2 mm beeswax sheets selection: raw beeswax (containing τ-fluvalinate, chlorfenvinphos, and coumaphos residues) for *test 1*, and decontaminated beeswax for *test 2*; ii) cutting 4 cm diameter beeswax disks using metal dies, and weighed (∼ 1.20 g); in the case of fortified samples, beeswax sheets were melted in a thermostated bath and the appropriate volume of coumaphos standard was added. Then, it was homogenized by vortex and poured on aluminum pans where it was left to solidify; iii) each beeswax disk was placed in an Erlenmeyer flask and honey was added until covered the disk surface of the disk (∼3 g); iv) Erlenmeyer flasks were placed in the orbital incubator with programmed shaking (200 rpm), and temperature (30 °C) conditions, which tried to simulate beehive conditions of temperature and bee movement (Ali et al., 2021; Altshuler et al., 2005; Gil-Lebrero et al., 2020); iv) at the end of the predefined incubation time (see Table 2) beeswax disks were separated from honey and rinsed with warm water to eliminate excess honey; v) both matrices were stored in Falcon tubes at 4 °C until analysis; vi) different extraction procedures were carried out to determine the acaricide concentration in both matrices and to monitor their evolution (see Subsections 2.3 and 2.4). It should be mentioned that three replicates were prepared for each assayed condition in both tests, and that they were injected in triplicate.

Table 2
Acaricides concentrations (mg/kg) in beeswax and honey (mean ± %RSD; Results obtained from three replicates injected in triplicate).

Experiment	Residence time (weeks)	τ-Fluvalinate	Chlorfenvinphos	Coumaphos
test 1				
Beeswax				
$W_0(1)$	0	0.70 ± 3.0	0.82 ± 2.6	5.7 ± 4.9
A	4	0.64 ± 4.2	0.73 ± 4.6	5.0 ± 2.4
B	8	0.57 ± 2.6	0.64 ± 2.7	4.6 ± 4.4
C	16	0.53 ± 4.2	0.59 ± 4.1	3.9 ± 2.1
D	20	0.47 ± 2.0	0.58 ± 2.1	3.1 ± 2.5
E	24	0.39 ± 1.6	0.56 ± 3.8	2.5 ± 2.8
Honey				
MH	0	ND	ND	ND
$MH_0(1)$	24	ND	ND	ND
A	4	ND	ND	ND
B	8	ND	ND	ND
C	16	ND	ND	0.014 ± 1.2
D	20	ND	ND	0.026 ± 2.5
E	24	ND	ND	0.036 ± 3.3
test 2				
Beeswax				
$W_0(2)$	0	ND	ND	ND
F	0	ND	ND	38.8 ± 3.5
F	8	ND	ND	31.0 ± 3.3
F	16	ND	ND	17.2 ± 2.2
F	24	ND	ND	13.9 ± 1.2
G	0	ND	ND	3.9 ± 1.9
G	8	ND	ND	3.3 ± 2.1
G	16	ND	ND	2.9 ± 2.0
G	24	ND	ND	2.5 ± 1.9
H	0	ND	ND	399.5 ± 3.4
H	1	ND	ND	346.0 ± 3.1
H	2	ND	ND	288.3 ± 2.9
H	4	ND	ND	207.7 ± 2.1
Honey		ND	ND	
F	8	ND	ND	ND
F	16	ND	ND	0.082 ± 1.4
F	24	ND	ND	0.247 ± 3.1
G	8	ND	ND	ND
G	16	ND	ND	0.011 ± 1.5
G	24	ND	ND	0.028 ± 2.8
H	1	ND	ND	0.102 ± 1.2
H	2	ND	ND	0.177 ± 2.2
H	4	ND	ND	0.241 ± 2.7
$MH_0(2)$	24	ND	ND	ND

A, B, C, D and E, beeswax and honey samples for test 1; F (40 mg/kg), G (4 mg/kg) and H (400 mg/kg), spiked beeswax with coumaphos and honey samples for test 2; MH, blank (acaricide-free) multifloral honey without incubator; $MH_0(1)$, blank and control multifloral honey in test 1; $MH_0(2)$, blank and control multifloral honey in test 2; ND, No detected; LODs-beeswax (*τ*-fluvalinate, chlorfenvinphos, and coumaphos: 2 μg/kg); LODs-honey (*τ*-fluvalinate: 0.3 μg/kg; chlorfenvinphos, and coumaphos: 0.2 μg/kg); $W_0(1)$, beeswax with endogenous acaricide content without incubation; $W_0(2)$, blank decontaminated beeswax (acaricide-free) without incubation.

