# Study of Acaricide Stability in Honey. Characterization of Amitraz Degradation Products in Honey and Beeswax

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A study on the possible degradation of amitraz, bromopropylate, coumaphos, chlordimeform, cymiazole, flumethrin, and tau-fluvalinate during the storage of honey was carried out by HPLC. Except amitraz, the other acaricides are stable in this medium for at least 9 months. Degradation studies of amitraz in honey and beeswax were carried out; the degradation products detected in both matrices were 2,4-dimethylphenylformamide (DMF) and *N*-(2,4-dimethylphenyl)-*N*′-methylformamidine (DPMF). The reaction rate constants and the half-lives of the amitraz degradation in honey and wax were calculated. Amitraz was nearly completely degraded within 1 day in beeswax and within 10 days in honey. When amitraz-spiked combs are recycled into new beeswax, DMF was found to be the principal degradation product left in pure wax.

**Keywords:** *Acaricide residues; amitraz; bromopropylate; cymiazole; coumaphos; chlordimeform; tau-fluvalinate; flumethrin; degradation products; kinetics; honey; beeswax*

## INTRODUCTION

Presently acaricides are used worldwide for the control of the honey bee parasite *Varroa destructor*, a mite that endangers beekeeping all over the world. Most synthetic acaricides are lipophilic and accumulate in beeswax, whereas honey residues are relatively low and lie mostly below the maximum residue limits (MRLs) fixed by laws (*1*, *2*).

Knowledge of the stability of the active ingredients of acaricides is very important, and residue studies should always include a sound investigation of the stability of the analyzed active ingredient. Amitraz residue methods in honey have been proposed, which determine only the active ingredient amitraz (*3*, *4*); however, due to the known instability of amitraz in honey (*5*, *6*), analytical methods for the determination of the total amitraz residues (i.e., including the metabolites) have been proposed (*6*, *7*). Acaricide registration authorities should be cautious to demand metabolism and stability studies clarifying whether the active ingredient is stable or not in honey and wax. Indeed, some varroacides seem to be unstable in aqueous media depending on the pH value. Cymiazole is stable at any tested pH (1−11); amitraz is unstable in all of them; bromopropylate, coumaphos, flumethrin, and tau-fluvalinate are unstable at basic pH, and chlordimeform is unstable in neutral and basic media (*8*−*10*). According to these results, amitraz is the unique acaricide that is unstable in acidic media. Honey is acidic, and breakdown of amitraz in honey has been reported (*5*, *6*, *11*). Dissipation in honey of other acaricides, such as coumaphos, malathion, and tau-fluvalinate, which are theoretically stable under acidic conditions, has also been reported, but some of these results are controversial. Degradation of tau-fluvalinate in honey has been reported (*12*, *13*). On the other hand, in other reports it was stated that fluvalinate is stable in honey (*14*−*16*). For coumaphos, too, contradictory reports have been made. Coumaphos dissipation during the acaricide treatment (*17*) and during honey storage (*18*, *19*) has been reported. On the other hand, other workers claim that coumaphos is stable in honey (*20*, *21*). For cymiazole (*22*) and flumethrin (*23*) stability upon storage for 3 months has been reported.

The chemical structures of the degradation products in honey have been elucidated for tau-fluvalinate (*13*) and amitraz (*5*, *6*, *11*). According to these authors the main breakdown products of amitraz were 2,4-dimethylaniline (DMA), 2,4-dimethylphenylformamide (DMF), and *N*-(2,4-dimethylphenyl)-*N*′-methylformamidine (DPMF), but the relative amounts of the degradation products in amitraz-spiked honey were different. One author found relative amounts of DPMF, DMF, and DMA of 50, 25, and 25% (*5*), whereas another found 0, 85, and 15% (*11*), respectively. Also, degradation kinetics was not carried out in these studies, which is important for the evaluation of the time of dissipation.

Beeswax is a highly lipophilic medium, containing high molecular weight acids. According to present knowledge synthetic acaricides such as coumaphos, bromopropylate, flumethrin, and tau-fluvalinate are stable and persistent in beeswax (*1*, *2*). To our knowledge there are no studies of the characterization of amitraz degradation products in beeswax. In one study amitraz residues in beeswax have been determined after chemical conversion to 2,4-dimethylaniline (DMA) (*24*), and in another (*25*) amitraz breakdown was found but the dissipation time and products were not studied.

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As cited above, there is some uncertainty regarding the stability of the acaricides in honey, and the breakdown products of amitraz have not been studied. Therefore, we set the following objectives for the present study: (1) to determine if amitraz, bromopropylate, chlordimeform, cymiazole, coumphos, flumethrin, and tau-fluvalinate degrade in honey; (2) to determine the degradation kinetics and identify the possible degradation products of amitraz in honey and beeswax; and (3) to determine the fate of the amitraz degradation products after recycling of old wax combs into new beeswax.

