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Journal of Chromatography A, 954 (2002) 207–215

JOURNAL OF CHROMATOGRAPHY A

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Extraction of thymol, eucalyptol, menthol, and camphor residues from honey and beeswax
Determination by gas chromatography with flame ionization detection[☆]

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Received 12 June 2001; received in revised form 7 February 2002; accepted 8 February 2002

Abstract

A gas chromatographic method to determine thymol, eucalyptol (cineole), menthol and camphor residues in honey and beeswax is proposed. To isolate the compounds, three methods involving liquid–liquid extraction with methylene chloride, distillation, or solid-phase extraction on octadecylsilica cartridges can be used. The GC separation is carried out on a 60 m×0.53 mm Stabilwax DA capillary column, using a flame ionization detector. The method is applied to the analysis of natural honey and also honey and beeswax samples from beehives treated with the above compounds. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Essential oils; Honey; Waxes; Extraction methods; Thymol; Eucalyptol; Menthol; Camphor

1. Introduction

Nowadays it is assumed among apiarists that the fight against *Varroa destructor,* an ectoparasite which is seriously infesting bee colonies, must be carried out in an integrated manner [1]. Treating beehives with only one compound leads to the appearance of strains of mites resistant to it [2,3]. This fact usually implies an increase in the dosage and, therefore, higher probabilities of finding residues of the compound in apiary products [4]. For this reason, the trend is to alternate the compounds used and, if possible, to select natural substances that do not carry risks for the consumer [5,6]. Among the alternative compounds [7], attention has been focussed on organic acids such as formic, lactic or oxalic, the last being preferred [8,9], and also on the use of some components of essential oils [10–12]. A product called Apilife VAR, which is a mixture of thymol, eucalyptol, menthol and camphor, first authorized for use in Italy, has shown good efficacy against varroosis in some regions and climates [12]. As thymol is the main active ingredient of this material, assays

[☆] Presented at the 30th Scientific Meeting of the Spanish Group of Chromatography and Related Techniques/1st Meeting of the Spanish Society of Chromatography and Related Techniques, Valencia, 18–20 April 2001.

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PII: S0021-9673(02)00153-X

have been carried out to obviate the use of the other ingredients and to reduce the quantity of thymol used in order to avoid negative effects on bees [13]. At the same time, new methods of application have been researched, such as the use of powdered thymol [14] or, more recently, the use of Frakno Thymol Frame. This device, used throughout the year, seems inadvisable because somewhat high (0.8 mg/kg) residue levels are found in honey after treatment [15]. Although such levels of residues do not imply a risk for the consumer, considering that the tolerance level is ~50 mg/kg, they can affect the taste of honey, which is forbidden by food regulations. The threshold for perception is ~1.1 mg/kg [16]. For this reason it is necessary to have at our disposal a simple and sensitive method that allows the determination of residues in apiary products, at the same time monitoring the efficacy of different treatments used, by controlling the residue levels, not only in honey but also in the beeswax, which will be used later in new hives.

Taking the chemical nature of the compounds involved into account, gas chromatography with a flame ionization detector seems to be an adequate technique [17], as has been stated by several groups [11,13,16], although the procedures, sometimes poorly detailed, are usually focussed on one compound only [18] or recommend two different columns for the analysis of the four compounds. The aim of this work has been to develop a method that, while avoiding toluene or similar toxic solvents, allows isolation of compounds from the samples and their simultaneous determination. The method has been applied to honey of different origins and samples from beehives where treatment with solid thymol or Apilife VAR had been assayed before.

2. Experimental

2.1. General

2.1.1. Chemicals

Analytical standard-grade thymol, menthol, eucalyptol, camphor and geraniol (used as internal standard) were obtained from Sigma–Aldrich (Madrid, Spain). Anhydrous sodium sulfate of analytical reagent-grade was supplied by Merck (Darmstadt, Germany). Residue analysis grade methanol and methylene chloride were provided by Labscan (Dublin, Ireland). Water was purified by the use of a Compact Milli-RO and Milli-Q system from Millipore (Milford, MA, USA). For solid-phase extraction, LiChrolut RP-18 cartridges containing 100 mg octadecylsilica were obtained from Merck.

