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Evaluation of Residues of Essential Oil Components in Honey after Different Anti-*Varroa* Treatments

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Apiary trials on the use of three different treatments (Apilife Var, thymol solution in olive oil, and thymol solution in ethanol) for the control of *Varroa destructor* were conducted in Aragon (northeastern Spain). For the evaluation of the presence of residues of these treatments in honey an analytical method was developed. The method is applied to analyze honey samples before and after treatments with the acaricides mentioned. A solid-phase extraction on trifunctional silane SPE C18 cartridge and gas chromatography separation using a flame ionization detector allow reliable and precise determination of residues of thymol, menthol, eucalyptol, and camphor in honey. The results indicate that camphor is present in only low concentrations, residues of eucalyptol or menthol were not found at all, and only thymol left residues in high concentrations. Residues of thymol found in honey collected from the beehives ranged from 0.75 to 8.20 μg/g for Apilife Var, from 0.03 to 6.30 μg/g for thymol solution in olive oil, and from 0.05 to 6.20 μg/g for thymol solution in ethanol. Even so, natural treatments can be considered to be good alternatives for synthetic acaricides, especially because they do not represent a sanitary risk.

KEYWORDS: Essential oils; menthol; eucalyptol; camphor; thymol; honey

INTRODUCTION

Varroa destructor Anderson and Trueman (formerly *Varroa jacobsoni* Oud.), origin of the varroosis, is the most damaging parasite in honey bees (*Apis mellifera* L.) and represents a serious infestation of bee colonies.

Use of synthetic acaricides causes their accumulation in the apiary products such as beeswax, honey, propolis, and royal jelly l (*1*−*5*) or their transfer from beeswax into the syrup and honey (*6*, *7*). In addition, *Varroa* mites can develop resistance against some of them (*8*−*13*).

For this reason the current trend is to find new alternatives in the fight against this parasite, such as mitosporic fungi *Hirsutella thompsonii* and *Metarhizium anisolplia*, as a potential microbial control agent of the parasitic mite (*14*, *15*) or to use almost nontoxic and nonpersistent acaricides such as organic acids or essential oils. Entomopathogenic fungi such as *H. thompsonii* have the definite advantage of infecting *Varroa* directly through the exoskeleton.

Organic acids such as formic, lactic, and oxalic acids have been investigated as a control strategy (*16*−*19*). The efficacy of these components depends on their ability to be dispersed within the hive. Some of them are temperature-dependent (*20*).

New methods of control based on the use of essential oils from different plants (*21*, *22*) have also been tested successfully against the mite. For example, uses of wintergreen oil as a thermal treatment, an aerosol treatment with a thyme−sage oil mixture, and a contact treatment with neem oil and the passive evaporation of oregano oil and marjoram oil have been described. However, for various reasons none of these treatments have been adopted in beekeeping. The main obstacles are the difficulties in obtaining standardized essential oils or the variations in local environmental and colony conditions.

However, components of these essential oils such as thymol, linalool, or camphor have been highly effective in controlling the varroosis. Different combinations of such components as well as different thymol application methods have been studied. As an example, the use of powdered thymol (*5*, *23*, *24*) and microencapsulated thymol (Apitimol) (*25*) has been reported. Good results have also been reported with the use of Frakno thymol frame (*26*, *27*), Thymovar wafer (*28*, *29*), thymol in a gelatin-like Apiguard (*29*, *30*), and homemade applications (*31*, *32*). The Italian product Apilife Var is a mixture of thymol, eucalyptol, menthol, and camphor. Its main active ingredient is thymol, and it showed good results against *Varroa* in different countries and climate zones (*29*, *30*, *32*−*37*).

Nevertheless, difficulties with natural treatments are the high level of residues (>0.8 mg/kg) found in honey after application (*26*, *27*, *38*). This does not represent a risk for human health but may change the taste of the honey. Bogdanov et al. (*39*) found that the taste threshold of thymol in acacia and rape honey was ∼1.1−1.3 mg/kg honey.

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The literature describes different analytical methods using gas chromatography with a flame ionization detector (GC-FID) as an adequate technique for the analysis of components of essential oils as stated by several groups (*30*, *35*, *38*, *40*, *41*), although the procedures were sometimes poorly detailed.

The objective of this work was to evaluate the presence of the essential oil components thymol, camphor, eucalyptol, and menthol in honey samples from hives treated with Apilife Var, thymol solution in olive oil, and thymol solution in ethanol. For this purpose, an analytical method, based on that described by Bogdanov et al. (*40*), was developed and validated in our laboratory.

MATERIALS AND METHODS

Acaricide Treatments. One commercial treatment (Apilife Var) and two homemade treatments (thymol solution in olive oil and thymol solution in ethanol) were applied.

Apilife Var (Chemicals LAIF, Vigonza, Italy) consisted of 10 g tablets containing 7.41 g of thymol, 1.60 g of eucalyptol, 0.37 g of menthol, and 0.37 g of camphor. Thymol solution in olive oil consisted of 15 g of thymol dissolved in 20 mL of olive oil, applied in a vermiculite pad of spongy material of 8 × 5 × 2 cm. Thymol solution in ethanol consisted of 15 g of thymol dissolved in 20 mL of ethanol, applied in a vermiculite pad of spongy material of 8 × 5 × 2 cm.