## MATERIALS AND METHODS

**Reagents and Aparatus.** Standards of amitraz, DMF, DMA, and DPMF (hydrochloride salt) were kindly supplied by Schering Agrochemicals Ltd. (Hauxton Cambridge, U.K.). Bromopropylate and cymiazole were provided by Ciba-Geigy (Basel, Switzerland). Tau-fluvalinate, coumaphos, and chlordimeform were from Cromlab S.L. (Barcelona, Spain), and flumethrin was from Bayer (Barcelona, Spain). Stock standard solutions of these compounds were prepared in acetonitrile at a concentration of 1000 $\mu$g/mL and were stored at 4 °C in the dark for a maximum of 1 year.

Methanol, ethanol, acetone, acetonitrile, and *n*-hexane were of HPLC grade. The water used in all experiments was purified on a Milli-Q system (Millipore, Bedford, MA). All other reagents were of analytical quality. The aqueous buffers used were 0.1 M sodium phosphate, pH 6−8.

The ultrasonic bath was a Knauer (Berlin, Germany) Bandelin Sonorex TK 52 or a Selecta (Barcelona, Spain) Ultrasons. For centrifugation a Sorvall RC5C centrifuge equipped with an M-24 rotor or a Kontron Instruments (Zurich, Switzerland) Centrikon T-124 centrifuge with a JA-20 rotor was used.

**Evaluation of the Stability of Acaricides in Honey and Beeswax.** *Fortification of Honey with Acaricides.* The experiments were carried out with honey samples spiked with acaricides at concentrations from 10 to 25 $\mu$g/g.

For spiked honey samples a multifloral commercialized honey was heated at 40 °C in a water bath for 20 min and then allowed to stand at room temperature for 10 min. After that time, 4 mL of a known concentration of an acaricide solution in acetonitrile was added to 20 g of this honey. The mixture was mechanically stirred with a blender and with a spatula and then left at room temperature until complete solvent evaporation (controlled by weight). This spiked honey was kept in the closed glass container at room temperature (20−25 °C) for 9 months, away from exposure to direct sunlight.

*Fortification of Beeswax with Amitraz.* To 100 g of chopped commercial beeswax, previously analyzed to ensure that it was free of amitraz residues, was added 10 mL of an amitraz solution in acetonitrile. Then the mixture was stirred by a blender to ensure homogeneity and left at room temperature until complete solvent evaporation (controlled by weight). The final concentration of amitraz in beeswax was 100 $\mu$g/g. The wax was kept in the closed glass container at room temperature, away from exposure to direct sunlight.

A model laboratory experiment was carried out to study the behavior of amitraz degradation products during the recycling process to produce new beeswax. Ten grams of beeswax, spiked with 100 $\mu$g/g of amitraz and stored for 2 weeks to ensure complete breakdown of amitraz, was mixed with 100 mL of water. The mixture was boiled for 1 h at 100 °C and then cooled, and the solid recycled wax was separated from water. Amitraz metabolites in the wax were analyzed by HPLC (in triplicate) before and after heating.

**Extraction of Acaricides.** *Honey.* One gram of the spiked honey was dissolved with 2 mL of the binary mixture of 0.1 M sodium phosphate buffer−acetonitrile (80:20 v/v). This solution was transferred to a volumetric flask and filled to 5 mL with the corresponding buffer−acetonitrile (80:20 v/v). The buffer pH was set at 6 for all of the acaricides except amitraz, for which the pH of the buffer was set at 8 due to the rapid hydrolysis of amitraz in acidic media. The percentage of acetonitrile used was necessary to get a good solubilization of the acaricides. After sonication during 5 min, this solution was filtered and directly injected into the HPLC-UV system.

For confirmation of the identity of the degradation products, the water of this solution was evaporated in a vacuum, and the remaining residue was subsequently extracted with 5 mL of acetonitrile and injected in GC-MS.

*Beeswax.* One gram of beeswax was placed into a glass centrifugation tube and 10 mL of the selected extraction solvent added. For amitraz extraction hexane was selected as extractant due to the instability of amitraz in solvents such as methanol (*26*), whereas for the extraction of its degradation products, which are more polar, methanol, ethanol, acetonitrile, or acetone was used. The extraction mixture was sonicated in an ultrasonic bath during 1 h. After this time, centrifugation at 20 °C and 9000*g* during 20 min separated the extract. The supernatant was collected in a new tube, which was placed into a freezer for at least 2 h, and then it was centrifuged at −4 °C and 9000*g* during 20 min. With this procedure, the greater part of the insoluble high molecular weight components of the beeswax is removed as a precipitate (*27*). After filtering, the supernatant was analyzed by HPLC or GC-MS.

