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Original article

Comparison of the transfer of coumaphos from beeswax into syrup and honey[1]

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Abstract – The organophosphate insecticide coumaphos has recently received emergency approval in the United States for control of fluvalinate-resistant *Varroa destructor* and the small hive beetle, *Aethina tumida* Murray. We investigated the transfer of coumaphos from wax into syrup and honey, using adsorption of coumaphos from diluted syrup or honey onto a solid-phase extraction cartridge, elution, and subsequent analysis. Coumaphos in syrup was quantitated using HPLC with UV detection, and we found that coumaphos migrates from wax into syrup, with low concentrations increasing over a few months. Concentrations reached 200–300 ppb in 100 g of syrup in contact with 10 g of wax containing 1000 ppm of coumaphos; contact with wax containing 100 and 10 ppm led to lower amounts. Impurities made HPLC determination of coumaphos in honey impossible, but the solid phase extract could be analyzed by gas chromatography/mass spectrometry. Concentrations in honey were similar to those in syrup, reaching 430 ppb after 26 weeks at 1000 ppm in wax.

coumaphos / wax / honey / residues / pesticide contamination

1. INTRODUCTION

Coumaphos ('Perizin', Bayer Corp.) was first investigated as an apicultural acaricide by Ritter (1985) and Neuhauser (1985) and approved for use against the parasitic mite *Varroa jacobsoni* Oudemans (now *Varroa destructor* Anderson and Trueman (Anderson, 2000; Anderson and Trueman, 2000) in Germany in 1985, as an emulsion applied directly to the bees and frames (Neuhauser and Krieger, 1988). The distribution of

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[1] Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

coumaphos within the colony and the effects on bees were studied by van Buren et al. (1992a).

The organophosphate insecticide coumaphos has recently received emergency approval in the United States for control of fluvalinate-resistant *V. destructor* and the small hive beetle, *Aethina tumida* Murray.

Since honey is used for human consumption, several groups have conducted coumaphos residue analyses on wax and honey. Neuhauser and Krieger (1988) claimed that residues in honey did not exceed 10 μg/kg (parts per billion, ppb, parts per 10^9) even after several years' use. Thrasyvoulou and Pappas (1988) found concentrations in Greek honey of up to 6 ppb and in wax of up to 2.83 mg/kg (parts per million, ppm, parts per 10^6). Similar residue levels were found in honey by others (Gallo and Genduso, 1986; Van Buren et al., 1992b; Fernandez Garcia et al., 1994; Garcia et al., 1996), and but were not detected in honey by Fernandez Muiño et al. (1997). In the laboratory, Wallner (1992) showed that coumaphos could be transferred into honey at detectable levels (0.5 ppb) from wax containing as little as 1 ppm of coumaphos, with concentrations increasing with higher wax concentrations. A later paper (Wallner, 1995) provided further data; concentrations of coumaphos in honey of from 0.7 to 94 ppb resulted from contact of honey with thin layers of wax containing from 1–400 ppm of coumaphos in 30 days at 30 °C. Analysis was carried out by solid-phase extraction and gas chromatography, but experimental details beyond that were not given. We decided to investigate the transfer of coumaphos from contaminated wax into syrup and honey under laboratory conditions.

2. MATERIALS AND METHODS

Syrup was prepared from SigmaUltra grade sucrose (Sigma). A syrup concentration of 67% w/w (sucrose:water 2:1 by weight, '2:1 syrup') was used. Unless otherwise specified, references to 'syrup' refer to this formulation. Honey was a blend of several commercial products obtained from a local grocery store. Analysis of this blend prior to experiments showed no coumaphos. Coumaphos was an analytical standard from Chemagro (now Bayer Animal Health, Shawnee Mission, KS), nominally 98.7% pure. Methanol and acetonitrile (both HPLC grade) and oxalic acid dihydrate (ACS reagent grade) were obtained from Aldrich. Beeswax was from a supply obtained from Betterbee (Greenwich, NY) before the registration of coumaphos in the US. No evidence was found for the presence of any coumaphos in this wax (absence of any coumaphos from samples of syrup or honey in contact with control wax). Water was purified with a Nanopure apparatus (Barnstead/Thermolyne, Dubuque, IA).

An initial concentrate of coumaphos was prepared by dissolving coumaphos (50 mg) in beeswax (50 g) to yield a concentration of 1 mg/g of wax (1000 ppm). This was used for the highest concentration, and dilutions of the concentrate were used for the 100 and 10 ppm concentrations, with enough untreated wax to make a total of 10.0 g in each case. The wax samples (10 g) were placed in the bottom of 250 mL Erlenmeyer flasks, melted on a hot plate, swirled to mix, and allowed to cool. Concentrations of coumaphos in the wax were not assayed directly. A model system was used instead. A solution of 1000 ppm of CI disperse blue 14 (1,4-bis(methylamino)anthraquinone) in wax was prepared; dilution of 0.5 g of this with 10 g wax gave a homogeneous light green color in a shorter time of swirling than that used for the coumaphos. Since the coumaphos was dissolved in the molten wax, it would not be expected that difficulties would occur in the mixing. In another study, coumaphos concentrations observed in wax were reported to be similar to those added (Fries et al., 1998).