Finally, the selection of beehive simulation conditions, agitation, and temperature was established based on the existing knowledge in the scientific literature. Altshuler et al. (2005) demonstrated that honeybees possess a significantly high wingbeat frequency of 230 Hz, although this value is largely influenced by the species. To illustrate that, *Apis mellifera*, with an average wing length of 9.7 mm, exhibits wing movements of 240 beats per second. In comparison, a much smaller fruit fly, *Drosophila melanogaster*, flaps its 2.5 mm wings at a rate of 200 times per second. Regarding temperature, Ali et al. (2021) conducted measurements at various locations within the hive and observed a range of 29.7–36.7 °C. Temperatures exceeding 38 °C could potentially result in the mortality of bees, particularly pupae (Vollet-Neto et al., 2015; Gil-Lebrero et al., 2020) also reported temperatures near 30 °C, specifically identifying average temperatures of 34.3 ºC in the brood nest and 29.9 °C in the food area. It is important to note that both parameters are not universally applicable in all scenarios, as both bee wingbeat and hive temperature are influenced by: **i**) climatic and environmental conditions; **ii**) bee size; **iii**) geographic location; **iv**) seasonal variations (Gil-Lebrero et al., 2020; Parmezan et al., 2021).

3. Results and discussion

3.1. Results of the migration studies

3.1.1. Test 1: beeswax samples with endogenous acaricide content

Test 1 was done using raw beeswax sheets, which presented residues of three acaricides (τ-fluvalinate, chlorfenvinphos, and coumaphos) monitoring the samples over four, eight, sixteen, twenty and twenty four weeks. A significant decrease in the coumaphos presence (concentration; see Table 2) was observed, indicating a decomposition or a possible migration to honey with which it was in contact (see Fig. 1A). The variation in the concentrations is less significant for the other two acaricides, τ-fluvalinate and chlorfenvinphos. By contrast, Fig. 1B shows the evolution of the acaricides in honey to study whether this decrease in concentration is due to a possible migration or to other factors such as their decomposition. However, currently there is a gap of information on the distribution, transfer, and degradation of acaricides. It was observed that the compounds, which were in lower concentration in beeswax (τ-fluvalinate and chlorfenvinphos), did not migrate to honey, as can be deduced for the practically constant straight line during the twenty four weeks of incubation. A progressive increase in coumaphos concentration was found proportionally over time. It might suggest a potential transfer from beeswax to honey from the first weeks of contact between both matrices. Thus, considering the results obtained in *test 1* in which only coumaphos migrated to honey, we decided to select this acaricide for performing *test 2*.

3.1.2. Test 2: spiked beeswax samples

Several experiments were carried out to obtain representative

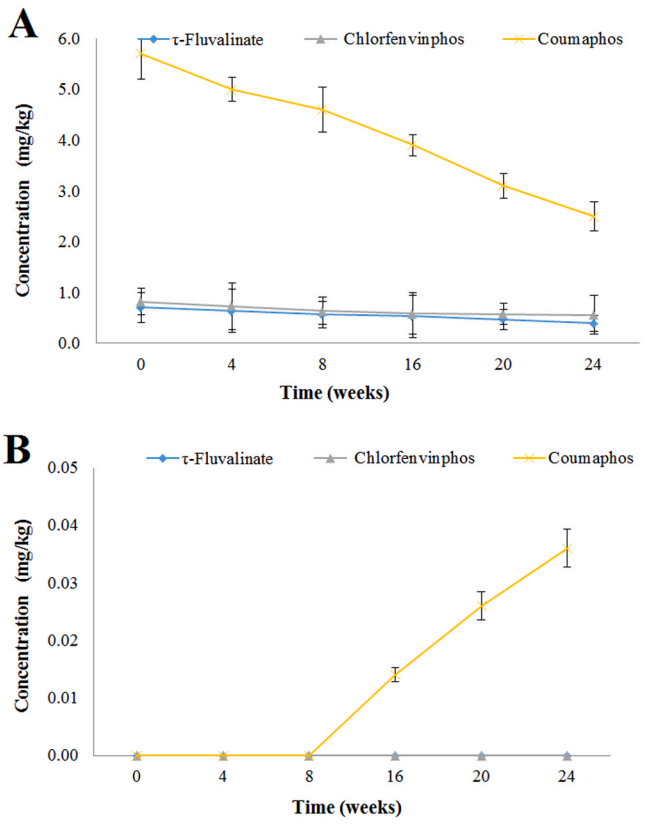

Fig. 1. - Evolution of acaricides' concentrations (mg/kg) in A) beeswax B) honey during *test 1*. Data represent the mean value of three replicates injected by triplicate ± the relative standard deviation of the mean (error bars).