**Instrumentation.** *HPLC.* HPLC was used in the kinetic study to measure the decrease of acaricide peak area as a function of time and for the study of the amitraz breakdown products in honey and wax. The chromatographic system consisted of two LKB (Bjorkgatan, Sweden) 2150 pumps, a high-pressure mixer LKB 2152-400, an HPLC Controller LKB 2152, a Rheodyne (Cotati, CA) 7125 sample injector with a 50 $\mu$L loop, and a Waters (Barcelona, Spain) 484 UV−vis detector. A Waters Nova-Pak C18 (15 cm × 3.9 mm i.d., 4 $\mu$m) column was used with a Waters Nova-Pak C18 (20 mm × 3.9 mm i.d., 4 $\mu$m) precolumn. The effluent was monitored at 210 and 250 nm. The flow rate was 1 mL/min. The elution was performed under binary gradient conditions. The gradient used was initially 1.5 min with an isocratic mobile phase of acetonitrile−0.01 M TEA, pH 6.1 (adjusted with 0.75 M $H_3PO_4$), 30:70 (v/v), and then a linear gradient was applied arriving at 100% acetonitrile at 23 min.

The detector had a linear response for the whole determination range. The concentrations of acaricides and their possible breakdown products were determined by comparison of the peak areas of the acaricides and the three degradation products of amitraz with standards.

*GC-MS.* GC-MS was used for the identification of the amitraz metabolites in wax. A Hewlett-Packard (Palo Alto, CA) 5890 series II gas chromatograph was directly coupled to a Hewlett-Packard 5971 mass spectrometer. The capillary column used was a DB-17 MS 30 m × 0.25 mm × 0.25 $\mu$m from J&W Scientific (Folsom, CA), and the precolumn used was a DB-XLB 50 cm × 0.53 mm × 0.050 $\mu$m from J&W Scientific. The injector and detector temperatures were 240 and 280 °C, respectively. The carrier gas was helium, and the headspace pressure was 40 kPa. The oven temperature program was as follows: 5 min at 50 °C, then a ramp to 300 °C at 5 °C/min, and finally 30 min at 300 °C. Two microliters was injected (splitless) with a Hewlett-Packard 7673A autosampler. The MS temperatures were 200 °C for the ion source and 100 °C for the quadrupole. Identification of the degradation products was carried out with electronic impact and full scan mode.

## RESULTS

**Stability of the Acaricides in Honey.** The stability at room temperature (20−35 °C) of chlordimeform, cymiazole, coumaphos, bromopropylate, amitraz, tau-fluvalinate, and flumethrin was examined during 9 months. With the HPLC conditions used the acaricides showed retention times of 9.8, 12.4, 16.6, 17.7, 19.0, 21.3, and 22.8 min, respectively. No significant decrease in