Syrup or honey (100 g) was added to the flasks, which were stored in an incubator at 34 °C in the dark. Flasks were not agitated during incubation. In the case of the honey samples, two flasks of each concentration flasks were prepared, and were sampled alternately at successive sampling times. Two sets of flasks were used to extend the duration of the experiment without changing the honey/wax ratio any more than necessary. In the syrup study, only 3 samples were taken over the 20 weeks, whereas in the honey study, 11 samples were taken over 26 weeks. Available incubator space limited the number of flasks we could use. Syrup samples were taken over 20 weeks before the samples developed mold; honey samples were taken over 26 weeks before the viscosity of the honey became too high for convenient sampling.

Flasks were sampled using a pipette to withdraw aliquots and weighing out 5.0 g of sample from the pipette into a 50-mL Erlenmeyer flask. Coumaphos was isolated from the samples using solid-phase extraction cartridges (Oasis HLB, 200 mg/6 mL, Waters, Inc., Medford, MA), with the aid of a vacuum manifold (Pierce, Rockford, IL). Cartridges were conditioned with methanol (1 mL), followed by water (1 mL). Honey or syrup (5 g) was diluted with water (10 mL) and passed through the conditioned cartridge. After passage of 5% methanol (1 mL), followed by 100% methanol (1 mL), the coumaphos was eluted with acetonitrile (1mL). The 100% methanol and acetonitrile fractions were collected separately, as the methanol fraction sometimes contained a little coumaphos. The two fractions were analyzed separately, and coumaphos concentrations were added to obtain the final result. We did not investigate adsorption of coumaphos onto the walls of the various glass and plastic objects involved in the assay.

Coumaphos was determined in syrup extracts by high performance liquid chromatography (HPLC) by direct injection of extract solutions onto a 3 mm × 250 mm column packed with C_{18} silica gel (Supelcosil LC-18-DB, 5 μm particle size, Supelco, Inc., Bellefonte, PA), using a full 20 μL injector loop. For the syrup runs, flow rate was 0.7 mL/min of methanol–acetonitrile–0.01 M oxalic acid (20:30:50 by volume) (Oka et al., 1994). The oxalic acid was necessary for other analyses being carried out at the same time, and caused no problems for the coumaphos analyses reported here. Coumaphos was detected at a wavelength of 315 nm with a SpectraSYSTEM UV2000 detector and quantitated with an SP4400 integrator (Thermo Separation Products, San Jose, CA). Range was 1.0 absorbance units full scale for all analyses. Each point is the average of three injections. Injections of a series of coumaphos standards in the range of 0.2 ng–2 μg gave a linear plot with an average detector response of 6438 detector units/ng.

This technique could not be used for analyses in honey, since some of the honey pigments coeluted with the coumaphos and made quantitation difficult. Coumaphos in honey was therefore analyzed by gas chromatography/mass spectrometry using a similar solid-phase extraction, but the final elution was with 1 mL of toluene. An aliquot of the toluene fraction (1.0 μL) was injected by an on-column injector (J&W Scientific, Folsom, CA) onto a Restek Rtx-5 MS capillary column (30 meter, 0.25 mm ID, 0.25 μm df, column head pressure 0.9 bar, Restek Corporation, Bellefonte, PA). The initial column temperature of 60 °C was held for 1 minute, then the temperature was raised 20 °C/min to 270 °C and held at 270 °C for 14.5 minutes (total run time 26 minutes). The column effluent was analyzed on a Finnegan GCQ mass spectrometer (Finnegan MAT, San Jose, CA) operated in positive electron impact mode (70 eV) at a source temperature of 165 °C. The transfer line between the GC and the source was held at 275 °C. The amount of coumaphos was determined by comparison of sums of ions at m/z 362, 334, and 306 in the

sample vs. the sum of the same ions in a 2.5 ng injection of authentic coumaphos. Injection of a series of coumaphos standards in the range of 0.1–25 ng gave a linear plot.

Estimated minimum quantifiable concentrations (using spiked samples) were 4 ppb in syrup (20 μL injection) and 10 ppb in honey (1 μL injection).

Data reduction and plotting were performed with GraphPad Prism ver. 3.0 (GraphPad Software, Inc. San Diego, CA). All concentrations in ppm refer to coumaphos in wax; concentrations in syrup and honey are expressed in ppb.