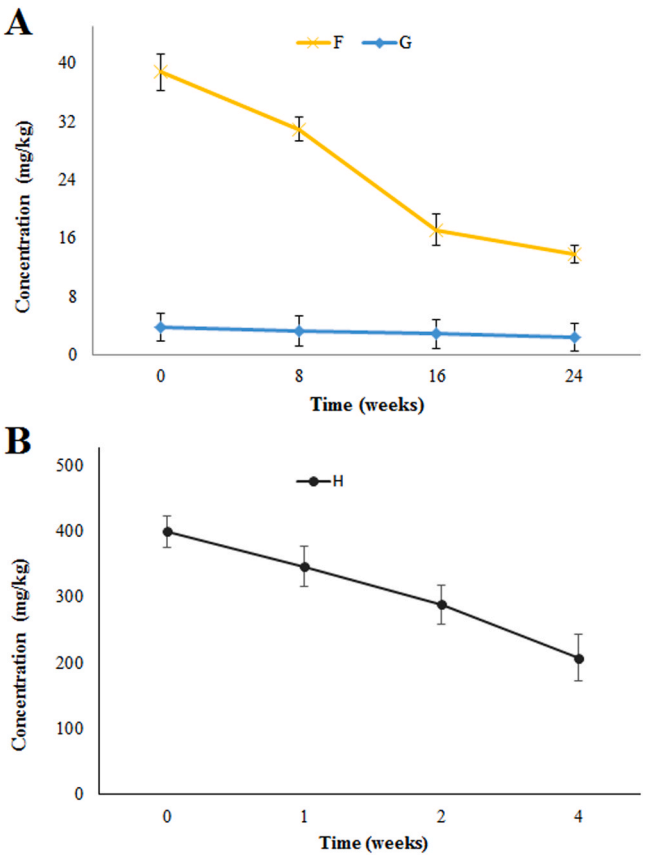

Fig. 2. - Evolution of coumaphos' concentrations (mg/kg) in spiked beeswax samples with at A) low (F and G samples) and B) high concentrations (H samples) during *test 2*. Data represent the mean value of three replicates injected by triplicate ± the relative standard deviation of the mean (error bars).

fortified beeswax samples from which discs could be cut and placed in contact with honey in *test 2*. Firstly, mass of beeswax to be fortified was calculated to get at least three wax discs (replicates) of approximately equal weight after cutting with the metal die. Furthermore, a small amount of beeswax was to be left over in the form of remnants that would also be analyzed to determine the concentration after fortifying. As mentioned above, the most appropriate methodology was melting wax and transferring it to aluminum pans and once solidified, three wax disks were cut. 10 g was the minimum weight of beeswax required for an adequate thickness and 28 g was the maximum beeswax to be melted in view of the capacity of the aluminum container. Preliminary tests were carried out by pouring melted wax on a watch glass and containers of different materials, but homogeneous discs that could be easily demolded were not achieved. Once the range of beeswax quantity needed to form the discs was determined, the incubator agitation and temperature conditions were optimized. Despite migration studies are generally slow processes in time, a first test was performed to assess the potential transfer of acaricides from beeswax to honey under accelerated high temperature and agitation conditions (60 °C and 300 rpm). The main purpose was to observe the distribution of acaricides after one month by analyzing both matrices over one, two and, four weeks. However, this test could not be completed successfully because during the first week beeswax discs began to disintegrate, and the honey carbonized. Consequently, that option was discarded, and it was proved that the simulation of the hive conditions using mild conditions (30 °C and 200 rpm) preserved the original state of both wax and honey.