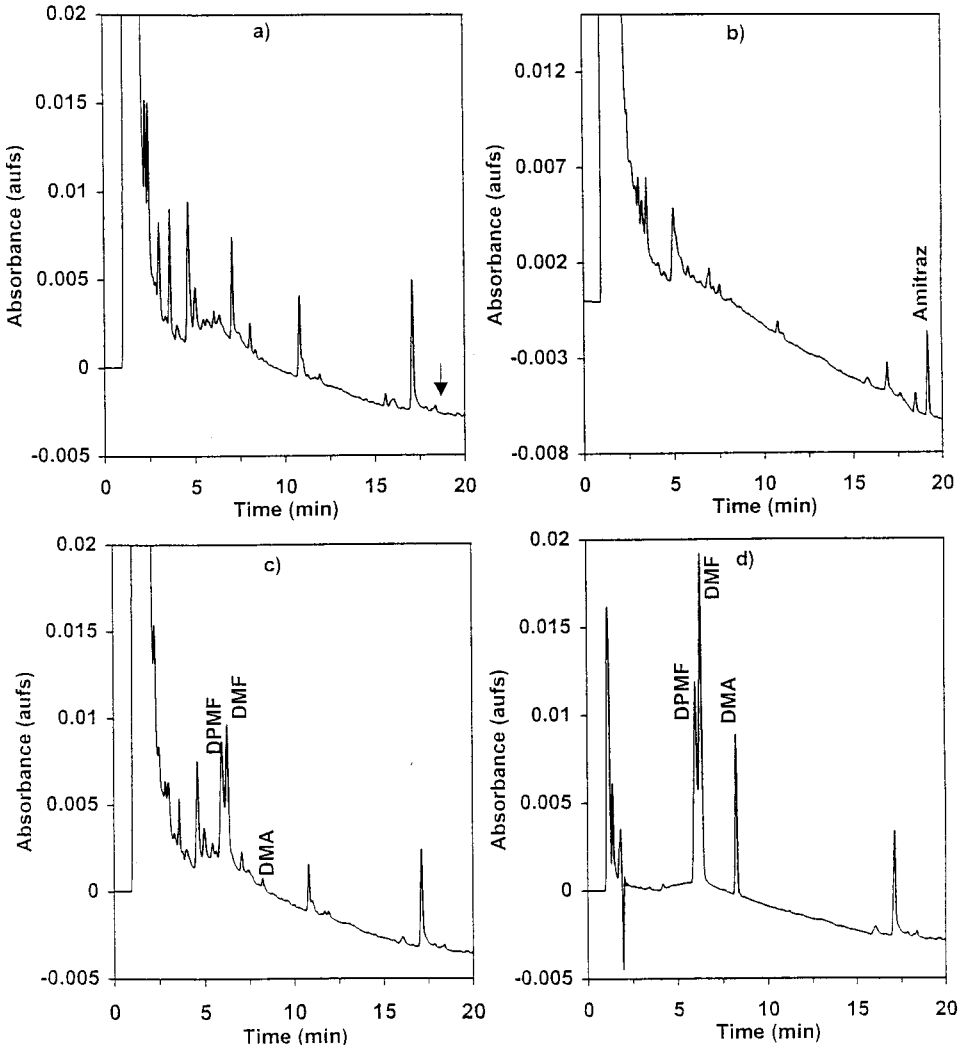


**Figure 1.** HPLC chromatograms (gradient elution and monitoring at 250 nm) of (a) blank honey, (b and c) 10 μg/g of amitraz-spiked honey analyzed just after spiking and after 15 days, respectively, and (d) standard solution of 2 μg/g of DPMF, DMF, and DMA.

**Table 1. Stability Study of Acaricides in Honey: Concentration of Acaricide as a Function of the Time in Honey Spiked with 10 μg/g of the Acaricide**

| | concn in honey (μg/g) | | |
|---|---|---|---|
| acaricide | initial | 3 months | 9 months |
| cymiazole | 10.0 | 9.9 | 10.0 |
| chlordimeform | 9.8 | 9.8 | 9.9 |
| coumaphos | 10.1 | 9.9 | 9.9 |
| bromopropylate | 9.7 | 10.1 | 9.9 |
| tau-fluvalinate | 10.1 | 9.9 | 10.0 |
| flumethrin | 9.8 | 10.1 | 9.9 |

peak area was observed for the acaricides during all of the study period, except for amitraz (Table 1).

HPLC analysis of amitraz-spiked honey versus time (Figure 1) reflected the nearly complete degradation of amitraz in honey within 10 days. Figure 1b shows the chromatogram corresponding to an amitraz-spiked honey analyzed just after spiking process with an amitraz peak at $t_R$ 19 min, and Figure 1c shows the extract after 15 days—the amitraz peak has disappeared and three new peaks were detected at $t_R$ 6.0, 6.3, and 8.0 min. These amitraz degradation peaks have been identified as DPMF, DMF, and DMA by comparison of their retention times with those of a standard solution of 2 μg/mL of DPMF, DMF, and DMA (Figure 1d). The identity of the breakdown products was confirmed by GC-MS analysis of the extract by comparison of the retention times and mass spectra of the degradation products with those of each individual standard.

The amitraz degradation curve versus time is given in Figure 2a, the degradation rate being of pseudo-first-order (Figure 2b). The apparent reaction rate constant and the amitraz half-life in honey were calculated (Table 2). After 15 days, in the honey spiked with 10 μg/g of amitraz, 4.9 μg/g of DPMF (52% w/w of the total breakdown products), 4.1 μg/g of DMF (44% w/w), and 0.4 μg/g of DMA (4% w/w) were detected (expressed in molar quantities: 0.034 μmol of amitraz/g decomposes into 0.030 mmol of DPMF/g, 0.027 μmol of DMF/g, and 0.003 μmol of DMA/g). These concentrations of DPMF and DMF were stable in honey for at least 45 more days.

Amitraz stability was studied in honeys from different geographical and botanical origins. In all honeys DPMF and DMF were the main degradation products, and the quantity of DMA was insignificant in all cases. Other breakdown products detected in small amounts in honey by other authors (*11*) were not found in our studies either by injecting the spiked honey extracts in HPLC or by GC-MS analysis. Amitraz half-lives that were calculated for four different honeys were from ap-

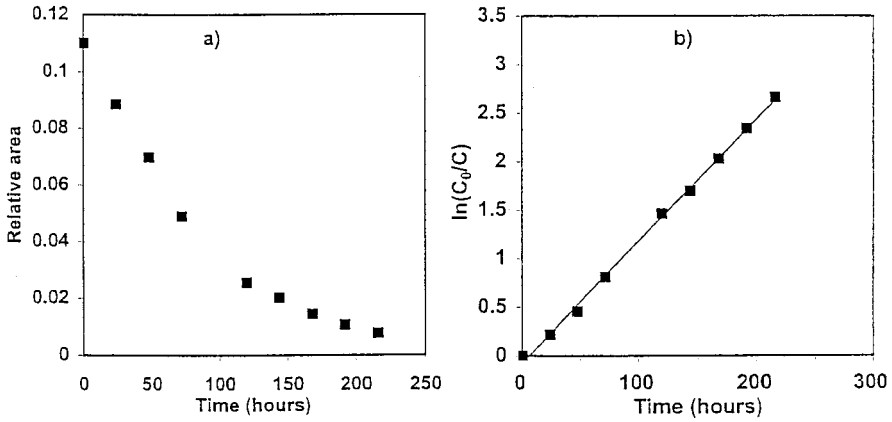


**Figure 2.** (a) Variation of amitraz peak area for a honey sample spiked with 10 $\mu$g/g of amitraz and (b) pseudo-first-order fit for the degradation of amitraz in honey.