The treatments were carried out in 20 Langstroht hives by seven apiarists in the region of Aragon (northeastern Spain).

The treatments consisted of two 10 g tablets of Apilife Var, two pads of thymol in olive oil, or two pads of thymol in ethanol. Apilife Var tablets and the thymol pads with olive oil and ethanol were placed on the top bars of the frames. All three treatments were applied during a period of 28 days, in the autumn and spring of two consecutive years (2001 and 2002) outside the honey flow. All of the treatments were renewed after 14 days. None of these treatments were used on any of the beehives for one year beforehand.

Honey Samples. Altogether, 126 samples of honeycomb with honey were analyzed. Sampling was as follows: Seven apiarists were selected for the study. Three hives from each apiarist were sampled (one for each treatment), and three samples from each hive were analyzed before and immediately after the treatment.

The samples were obtained by cutting pieces of comb to 5 × 10 cm strips, stored in plastic bags, vacuum-sealed, and deep-frozen until analyzed.

Materials and Standards. Thymol for acaricide treatment was supplied by Panreac. Analytical standard-grade thymol, menthol, eucalyptol, and camphor for GC determination were from Extrasynthese (Genay, France). HPLC analysis grade acetone and hexane were provided by Labscan (Dublin, Ireland). HPLC analysis grade ethanol 96% v/v was provided by Panreac Quimica S.A. (Barcelona, Spain). Anhydrous sodium sulfate of analytical reagent grade was supplied by Panreac Quimica S.A. Water was deionized by means of a Milli-Q system. For solid-phase extraction, C18 (EC) 6 mL cartridges containing 500 mg of octadecylsilica were from Isolute (International Sorbent Technology U.K.). All gases for gas chromatography analysis were supplied by Carburos Metalicos (Barcelona, Spain), with a purity degree of 99.99%.

Apparatus. A Meditronic 7000599 centrifuge was supplied by Selecta P (Barcelona, Spain). A SPE vacuum manifold for solid-phase extraction was supplied by Supelco. GC-FID analysis was performed with an HP 5890 series II gas chromatograph equipped with an HP 7673A autosampler, all controlled by an HP3365 series II Chemstation from Hewlett-Packard (Avondale, PA).

Sample Preparation and Extraction. Frozen honeycomb samples were left to melt at room temperature. For the desoperculation the honeycombs were cut with a knife. To separate the honey, samples were put in a sieve and scraped with the help of a glass stick, letting the honey drop afterward. The honey was collected in a beaker.

Briefly, solid-phase extraction, based on that described by Bogdanov et al. (*40*), was as follows: 10 g of honey was dissolved in 20 mL of a 20% (v/v) aqueous ethanol solution. The SPE C18 cartridge packed with 500 mg of the stationary phase was preactivated with 6 mL of ethanol (96%) and 6 mL of deionized water. Then, the honey/ethanol solution was passed through the activated SPE C18 cartridge, being washed afterward with 6 mL of aqueous ethanol solution (20%). Finally, the essential oil components were eluted with 2 mL of acetone. Approximately 0.15 g of anhydrous sulfate (Na_2SO_4) was added to the eluate to eliminate the water. The Na_2SO_4 was then separated from the extract with a centrifuge (2500 rpm, 5 min). This extract was kept in autosampler vials in the freezer until GC analysis.