Test 2 was performed fortifying beeswaxes at different acaricide levels, and a decrease in the coumaphos concentration was again observed in all samples (see Table 2, Fig. 2A and B), and the variation was more significant for the samples spiked at higher concentration (H, 400 mg/kg). However, it should be mentioned that H samples although spent less time in the incubator (maximum four weeks) showed a much greater loss of concentration or coumaphos migration in a shorter time interval, which could be related with the higher spiking concentration. When the evolution of the coumaphos presence in honey was analyzed (see Fig. 3A), it was observed that for the tests with low spiked levels (F and G), migration did not occur or not was detected until sixteen weeks of the study (see Table 2), and in a similar ratio than that observed in *test 1*. On the contrary, a slight increase in the coumaphos concentration was found in H samples since the first week (see Table 2 and Fig. 3B), and it was proportionally higher over time. Indeed, a concentration 0.241 mg/kg of coumaphos was detected after only four weeks. Consequently, it has been corroborated that the migration of coumaphos form beeswax to honey can occur by simulating the beehive conditions, which is in good agreement with the findings of previous works (Albero et al., 2023; Baša Česnik et al., 2019; Kast et al., 2021; Murcia-Morales et al., 2020; Shimshoni et al., 2019). In addition, it can be also tentatively concluded that the migration of coumaphos from beeswax to honey depends on the initial level of this compound in the beeswax, not only in relation to the overall amount but also in the percentage of compound migrated to honey. Thus, acaricide contents lower or equal than 40 mg/kg do not migrate significantly into the honey until they have passed sixteen weeks (see Table 2). This finding is in good agreement with a previous study (Karazafiris et al., 2022) in which it was stated that coumaphos may be transferred from wax to other beehive matrices such as royal jelly even in low concentrations. By contrast, a migration of coumaphos was fastest at highest concentrations (400 mg/kg), as residues of this acaricide were already observed in the first week of contact. However, such high concentrations, simulated by spiking the samples, are not generally reported in decontaminated beeswax sheets (Nozal et al., 2021).

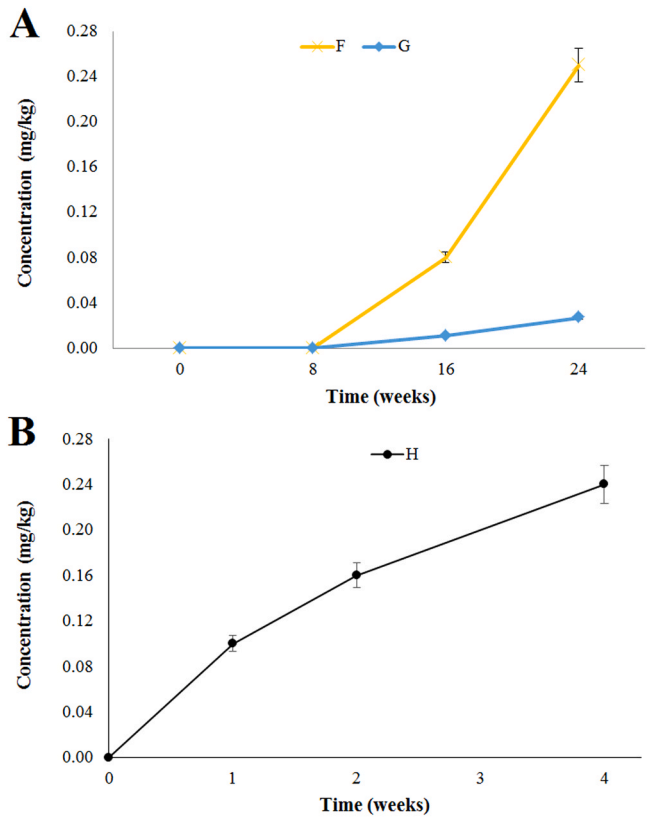

Fig. 3. - Evolution of coumaphos´ concentrations (mg/kg) in honey samples, which were in contact with beeswax samples spiked at A) low (F and G samples) and B) high concentrations (H samples) during *test 2*. Data represent the mean value of three replicates injected by triplicate ± the relative standard deviation of the mean (error bars).

3.2. Overall discussion of the results

The transfer of acaricides, particularly coumaphos, from wax to honey had already been reported years ago. For example, Wallner (1992) showed that coumaphos could be transferred to honey at detectable levels from wax. Migration of coumaphos from fortified beeswax (up to 1000 mg/kg) into honey was also found by Wallner (1999) and Kochansky et al., (2001) under laboratory conditions for twenty six weeks. Lodesani et al. (2008) also agreed with those findings confirming that high concentrations of coumaphos in wax cause the transfer of detectable levels of residues to the honey extracted. Indeed, our study confirms the previous findings (Kochansky et al., 2001), as the coumaphos load on beeswax particles became significant at high concentration, and the evolution in honey reflects the situation in beeswax. However, it should be mentioned that to our knowledge, no study was performed by simulating the beehive conditions as we have done in the present work.