**Table 2. Apparent Reaction Rate Constants ($k_{obsd}$), Square Correlation Coefficient ($r^2$), and Half-Lives ($t_{1/2}$) for the Degradation of Amitraz in Honey and Beeswax**

| matrix | $k_{obsd}$ ($h^{-1}$) | $r^2$ | $t_{1/2}$ (h) |
|---|---|---|---|
| honey | 0.0125 | 0.998 | 55.2 |
| beeswax | 0.1105 | 0.9998 | 6.3 |

**Table 3. Degradation of Amitraz in Different Honeys: Relative Mass Percentage for the Different Breakdown Products after Complete Amitraz Degradation**

| honey | pH | $t_{1/2}$ (h) | % DMF | % DPMF | % DMA |
|---|---|---|---|---|---|
| mixed blossom honey | 3.93 | 55 | 44 | 52 | 4 |
| mixed blossom honey | 3.80 | 35 | 50 | 50 | 0 |
| mixed blossom honey | 3.55 | 25 | 52 | 48 | 0 |
| Erica honey | 3.90 | 12 | 46 | 46 | 8 |

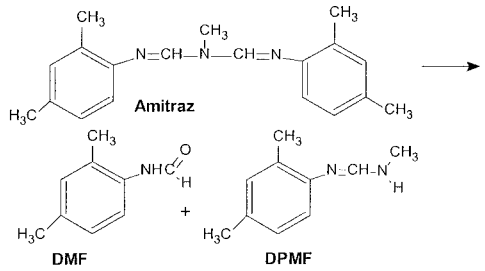


**Figure 3.** Reaction proposed for the degradation of amitraz in honey.

proximately 12 to 55 h (Table 3). The similar molar quantities of DPMF and DMF found in all honeys suggest a simple hydrolysis reaction due to the breakdown of the amino group of amitraz (Figure 3).

**Degradation of Amitraz in Beeswax.** Preliminary experiments showed a complete degradation of amitraz (to DPMF, DMF, and DMA) and a partial degradation of DPMF (~30%) (to DMF and DMA) when they were injected into the gas chromatograph in beeswax matrix, whereas when standard solutions were directly injected (without matrix), no degradation was observed. This unusual behavior forced us to measure the amitraz degradation by HPLC, and the GC-MS was used only for confirmation of the identity of the breakdown products.

HPLC chromatograms of hexane extracts obtained just after beeswax spiking showed an amitraz peak at 19 min (Figure 4b) that after 1 day of storage practically disappeared (Figure 4c) (the hexane extraction efficiency for amitraz in beeswax was >90%). To clarify if this degradation was due to the beeswax or the extraction procedure, a control amitraz sample without the presence of beeswax was extracted using the same procedure. As no degradation took place, we conclude that the degradation was due to beeswax. The degradation rate was monitored and showed a pseudo-first-order character (Figure 5b). The apparent reaction rate constant and the amitraz half-life were calculated by the kinetic data (Table 2). The half-life of amitraz in beeswax was 6.3 h, which means that a complete degradation takes place after 1 day.

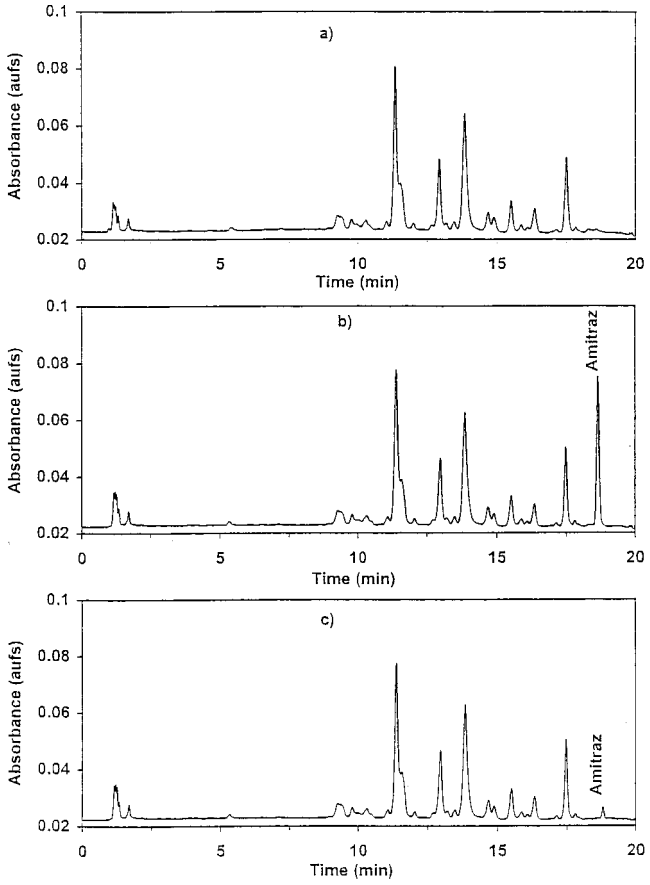


**Figure 4.** HPLC chromatograms (gradient elution and 210 nm) of the hexane extracts for (a) blank beeswax and (b and c) beeswax spiked with 100 $\mu$g/g of amitraz just after spiking and after 1 day, respectively.