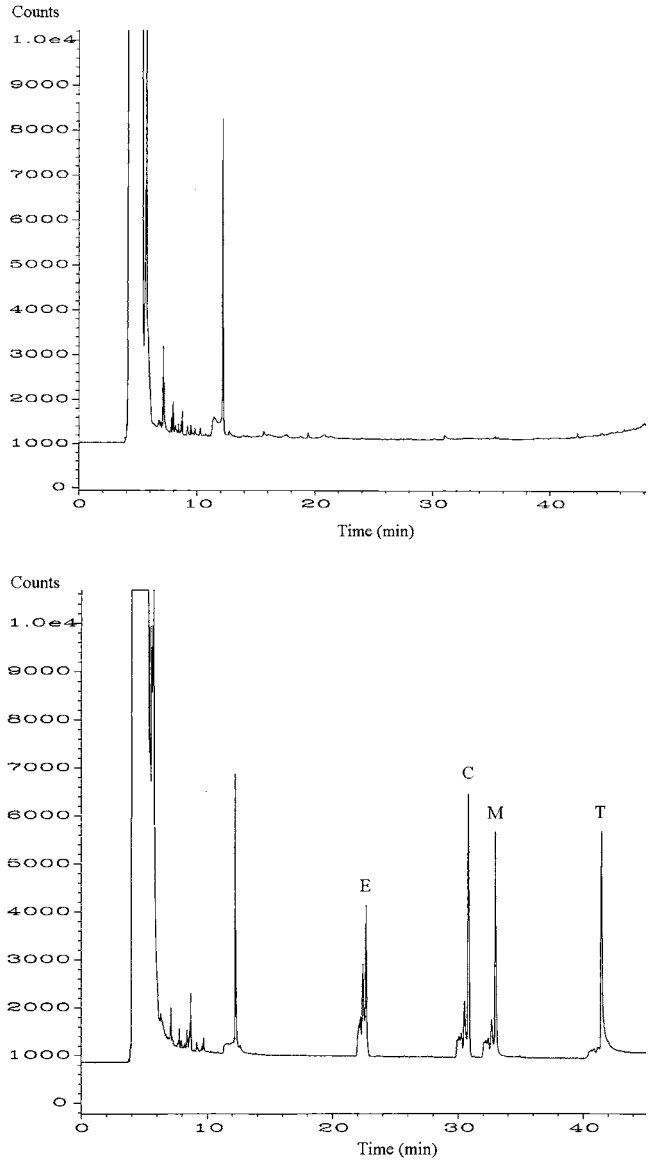

Figure 1. Chromatograms of a blank honey extract and blank honey extract containing 3 μg/g of eucalyptol (E), camphor (C), menthol (M), and thymol (T).

Gas Chromatography Analysis. A fused silica capillary column Quadrex 5% phenyl methyl (50 m × 0.25 mm i.d. × and 0.250 μm film thickness) supplied by RW was used. Operating conditions were as follows: injector port temperature, 250 °C; helium as carrier gas at a flow rate of 1 mL/min. The flame ionization detector temperature (FID) was 300 °C. The column temperature was maintained at 50 °C for 1 min and then programmed to increase at 2 °C/min to 130 °C, then at a rate of 10 °C/min to 220 °C, followed by a final rate of 30 °C/min to 280 °C. Total time of analysis was 60 min. A 2 μL sample was injected, in splitless mode.

To avoid a matrix effect, honey sample extracts free of natural acaricide treatments were used for calibration by spiking them with a known amount of each analyte (**Figure 1**). Calibration standards were

Table 1. Method Sensitivity: Absolute Detection Limit (ADL), Limit of Detection (LOD), and Limit of Quantification (LOQ)

analyte	ADL (pg)	LOD (μg/g)	LOQ (μg/g)
eucalyptol	32	0.3	0.5
camphor	42	0.1	0.2
menthol	180	0.1	0.3
thymol	172	0.2	0.2

Table 2. Results from Studies of Recovery (Percent), Repeatability (RSD),[a] and Reproducibility (RSD)

	recovery		repeatability		reproducibility	
analyte	2 μg/g	0.5 μg/g	2 μg/g	0.5 μg/g	2 μg/g	0.5 μg/g
eucalyptol	73.8	89.6	7.6	17.0	16.1	6.3
camphor	96.0	89.3	6.8	14.8	10.1	29.8
menthol	100.3	83.9	6.3	10.9	11.9	23.7
thymol	102.8	78.1	12.3	9.4	20.4	14.5

[a] Relative standard deviation.

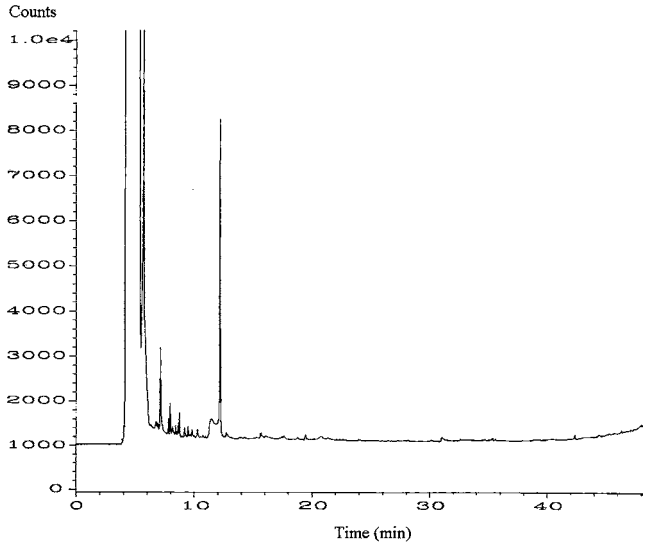

Figure 2. Chromatogram of a honey extract before treatment.

prepared at three different levels. The regression of the calibration curves was $r^2 \geq 0.999$ for all analytes.

Method Validation. For method validation, sensitivity, repeatability, reproducibility, and recovery studies were carried out, using blank sample extracts free of analytes to prevent a matrix effect. According to ORA (*42*) recommendations, for the sensitivity study, the absolute detection limit, detection limit, and quantification limit were determined. For the repeatability study, eight samples were analyzed by the same operator, with the same equipment, in the same laboratory, on the same day. The conditions for the study of reproducibility were as follows: analysis of three samples by the same operator on the same equipment, in the same laboratory, on three different days within two weeks. Finally, for the recovery study, 10 samples were analyzed. Studies of repeatability, reproducibility, and recovery were made by spiking extracts at two levels (2 and 0.5 μg/g).