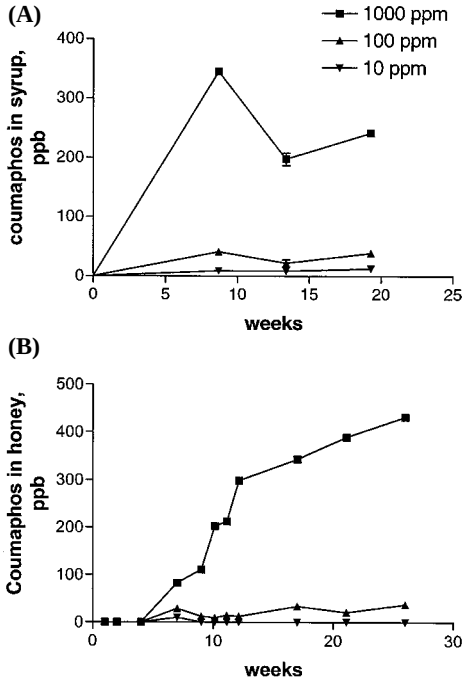

Figure 1. Transfer of coumaphos from wax into syrup and honey (legend is concentration of coumaphos in wax). (**A**) Plot of coumaphos concentration vs. time in sucrose syrup (100 g) in contact with wax (10 g) containing coumaphos at 10, 100, and 1000 ppm, determined by HPLC. (**B**) Plot of coumaphos concentration vs. time in honey (100 g) in contact with wax (10 g) containing coumaphos at 10, 100, and 1000 ppm, determined by GC-mass spectrometry.

3. RESULTS AND DISCUSSION

Coumaphos was extracted from wax into syrup at low but detectable concentrations, increasing to an apparent equilibrium after 2–3 months. An initial experiment using 75% syrup (3:1 syrup) in contact with wax containing 1000 ppm of coumaphos gave a concentration of up to 360 ppb (data not shown), but since crystals of sucrose were deposited on the wax, this concentration was not investigated further. Syrup (2:1) in contact with wax containing 1000 ppm of coumaphos reached concentrations of 200–340 ppb after 19 weeks (Fig. 1a). Wax containing 100 and 10 ppm of coumaphos gave concentrations in syrup of 20–40 and 8–12 ppb, respectively after the same time interval. We have no explanation for the apparent high coumaphos concentration at 8 weeks. It may have been a sampling artifact. The syrup became slightly moldy after 3 months, so the experiment was terminated.

Honey in contact with coumaphos-treated wax similarly extracted the insecticide from the wax (Fig. 1b). After 12 weeks, honey in contact with wax containing 1000 ppm of coumaphos had extracted 300 ppb. After the same interval, honey extracted 15 ppb coumaphos from wax containing 100 ppm. After 26 weeks, the concentration of coumaphos in honey exposed to wax containing 1000 ppm had increased to 430 ppb and was still increasing slowly. Concentration in honey exposed to wax containing 100 ppm had reached 37 ppb after 26 weeks, and was also still increasing slowly. Coumaphos could not be detected in honey in contact with wax containing 10 ppm. Greater contact area would presumably have allowed equilibrium to be approached more rapidly.

In contact with uncontaminated wax, neither syrup nor honey showed any coumaphos; the controls are not shown in the figures. This supports the assumption that the wax purchased was free of serious coumaphos contamination.

Honey and syrup sample sizes were in each case 5 g. Larger samples would have given greater sensitivity to coumaphos at the expense of decreased number of sampling points. The extreme of this was the type of study reported by Wallner (1992, 1995) in which the wax was cast in a thin layer in a petri dish, covered with honey, and the entire honey layer was analyzed. We decided to sacrifice the sensitivity of this method for the ability to follow the extraction over time.

It is clear from our results and those of Wallner that coumaphos can be transferred from wax into either honey or syrup. Concentrations are < 1 ppm, even with high concentrations in wax, but they are readily detectable using the proper techniques. When this work was started, there was no tolerance for coumaphos in either wax or honey in the US, so transfer of coumaphos into honey was a potentially serious problem. In August 2000, the US EPA granted a tolerance for coumaphos of 100 ppb in honey and 100 ppm in beeswax (Environmental Protection Agency, 2000). Both our results and those of Wallner suggest that for any concentrations of coumaphos in wax below the tolerance, there will not be a level in honey that exceeds 100 ppb. Nonetheless, in order to minimize potential contamination, it would be prudent to avoid using any combs having had any contact with coumaphos for extracted honey, reserving them only for use in the brood chamber.