As Figure 4c shows, no chromatographic peaks that could be attributed to degradation products were found in the hexane extracts. That is why, after confirmation that amitraz had been completely degraded in the

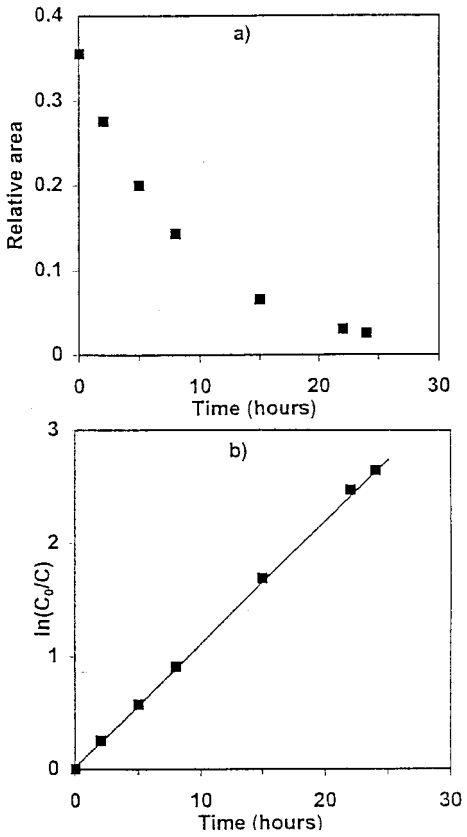


**Figure 5.** (a) Variation of amitraz peak area for beeswax spiked with 100 $\mu$g/g of amitraz and (b) pseudo-first-order fit for the degradation of amitraz in beeswax.

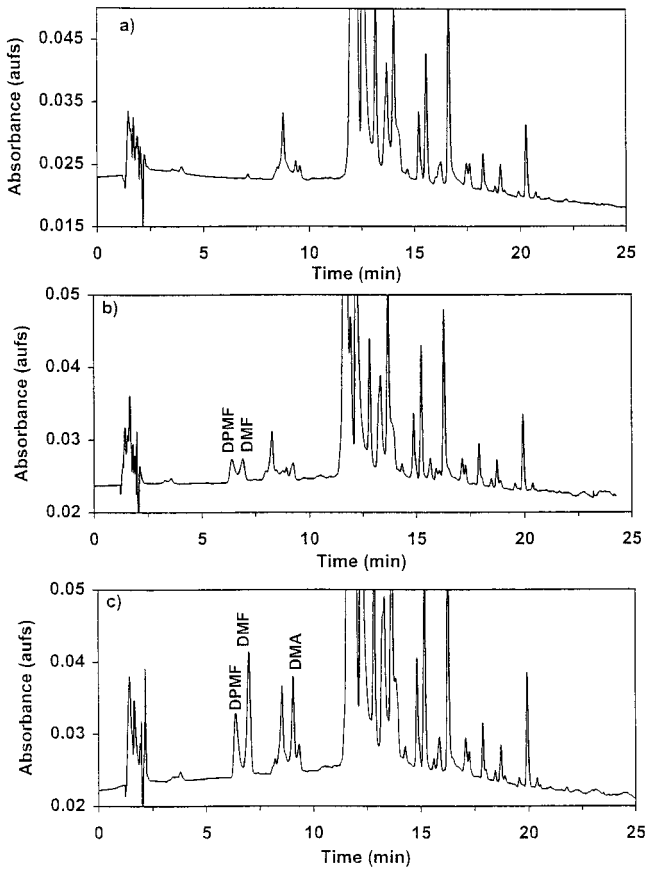


**Figure 6.** HPLC chromatograms (gradient elution and monitoring at 210 nm) of the methanol extracts for (a) blank beeswax, (b) beeswax spiked with 100 $\mu$g/g of amitraz after 1 week, and (c) blank beeswax extract spiked with 10 $\mu$g/g of DPMF, DMF, and DMA.

beeswax (after 1 week of the spiking process), more polar solvents (methanol, ethanol, acetone, and acetonitrile) were tested as extractants in order to detect the more polar amitraz degradation products, which could not be extracted with hexane. As Figure 6 shows, the comparison of the chromatograms for the methanol extracts of blank beeswax (Figure 6a) and amitraz-spiked beeswax after 1 week (Figure 6b) shows that two new peaks appeared in the spiked beeswax chromatograms at $t_R$ 6.3 and 6.9 min, tentatively identified as DPMF and DMF by comparison with retention times of standards. These identities were confirmed by the injections of the extracts into the GC-MS system and comparison of the retention times and mass spectra with those of standards (Figure 7). No degradation peaks other than those mentioned were detected when the beeswax extracts obtained with the different extractants were injected in both chromatographic systems.