2.1.2. Equipment

An HP 5890 Series II gas chromatograph equipped with an HP7673 autosampler and a flame ionization detection (FID) system, all controlled by an HP 3365 Series II Chemstation from Hewlett-Packard (Avondale, PA, USA), was used. An ultrasonic water bath and a vortex-mixer were obtained from Selecta (Barcelona, Spain). The semimicro Kjeldahl unit, the reflux dispositive and the Kuderna–Danish concentrator were obtained from Afora (Madrid, Spain). A model 5810R refrigerated centrifuge was supplied by Eppendorf (Hamburg, Germany). The HB140 thermostatic water bath was supplied by Büchi (Flawil, Switzerland). To purge the vapor produced during distillation, a Gillian personal air sampler pump, calibrated with a Gilibrator-2TM, from Sensidyne (Wayne, FL, USA) was used.

2.1.3. Gas chromatography

A 60 m×0.53 mm, 1.5 μm Stabilwax DA widebore capillary column from Restec (Bellefonte, PA, USA) was used. The carrier gas (N_2) flow-rate was kept constant during the run (5 ml/min, measured at 50 °C). Nitrogen (30 ml/min), hydrogen (35 ml/min) and synthetic air (350 ml/min) were used as auxiliary gases for the flame ionization detector. All gases were supplied by Carburos Metálicos (Barcelona, Spain).

The oven temperature program was as follows: initial temperature 50 °C, held for 1 min, a 30 °C/min ramp to 110 °C, held for 15 min, then a 30 °C/min ramp to 155 °C, held for 20 min, and finally a 30 °C/min ramp to 220 °C, held for 15 min. The temperature of the injection port was 250 °C, while that of the detector was 300 °C.

A 3-μl sample was injected in splitless mode. The purge valve was turned on at 1 min after the injection.

2.2. Samples

Honey samples of different botanical origin were generously given by the Asociacion Soriana de Apicultores (Soria, Spain). A total of 12 beehives were used to study the action of solid thymol. Two of the beehives were not treated and were employed as controls, while the other ten were spiked with 8 g of powdered thymol, once a week, for 4 weeks. Samples were taken 1 week after the last treatment. Another eight beehives were treated in a similar way but using Apilife VAR (74.08% thymol, 16.00% cineole, 3.7% camphor and 3.7% menthol). Samples of raw honey and beeswax from the beehives were taken on the 7th, 14th and 21st days after the end of the treatment. All samples were kept at −10 °C until analysis to evaluate thymol residues. This experiment was carried out in the apiary (300 hives) of the Centro Apícola Regional of Marchamalo (Guadalajara, Spain).

Raw honey samples were centrifuged at 16 000 *g* and 4 °C to remove extraneous material before use. Essential oil components were isolated by solvent extraction, solid-phase extraction (SPE) or distillation, prior to chromatographic analysis.

2.3. Solvent extraction

A 10-g sample of honey was diluted with 100 ml of water, the internal standard was added, and the mixture was homogenized by mechanical shaking. The essential oil components were extracted by refluxing with 20 ml of methylene chloride for 20 min. After cooling and washing, the two phases were separated. The collected organic phase was dried with anhydrous sodium sulfate and the extract was concentrated in a Kuderna–Danish concentrator at 70 °C. The residue was diluted with methanol to 2 ml and analyzed by gas chromatography.

2.4. Solid phase extraction

A 2.5-g sample of honey was diluted with 5 ml of water, the internal standard was added, and the mixture was homogenized by mechanical shaking. The mixture was passed through an SPE LiChrolut RP-18 cartridge packed with 100 mg of the stationary phase that had been pre-activated with 5 ml of methanol and 5 ml of deionized water. After rinsing with 5 ml of water, the compounds of interest were eluted with 0.5 ml of methanol and aliquots injected into the chromatograph.

2.5. Distillation

A 2.5-g sample of honey was diluted with 25 ml of water and the internal standard was added. The mixture was placed in a flask (Kjeldahl type or reactor) which was heated at 70 °C for 20 min. The distillate was trapped into a SPE LiChrolut RP-18 cartridge, packed with 100 mg of the stationary phase, which was pre-activated with 5 ml of methanol and 5 ml of deionized water, using gentle suction with the aid of a pump, at a rate of 5 ml/min (Fig. 1). Then, the cartridge was washed with 5 ml of water and afterwards compounds were eluted with 0.5 ml of methanol and injected into the gas chromatograph.