RESULTS AND DISCUSSION

Analytical Method. The chromatographic conditions resulted in a good analytical resolution. The sequence of the elution was eucalyptol > camphor > menthol > thymol. **Table 1** shows the results of the study of sensitivity. The detection limits for the four components were between 0.1 and 0.3 μg/g of honey, and the quantification limits were between 0.2 and 0.5 μg/g of honey.

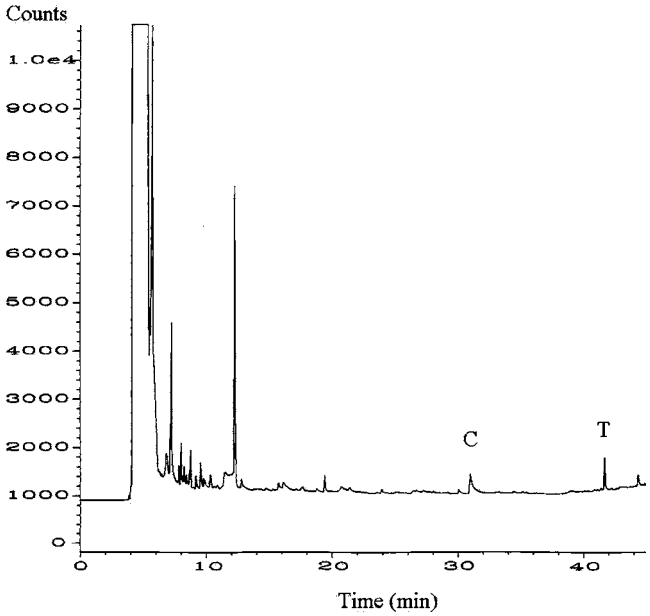

Figure 3. Chromatogram of a honey extract after treatment with Apilife Var. Acaricide peaks: camphor (C) and thymol (T).

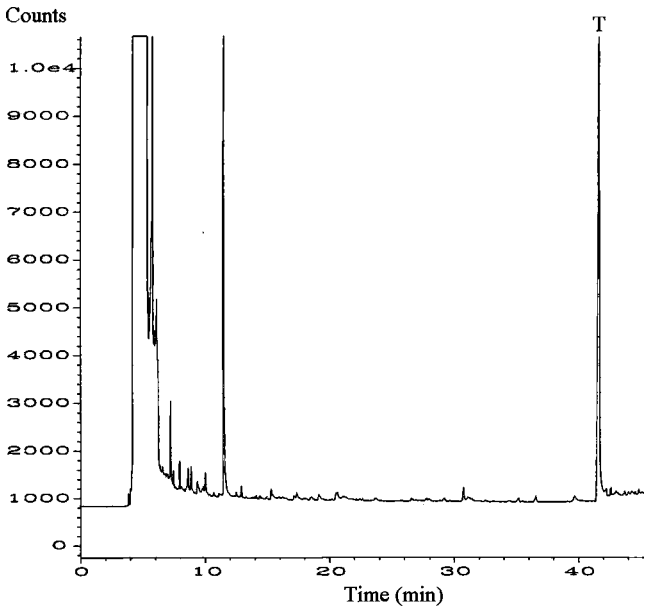

Figure 4. Chromatogram of a honey extract after treatment with thymol in olive oil. Acaricide peaks: thymol (T).

The repeatability and the reproducibility of the method, expressed as relative standard deviation (RSD), were in accordance with the recommendations of Horwitz (*43*) (repeatability RSD <15% and reproducibility RSD <31%) (**Table 2**).

The recovery percentages in spiked samples ranged between 74 and 103% for both tested levels (2 and 0.5 μg/g of honey). This is satisfactory, in accordance with the European Commission (*44*) that accepts recovery ranges between 70 and 110% (**Table 2**).

Results of Honey Sample Analysis. Residues of the components of essential oils before and after the treatments with Apilife Var, thymol solution in olive oil, and thymol solution in ethanol were evaluated. **Figures 2**−**5** show chromatograms of honey samples before and after the treatments. A total of 126 honey samples were analyzed according to the previously described method. The results of the samples analyzed are reported in **Tables 3** and **4**.

Table 3. Natural Essential Oil Components in Honey from Aragon before Treatments (63 Samples)

		range (% samples)			
essential oil component	% of positive samples	<LOD[a]–0.3 μg/g	>0.3–0.6 μg/g	>0.6–2 μg/g	>2 μg/g
thymol	36.5	74.5	1.6	17.5	6.4
camphor	39.7	68.2	14.3	15.9	1.6
menthol					
eucalyptol					

[a] <LOD = less than detection limit.

Table 4. Levels of Residues of the Essential Oil Components after Different Treatments

treatment	% of positive samples	mean (μg/g of honey)	range (μg/g of honey)	RSD
		Apilife Var ($n = 21$)[a]		
thymol	86	2.8	0.5–8.2	95.3
camphor	10	0.3	0.2–0.5	79.3
menthol		<LOD[b]	<LOD	
eucalyptol		<LOD	<LOD	
		Thymol in Ethanol ($n = 21$)		
thymol	95	2.5	0.2–6.2	79.9
		Thymol in Olive Oil ($n = 21$)		
thymol	86	2.6	0.1–6.3	82.3

[a] n = number of samples. [b] <LOD = less than detection limit.