After 1 week (when amitraz was completely degraded) in beeswax spiked with 100 $\mu$g of amitraz/g, 24.5 $\mu$g of DPMF/g and 20 $\mu$g of DMF/g were quantified by HPLC, whereas no DMA was found. These results indicate that the recovery of the amitraz in beeswax as its degradation products was ~50%; this recovery could not be increased, although many different solvents and solvent combinations were tried for the extraction. This disagreement could not be explained as extraction with methanol of beeswax spiked with 100 $\mu$g of DPMF, DMF, or DMA/g resulted in recoveries >90% and no other metabolites as new peaks could be identified in the GC or HPLC chromatograms.

*Behavior of Degradation Products during Recycling.* The behavior of amitraz degradation products in a model experiment to produce new beeswax after melting of old beeswax was studied (see Materials and Methods) using methanol as extractant. The chromatograms of beeswax spiked with 100 $\mu$g of amitraz/g before and after wax melting are presented in Figure 8. The conclusion that can be drawn from this experiment is that DPMF disappears nearly completely after recycling, whereas the DMF concentration stays invariable before (19 $\mu$g/g, $n = 2$) and after (20 $\mu$g/g, $n = 2$) the recycling process. Thus, the only residue present in recycled beeswax was DMF.

## DISCUSSION

Our results show that bromopropylate, cymiazole, coumaphos, chlordimeform, flumethrin, and tau-fluvalinate are stable in honey for ~9 months. Thus, a determination method of these acaricides in honey can be based on analysis of these compounds only.

Amitraz is the only studied acaricide that breaks down in honey, being completely degraded within 10 days by the breakdown of the amitraz amino bond (1-to-1 stoichiometry). This reaction proposed in honey was in concordance with the mechanism in aqueous media at pH values from 3 to 6 (*9*) taking into account that honey has a similar pH range. Our conclusion in honey is that the main residues found in honey after complete breakdown of amitraz are DPMF and DMF, and only very small amounts (<5%) of DMA can be found. The nonconcordant results obtained in the literature in relation to the relative amounts of degradation products