Migration questions also stem from the fact that in the hive all matrices are connected, thus contaminants can migrate by diffusion/partition from the beeswax to the stored honey or even to the bees themselves. Regarding acaricides evolution, the different accumulation patterns can be explained according to physicochemical properties with the value of log P, although it should be considered that the compounds are with similar physicochemical characteristics. For example, τ-fluvalinate (log P 7.0) is more lipophilic than coumaphos (log P 3.93), and therefore has a greater tendency to accumulate in matrices with a high content of nonpolar components such as beeswax. It is also essential to underline that beeswaxes that have not undergone a decontamination process reveal high concentrations of pesticides (Nozal et al., 2021). Focusing on honey, Murcia-Morales et al. (2022) emphasized that this matrix is generally the least contaminated. The high polarity of its components, mainly water and carbohydrates, causes low migration and accumulation of most pesticides, which are of intermediate/low polarity. Nevertheless, although acaricide-positive bee samples have been detected, the concentrations found in honey were not significant or the levels were below MRL (Amulen et al., 2017). However, it does not necessarily mean that honey is not contaminated, as the detection of pesticides in bee products is influenced by factors such as: physicochemical properties, pesticide application, sensitivity, and extent of the analytical method, or even sample preparation. In this sense, a previous study in which the behavior and distribution of the acaricide coumaphos released from strips in treated colonies was assessed (Karazafiris et al., 2008). The experimental results of this study showed considerable variability in coumaphos levels in honey. This variability could be explained because of floating particles contaminated with coumaphos in honey, and if they would be analyzed beeswax samples instead of honey samples, the variability could be neglected. Finally, it is of utmost importance to highlight that this work is a pilot study, and it would be recommended to continue carrying out more experimental designs optimizing all the steps and considering the difficulty of reproducing beehive conditions. There are scarce studies in the scientific literature evaluating the potential transfer of acaricides from beeswax to honey and although most publications analyzed pesticide residues in both matrices, they did not simulate beehive parameters.

We are aware of the difficulty of simulating hive conditions given their complexity, and that there are many other factors that can potentially affect the migration of acaricide residues such as the work of the bees or humidity. Therefore, this research is a pilot study that takes into consideration temperature, wing movement, and the degree of contamination of the beeswax sheets. We support the fact that more complementary studies are needed where these other parameters must be studied and a larger number of analytes should be monitoring, but our research sheds light on this potential transfer between both matrices.

4. Conclusions

The potential migration of acaricides from stamped beeswax to honey by simulating beehive conditions has been tested through this pilot study. Raw beeswaxes with endogenous acaricide content, and decontaminated waxes fortified with acaricide were placed in contact with acaricide-free honey in an incubator. Mild temperature and agitation conditions were set, and acaricide residues were monitored throughout several weeks. Beeswax and honey were analyzed using previously validated GC-MS methods. It was found that coumaphos was the only acaricide of the three initially present in beeswax that migrated to honey, although at lower concentrations; meanwhile, it was not detected a migration of τ-fluvalinate and chlorfenvinphos during the period the time of the study. Subsequently, it was selected for the second part of the study in which it was studied the potential migration of acaricides from spiked beeswax to honey. Results showed that migration was observed from the first week of the study when coumaphos was spiked at the highest concentration in beeswax; meanwhile, for lower concentrations, the transfer of coumaphos from beeswax to honey did not begin until sixteen weeks. Consequently, it can be tentatively concluded that the initial acaricide concentration in beeswax significantly influences the transfer to the honey. However, it should be considered that this study constitutes a first approach to evaluate the migration of acaricides from beeswax to honey by simulating beehive conditions, and therefore it is suggested in the future to conduct further studies and designs of experiments using waxes of different origins, more and different acaricides and concentrations, as well as varying the experimental conditions.

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CRediT authorship contribution statement

Adrián Fuente-Ballesteros: Conceptualization, Formal analysis, Methodology, Investigation, Visualization, Writing – original draft; Writing – review & editing. **María J. Nozal:** Conceptualization, Resources, Supervision, Visualization. **Ana M. Ares:** Conceptualization, Investigation, Supervision, Visualization, Writing – original draft. **José Bernal:** Conceptualization, Project administration, Resources, Supervision, Visualization, Writing – original draft; Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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