The analysis of samples of honey from hives before any acaricide treatment revealed the presence of aromatic components (**Table 3**). Thymol, camphor, menthol, and eucalyptol are natural components of essential oils from some aromatic plants (*45*), and so they may occur in honey types (*38*, *46*, *47*). In fact, from the honey samples taken in our study, 40% contained camphor and 40% contained thymol. This finding agrees with the fact that Aragon is rich in aromatic plants such as thyme, rosemary, salvia, and lavender (*48*, *49*), which contain the essential oil components. Therefore, the honey samples contained thymol and camphor as natural ingredients.

With regard to the treatments applied, the statistical analysis of our results showed that both commercial and homemade preparations left residues in honey. After the Apilife Var treatment, thymol residues were found in 86% of the honey samples from hives at concentrations between 0.5 and 8.2 μg/g of honey (mean level of 2.8 μg/g of honey). Only two samples showed residues of camphor between 0.2 and 0.5 μg/g of honey (mean level concentration of 0.3 μg/g of honey). The RSD for the two components shows a high variability. Residues of eucalyptol and menthol were not found above the limit of detection.

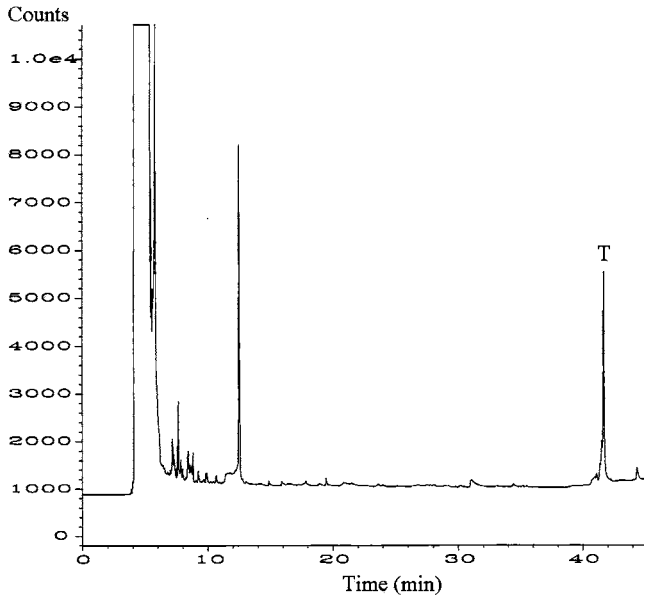

Figure 5. Chromatogram of a honey extract after treatment with thymol in ethanol. Acaricide peaks: thymol (T).

Other researchers have also found residues in honey after treatment with Apilife Var (**Table 5**). In one study carried out in Guadalajara (Spain) (*38*), Apilife Var treatment was applied for up to 21 days, and samples were taken 1 week after the last day of treatment. This region is comparable, in terms of climatic conditions, with Aragon. The residues of thymol found in honey in this case ranged between 0.8 and 2.3 μg/g. These levels are lower than ours, but it must be taken into account that we took samples immediately after the treatments and that we applied the treatments for 28 days. They also found eucalyptol residues around 0.2 μg/g after the Apilife Var treatment. In other studies carried out in Switzerland (*33*, *35*, *40*), where treatments with Apilife Var were applied during 4−8 weeks and the samples were taken 6−10 months later, the highest residue of thymol found in honey was 0.5 μg/g. In Italy (*30*), where Apilife Var treatments were applied for 2 weeks, thymol residue levels were not higher than 2 μg/g.

Similarly, the two homemade thymol preparations resulted in a high incidence of thymol residues in honey. In 86% of the samples, thymol residues were found after treatment with thymol solution in olive oil and in 95% after treatment with thymol

Table 5. Levels of Residues in Honey after Apilife Var Treatment (Other Studies)

application time (weeks)	honey sampling after the end of treatment	eucalyptol residues found (μg/g)	thymol residues found (μg/g)	ref
7	nd[a]	not detected	0.1–0.3	*34*
4–8	32 weeks	not detected	0.03–0.1	*35*
6–8	nd	not detected	0.1–0.5	*33*
6–8	28–34 weeks	not detected	0.02–0.5	*40*
4	1 week	0.19–0.22	0.5–1.7	*38*
	2 weeks	0.20–0.24	1.1–2.7	
	3 weeks	0.16–0.18	0.8–2.3	
2 (first application)	1 week before	not detected	2.0 (mean)	*30*
	last day of application	not detected	0.8 (mean)	
2 (second application)	1 week before	not detected	1.1 (mean)	
	last day of application	not detected	0.6 (mean)	

[a] No data.