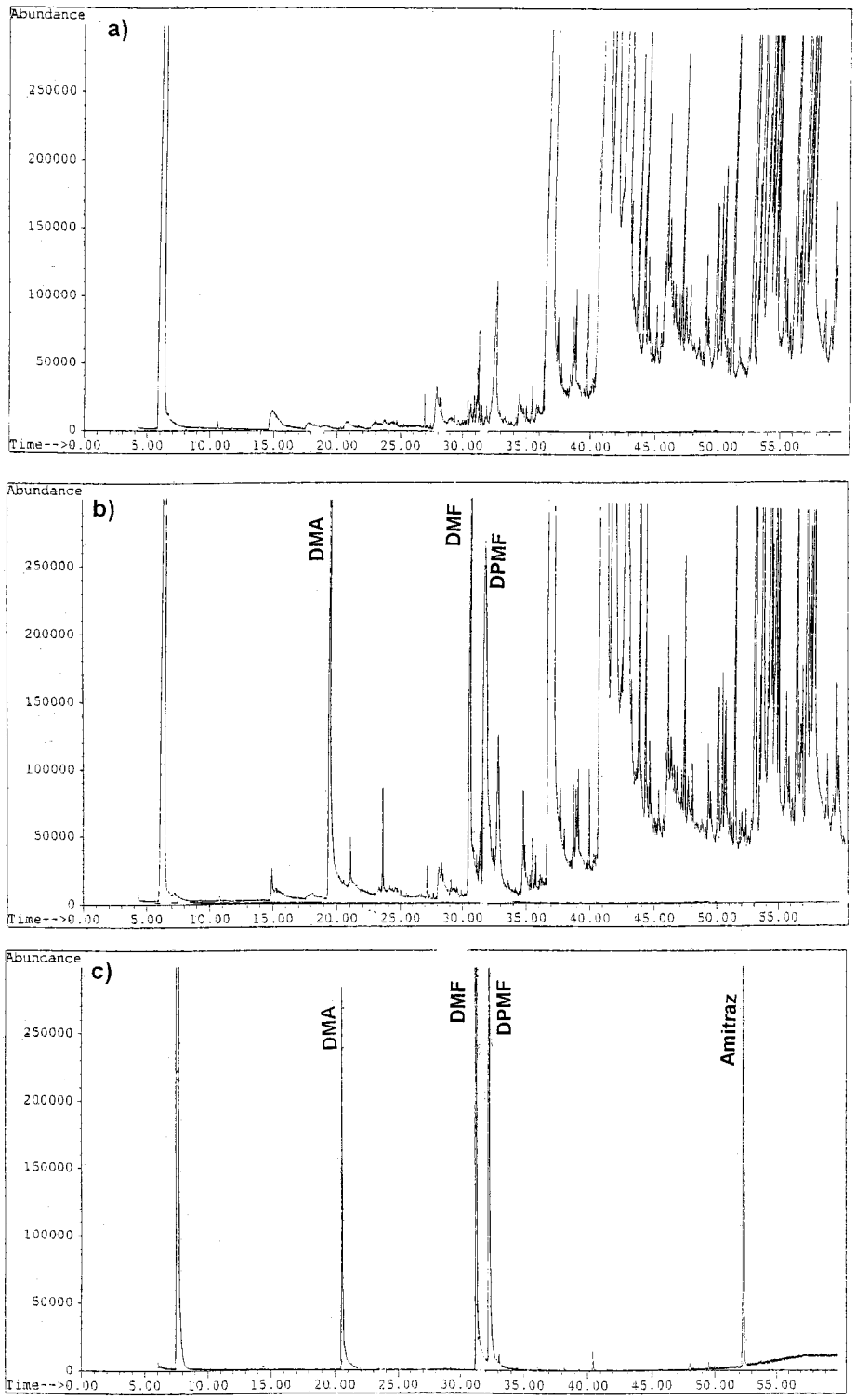


**Figure 7.** GC-MS chromatograms (SCAN mode) of the extracts for (a) blank beeswax, (b) beeswax spiked with 100 $\mu$g/g of amitraz after 1 week, and (c) standard solution of 20 $\mu$g/g of DPMF, DMF, and DMA.

found (*5*, *6*, *11*) could be explained due to the degradation of DPMF to yield DMF and DMA. We observed this degradation when DPMF in wax matrix was chromatographed by GC or at aqueous basic conditions ($t_{1/2} = 2$ h at pH 10).

The most frequently used methods for the determination of amitraz residues in honey are based on the hydrolytic conversion of amitraz and its degradation products to DMA and a subsequent determination of this compound (*7*, *28*, *29*). Taking into account the results obtained in this work, a direct quantification of DMF and/or DPMF amounts, without their hydrolytic conversion to DMA, can be sufficient to have a good estimation of the total residue of amitraz in honey.

In relation to beeswax, amitraz is completely degraded within 1 day, so it is the only known acaricide that is unstable in beeswax. Indeed, all known synthetic lypophilic acaricides are stable in this matrix and their concentration in beeswax increases with increasing numbers of acaricide treatments (*1*, *2*). The persistent presence of subtoxic amounts of acaricides in wax promotes the development of acaricide-resistant varroa

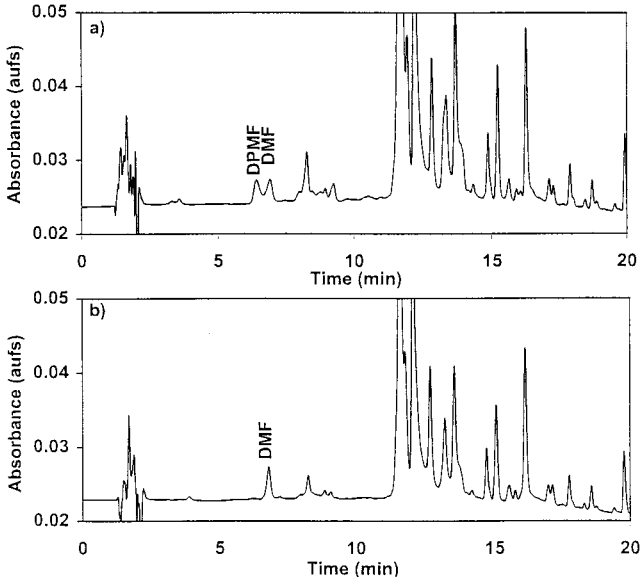


**Figure 8.** HPLC chromatograms (gradient elution and monitoring at 210 nm) of methanol extracts of a beeswax spiked with 100 $\mu$g/g of amitraz before (a) and after (b) recycling.

mites. Indeed, resistance against acaricides such as fluvalinate, flumethrin, coumaphos, and bromopropylate is common and has been reported (*30*). In ref *30* one report of amitraz resistance in the former Yugoslavia is cited. However, because of the degradation of amitraz in wax the development of resistance against this acaricide seems to be less probable. DPMF has also acaricide and insecticide properties (*31*) but has not been used as a varroacide. Its acute toxicity in mammals is higher than that of amitraz (*32*). DMF has no acaricide activity, and its acute toxicity value is lower than that of amitraz (*33*).

No residues of amitraz in beeswax have been reported until now. One reason might be that these might have remained unnoticed due to the lack of knowledge on the rapid amitraz degradation. In another work we describe a method for the determination of residues in wax of DMF from the acaricides chlordimeform, chlorfenvinfos, cymiazole, and bromopropylate (*34*). There, we report for the first time on residues of amitraz in beeswax in the form of DMF.

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