Résumé – Étude comparative du transfert du coumaphos dans la cire à partir du sirop ou du miel. L'insecticide organophosporé coumaphos est utilisé en Europe contre l'acarien *Varroa destructor*. Il a reçu récemment une autorisation d'urgence aux États-Unis pour lutter contre *V. destructor* résistant au fluvalinate et contre le petit coléoptère des ruches, *Aethina tumida*. Lorsque l'expérience a démarré, aucun résidu de cet insecticide n'était autorisé dans les produits de la ruche. Nous avons utilisé une méthode analytique qui fait intervenir l'adsorption de coumaphos à partir d'échantillons de sirop/miel dilués sur une cartouche d'extraction en phase solide ; on procède ensuite à l'élution puis à une analyse pour déterminer l'extraction du coumaphos à partir de cire contaminée et son incorporation dans le sirop ou le miel. Le coumaphos dans le sirop a pu être quantifié par HPLC et détection UV, mais les impuretés présentes dans le miel ont rendu l'analyse par HPLC du coumaphos difficile. Le coumaphos dans les extraits de miel a été déterminé par chromatographie en phase gazeuse couplée à la spectrométrie de masse. Nos résultats montrent que le coumaphos peut être transféré dans la cire à partir du sirop ou du miel avec des concentrations qui augmentent sur quelques mois. La concentration finale est néanmoins faible avec seulement 200 à 300 ppb pour 100 g de sirop en contact avec 10 g de cire renfermant 1 000 ppm de coumaphos au bout de 19 semaines. Le sirop en contact avec de la cire renfermant 100 ou 10 ppm de coumaphos contenait aussi des quantités décelables de coumaphos, mais elles n'étaient respectivement que de 30 et 10 ppb (Fig. 1a). Les concentrations dans le miel étaient semblables à celles du sirop, atteignant 430 et 37 ppb au bout de 26 semaines pour des miels en contact avec de la cire renfermant respectivement 1 000 et 100 ppm de coumaphos, mais nous n'avons pas pu détecter de coumaphos dans le miel en contact avec de la cire renfermant 10 ppm de coumaphos. (Fig. 1b). Pour de la cire présentant des résidus inférieurs au nouveau seuil toléré de 100 ppm, les taux de coumaphos dans le miel ne devraient pas excéder la limite de 100 ppb tolérée aux USA par l'Agence de protection de l'Environnement (EPA).

coumaphos / résidu / contamination / miel / cire / pesticide

Zusammenfassung – Ein Vergleich des Transfers von Coumaphos aus Bienenwachs in Zuckerlösung oder Honig. Das auf Organophosphat beruhende Pestizid Coumaphos wird in Europa zur Bekämpfung der Milben *Varroa destructor* benutzt. Vor kurzem erhielt es eine „Notregistrierung“ in den Vereinigten Staaten, um es als Bekämpfungsmittel gegen Fluvalinat widerstandsfähige *Varroa* Milben und gegen den kleinen Beutenkäfer einzusetzen. Zur Zeit des Versuchsbeginns waren keine Rückstände von Pestiziden in Bienenprodukten zugelassen. Um den Übergang von Coumaphos von kontaminiertem Wachs in Zuckerwasser und Honig zu bestimmen, führten wir die Analyse mit Methoden durch, die eine Adsorption von Coumaphos aus verdünnten Zuckerwasser- bzw. Honigproben auf eine Festphasenextraktionssäule sowie eine anschließende Elution ermöglichten. Im Zuckerwasser konnte Coumaphos im HPLC (Hochdruckflüssigchromatograph) mit UV – Detektor quantifiziert werden, aber im Honig machten Verunreinigungen die HPLC – Analyse schwierig. Deshalb wurde Coumaphos aus Honigproben nur mit dem GCMS (Gaschromatograph mit Massenspektrographen) bestimmt.

Wir stellten fest, dass Coumaphos von Wachs auf Zuckerwasser oder Honig übergeht, wobei sich die Konzentrationen im Laufe mehrerer Monate des Kontakts erhöhten. Insgesamt ist die Konzentration in 100 g Zuckerwasser nach einer Kontaktzeit von 19 Wochen mit 10 g Wachs, das 1 000 ppm Coumaphos enthält, mit nur 200–300 ppb jedoch gering. Zuckerwasser, das mit Wachs mit 100 ppm bzw. 10 ppm Coumaphos in Kontakt stand, enthielt ebenfalls nachweisbare Mengen von Coumaphos, aber nur 30 bzw. 10 ppb (Abb. 1a). Die Konzentrationen im Honig waren ähnlich wie die im Zuckerwasser, sie erreichten 430 und 37 ppb nach 26 Wochen, mit einer Ausnahme: wir konnten kein Coumaphos im Honig nachweisen, der mit Wachs in Kontakt stand, das nur 10 ppm Coumaphos enthielt (Abb. 1b). Bei Wachs, dessen Gehalt an Coumaphos unterhalb der neu bestimmte Toleranzgrenze von 100 ppm Coumaphos liegt, dürfte die Toleranzgrenze von 100 ppb im Honig nicht überschritten werden.

Coumaphos / Wachs / Honig / Rückstände / Kontamination durch Pestizide

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