Table 6. Levels of Residues in Honey after Different Thymol Treatments (Other Studies)

treatment	application time (weeks)	honey sampling after the end of treatment	thymol residues found (μg/g)	ref
powdered thymol	3 (treated three times)	20 weeks	0.02–1.5	*5*
		28 weeks	<0.001–0.7	
		36 weeks	<0.001	
		40 weeks	<0.001	
Frakno thymol frame	52[a]	nd[b]	0.02–0.3	*26*
Frakno thymol frame	52	last day of application	0.1–1.1	*26*
	104	last day of application	0.4 (mean)	
powdered thymol	4	1 week	0.1	*38*
Apiguard	2 (first application)	1 week before	3.1 (mean)	*30*
		last day of application	0.9 (mean)	
	2 (second application)	1 week before	2.6 (mean)	
		last day of application	1.0 (mean)	

[a] Treatment interrupted during honey flow. [b] No data.

solution in ethanol. One possible reason for this difference is the higher carry-over effect of ethanol, due to its high volatility. The level of thymol residues after treatment with thymol solution in olive oil was between 0.1 and 6.3 μg/g of honey (mean level of 2.6 μg/g of honey). After the second treatment, thymol solution in ethanol, thymol residues were found at levels between 0.2 and 6.2 μg/g of honey (mean level of 2.5 μg/g of honey). The variability of results was also very high for both treatments.

Results of other studies on residues in honey after treatment with thymol blends are shown in **Table 6**. In Switzerland, residues of thymol not higher than 1.1 μg/g were found after different treatments with Frakno thymol frames (*26*, *27*). Thymol residues after different treatments with powdered thymol applied in Spain and Italy were similar and not higher than 1.5 μg/g (*5*, *38*). Treatments with other thymol-based products such as Apitimol and Apiguard (*25*, *30*) were slightly higher (3.1 μg/g). Although the majority of these treatments were applied for longer than in our study (up to one year), they did not result in higher thymol residue levels. An explanation for such differences is the better controllability of commercial products compared to homemade solutions, the different application methods of the treatments, and the duration of the application as well as the fact that in our study samples were taken directly after the treatments.

In accordance with the European Agency for Evaluation of Medical Products (EMEA) (*50*), thymol is not likely to be of toxicological concern to humans. What is more, thymol is permitted as a flavoring agent, with an upper limit for inclusion in food of 50 mg/kg. The EMEA (*51*) reports that camphor did not give rise to worries about consumer health, either.

Furthermore, European Union Regulation 2377/90 EEC (*52*) includes thymol, as well as camphor, menthol, and eucalyptol, in Annex II corresponding to nontoxic veterinary drugs, which do not have a maximal residue limit (MRL).

Nevertheless, Bogdanov et al. (*39*) found that the taste threshold of thymol in acacia and rape honey was between 1.1 and 1.3 μg/g with an astringent and medicine-like taste. For this reason, Swiss food legislation has established a maximal limit for thymol of 0.8 μg/g in honey. In our study 74% of the samples exceeded this threshold for thymol. The taste threshold of camphor in acacia honey is 5−10 μg/g, and not one of the honey samples analyzed contained this amount of camphor.

In conclusion, the presence of residues of essential oil components in our honey samples does not represent a sanitary risk or a risk for human health, but may change the taste of the honey. However, these results should be completed with a sensorial analysis.

LITERATURE CITED

(1) Wallner, K. Varroacides and their residues in bee products. *Apidologie* **1999**, *30*, 235−248.

(2) Bogdanov, S.; Kilchenmann, V.; Imdorf, A. Acaricide residues in some bee products. *J. Apic. Res.* **1998**, *37* (2), 57−67.

(3) Thrasyvoulou, A. T.; Pappas, N. Contamination of honey and wax with malathion and coumaphos used against the *Varroa* mite. *J. Apic. Res.* **1988**, *27* (1), 55−61.

(4) Balayannis, P. G. Gas chromatographic determination of coumaphos and tau-fluvalinate residues in royal jelly produced under commercial conditions. *J. Apic. Res.* **2001**, *40* (2), 71−78.

(5) Lodesani, M.; Pellacani, A.; Bergomi, S.; Carpana, E.; Rabitti, T.; Lasagni, P. Residue determination for some products used against *Varroa* infestation in bees. *Apidologie* **1992**, *23*, 257−272.

(6) Wallner, K. Diffusion varroazider Wirkstoffe aus dem Wachs in den Honig. *Apidologie* **1992**, *23*, 387−389.

(7) Kochansky, J.; Wilzer, K.; Feldlaufer, M. Comparison of the transfer of coumaphos from beeswax into syrup and honey. *Apidologie* **2001**, *32*, 119−125.

(8) Milani, N. The resistance of *Varroa jacobsonni* Qud. to pyrethroids: a laboratory assay. *Apidologie* **1995**, *26*, 415−429.

(9) Milani, N.; Della Vedova, G. Decline in the proportion of mites resistant to fluvalinate in a population of *Varroa destr*uctor not treated with pyrethroids. *Apidologie* **2002**, *33*, 417−421.

(10) Thompson, H. M.; Brown, M. A.; Ball, R. F.; Medwin, H. B. First report of *Varroa destructor* resistance to pyrethroids in the UK. *Apidologie* **2002**, *33*, 357−366.

(11) Colin, M. E.; Vandame, R.; Jourdan, P.; Di Pasquale, S. Fluvalinate resistance of *Varroa jacobsoni* Oudemans (Acari: Varroidae) in the Mediterranean apiaries of France. *Apidologie* **1997**, *28*, 375−384.

(12) Elzen, P. J.; Baxter, J. R.; Spivak, M.; Wilson, W. T. Control of *Varroa jacobsoni* Oud. resistant to fluvalinate and amitraz using coumaphos. *Apidologie* **2000**, *31*, 437−441.

(13) Mozes-Koch, R.; Slabezki, Y.; Efrat, H.; Kalev, H.; Kamer, Y.; Yakobson, B. A.; Dag, A. First detection in Israel of fluvalinate resistance in the varroa mite using bioassay and biochemical methods. *Exp. Appl. Acarol.* **2000**, *24*, 35−43.

(14) Shaw, E. K.; Davidson, G.; Clark, S. J.; Ball, B. V.; Pell, J. K.; Chandler, D.; Sunderland, K. D. Laboratory bioassays to assess the pathologenicity of mitosporic fungi to *Varroa destructor* (Acari: Mesostigmata), an ectoparasitic mite of honeybee, *Apis mellifera*. *Biol. Control* **2002**, *24*, 266−276.

(15) Kanga, L. H. B.; James, R. R.; Boucias, D. G. *Hirsutella thompsonii* and *Metarhizium anisopliae* as potencail microbial control agents of *Varroa destructor*, a honey bee parasite. *J. Invertebr. Pathol.* **2002**, *81*, 175−184.

(16) Gregorc, A.; Planinc, I. The control of *Varroa destructor* using oxalic acid. *Vet. J.* **2002**, *163*, 306−310.

(17) Charriere, J. D.; Imdorf, A.; Fluri, P. Ácido oxálico ×c0Qué podemos esperar de su empleo en la lucha contra Varroa? *Vida Apic.* **1999**, *96*, 18−20.

(18) Imdorf, A.; Charriere, J. D. Alternative Varroabekämpfung, Separatdruck. *Eidgenoessische Forschungsanstalt fuer Milchwirtschaft Liebefeld (FAM)* **1998**, 1−8.

(19) Pajuelo, A. G.; Segura, I. Ácido fórmico y Mite gone. Pruebas de campo para el control invernal de Varroa destructor. *Biol. Control* **2004**, *126*, 28−31.

(20) Imdorf, A.; Charriere, J. D.; Rosenkranz, P. Varroa Control with Formic Acid, Commission on the European Communities. Concerted Action 3686 "Coordination in Europe of research on integrated control of *Varroa* mites in honey bee colonies", Meeting Nov 13−14, Agricultural Research Centre-Ghent Merelbeke, Belgium, Meetingbooket, 1999; pp 22−27.

(21) Imdorf, A.; Bogdanov, S.; Ibañez Ochoa, R.; Calderone, N. W. Use of essential oils for the control of *Varroa jacobsoni* Oud. in honey bee colonies. *Apidologie* **1999**, *30*, 209−228.

(22) Schenk, P.; Imdorf, A.; Fluri, P. Wirkung von Niemoel auf Varroa-Milben und Bienen. *Schweiz. Bienenztg.* **2001**, *124* (3), 25−27.

(23) Higes, M.; Llorente, J. Timol. Ensayo de eficacia en el control de la varroosis en colmenas de producción. *Vida Apic.* **1997**, *81*, 14−17.

(24) Chiesa, F. Effective control of varroatosis using powdered thymol. *Apidologie* **1991**, *22*, 135−145.

(25) Higes, M.; Martín, R.; Sanz, A.; Jiménez, J. J.; Bernal, J. L.; Nozal, M. J. Spring efficacy of Apivar, Apistan and Apitimol in the control of varroosis in honeybees in Mediterranean climate. *First European Conference of Apidology*, Udine, Italy, Sept 19−23, 2004; web.uniud.it/eurbee, consulted Dec 7, 2004; 107.

(26) Bogdanov, S.; Imdorf, A.; Kilchenmann, V.; Fluri, P. Frakno Thymol Frame for controlling *Varroa jacobsonia* a field trial. Swiss Bee Research Centre, 1999; www.apis.admin.ch, Jan 1, 2005; 1−7.

(27) Bogdanov, S.; Kilchenmann, V.; Imdorf A.; Fluri, P. Residues in honey after application of thymol against *Varroa* using the Frakno Thymol frame. *Am. Bee J.* **1998**, *138*, 610−611.

(28) Bollhalder, F. "Thymovar" zur Varroabekaempfung. *Schweiz. Bienenztg.* **1998**, *121* (3), 148−151.

(29) Marinelli, E.; De Pace, F. M.; Ricci, L.; Persano Oddo, L. Use of different formulated with thymol for summer treatment antivarroa in the Mediterranean environment. Experimental Institute for Agricultural Zoology, Section of Beekeeping, Rome, Italy; www.apicoltura.org/timolo-web/, consulted Jan 1, 2001; 1−5.

(30) Floris, I.; Satta, A.; Cabras, P.; Garau, V. L.; Angioni, A. Comparison between two thymol formulations in the control of *Varroa destructor:* effectiveness, persistence, and residues. *J. Econ. Entomol.* **2004**, *97* (2), 187−191.

(31) Carmona, M.; Valero, A.; Zalacaín, I.; Zalacaín, A.; Salinas, M. R. Influencia del Timol en la Puesta de la cría de la abeja melífera. *Vida Apic.* **2002**, *113*, 35−43.

(32) Van der Steen, J. Der Effect einer Mischung aetherischer Oele auf die Varroainfektion in Bienenvoelkern. *Apidologie* **1992**, *23* (4), 383−385.

(33) Imdorf, A.; Bogdanov, S.; Kilchenmann, V.; Maquelin, C. Apilife VAR: A new varroacide with thymol as the main ingredient. *Bee World* **1995**, *76*, 77−83.

(34) Mutinelli, F.; Irasara, A.; Cremasco, S.; Piro, R. Utilizzo di APILIFE-VAR sul vassoio di fondo per il controllo della varroasi. *Apic. Mod.* **1993**, *84*, 111−117.

(35) Imdorf, A.; Kilchenmann, V.; Maquelin, C.; Bogdanov, S. Optimierung der Anwendung von Apilife VAR zur Bekämpfung von *Varroa jacobsoni* Qud. In Bienenvölkern. *Apidologie* **1994**, *25*, 49−60.

(36) Melathopoulos, A. P.; Gates, J. Comparison of two thymol-based acaricides, Apilife Var and Apiguard, for the control of *Varroa* mites. *Am. Bee J.* **2003**, 489−493.

(37) Rickli, M.; Imdorf, A.; Kilchenmann, V. Varroa-Bekämpfung mit Komponeten von ätherischen Oelen. *Apidologie* **1991**, *22*, 417−421.

(38) Nozal, M. J.; Bernal, J. L.; Jiménez, J. J.; González, M. J.; Higes, M. Extraction of thymol, eucalyptol, menthol, and camphor residues from honey and beeswax. Determination by gas chromatography with flame ionization detection. *J. Chromatogr. A* **2002**, *954*, 207−215.

(39) Bogdanov, S.; Kilchenmann, V.; Fluri, P.; Bühler, U.; Lavanchy, P. Influence of organic acids and components of essential oils on honey taste. *Am. Bee J.* **1999**, 61−63.

(40) Bogdanov, S.; Imdorf, A.; Kilchenmann, V. Residues in wax and honey after Apilife VAR treatment. *Apidologie* **1998**, *29*, 513−524.

(41) Li, M.; Nelson, D. L.; Sporns, P. Determination of menthol in honey by gas chromatography. *J. AOAC Int.* **1993**, *76* (6), 1289−1294.

(42) Haagsma, N.; Ellen, G.; Stephany, R. W.; Ruig, W. G. ORA, Dutch Discussion Group for Residue Analysis. Definitions for the determination of drug residues in food of animal; 1995.

(43) Horwitz, W.; Albert, R. Reliability of the determination of polychlorinated contaminants (biphenyls, dioxins, furans). *J. AOAC Int.* **1996**, *77* (1), 79−86.

(44) European Commission (Documento SANCO/3103/2000). Quality control procedures for pesticide residues analysis. *Guidelines for Residues Monitoring in the European Union*, 2nd ed.; 1999/2000.

(45) Guillen, M. D.; Cabo, N.; Burillo, J. Characterisation of the essential oils of some cultivated aromatic plants of industrial interest. *J. Sci. Food* **1996**, *70*, 359−363.

(46) Guyot, C.; Bouseta, A.; Scheirman, V.; Collin, S. Floral origin markers of chestnut and lime tree honeys. *J. Agric. Food Chem.* **1998**, *46*, 625−633.

(47) Tschogowadse, S. K.; Koblianidse, G. L. Die aromatischen Stoffe des Honigs. *Lebensm.-Ind.* **1973**, *20* (H.5), 225−228.

(48) Pérez-Arquillué, C.; Ucar Casorrán, A.; Herrera, A. Análisis polínico aplicado a la determinación del origen botánico de mieles de Monegros (España). *Anal. Bromatol.* **1988**, *XL-2*, 265−277.

(49) Pérez-Arquillué, C.; Conchello, P.; Ariño, A.; Juan, T.; Herrera, A. Physicochemical attributes and pollen spectrum of some unifloral Spanish honey. *Food Chem.* **1995**, *54*, 167−172.

(50) Thymol Summary Report. Committee for veterinary medicinal products. The European Agency for the Evaluation of Medicinal Products. Veterinary Medicines evaluation Unit, 1996; www.emea.eu.int, consulted Jan 7, 2005.

(51) Camphora Summary Report. Committee for veterinary medicinal products. The European Agency for the Evaluation of Medicinal Products. Veterinary Medicines evaluation Unit, 1999; www.emea.eu.int, consulted Jan 7, 2005.

(52) European Union Council Regulation (EEC) 2377/90. *Off. J. Eur. Union* **1990**, 224.

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