The beeswax samples were analysed similarly: 30 ml of water and the internal standard were added to a sample of 0.5 g. The mixture was placed in the distillation flask, heated at 70 °C for 20 min, and the distillate trapped in the cartridge and finally eluted with 0.5 ml of methanol.

2.6. Internal standard calibration

Stock standard solutions of the compounds of interest and of the internal standard at 1 g/l were made in methanol. Mixtures of different concen-

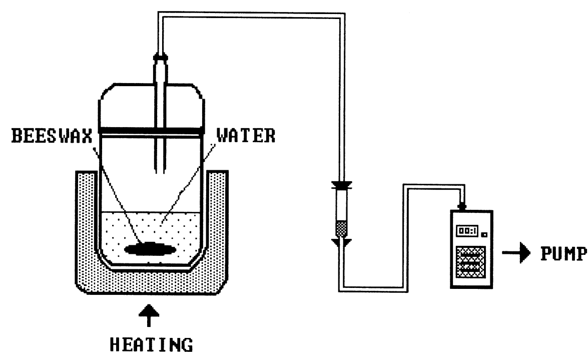

Fig. 1. Scheme of the system used to trap the essential oil components from beeswax or honey samples.

trations were made from the solutions with Nanopure water and stored in a refrigerator until analysis.

Samples free of the essential oil components were spiked with known amounts of the compounds of interest and an appropriate internal standard (geraniol), and analyzed concurrently with each set of unknown samples. At least eight different concentrations of analytes across the working range were measured in quintuplicate. Calibration graphs were obtained by using the linear least-squares method. Peak-height ratios between each analyte and its corresponding internal standard were used to construct the least-squares regression lines and were found to be linear over the range of 0.2–100 μg/ml. The concentrations of essential oil components were determined by interpolation from the graphs using peak-height ratios.

3. Results and discussion

3.1. Extraction and clean-up

The influence of parameters potentially affecting the extracting processes were studied in order to establish the optimal conditions for maximum recoveries and cleanest chromatograms.

3.1.1. Honey samples

3.1.1.1. Solvent extraction

Of all the solvents studied, methylene chloride gave the best results. Therefore, this solvent was selected to optimize the extractant volume, extraction time, temperature and the final elution volume. To carry out this study the procedure was always the same: different amounts of honey (between 5 and 20 g) were mixed with 100 ml of water, adding, when necessary, either the mixture of standards or the internal standard, at 2 μg/l. A parameter was varied, keeping the others constant. As starting conditions, an extraction time of 60 min, a temperature of 80 °C in a Kuderna–Danish apparatus, and a final volume of 5 ml of methanol were chosen. The results obtained are summarized in Table 1. From these data, an extractant volume of 20 ml, an extraction time of 30 min and a temperature of 70 °C were

Table 1
Effects of the methylene chloride volume used, the time and the temperature on the recoveries of essential oil components

	Recovery (%)				
	Eucalyptol	Camphor	Menthol	Geraniol	Thymol
CH_2Cl_2 (ml)[a]					
10	65.4	67.4	66.8	83.9	85.6
20	75.3	74.2	71.4	90.5	90.6
50	79.2	75.6	72.3	91.2	92.3
100	79.8	76.2	73.2	91.8	93.5
Time (min)[b]					
15	82.0	77.8	78.1	89.9	91.3
30	86.1	80.5	83.1	93.8	95.3
60	79.7	75.5	79.0	94.5	95.8
90	68.7	74.5	78.7	94.8	95.3
120	76.8	74.5	71.2	94.6	95.8
T (°C)[c]					
50	96.2	98.2	97.9	94.5	95.6
60	95.9	97.6	97.3	94.3	95.5
70	94.7	96.1	95.2	94.6	95.9
80	86.3	81.2	83.1	94.1	95.4

[a] Temperature: 80 °C, time: 2 h, final volume: 5 ml.
[b] Extractant volume: 20 ml, temperature: 80 °C, final volume: 5 ml.
[c] Extractant volume: 20 ml, time: 30 min, final volume: 5 ml.

finally selected. A sample volume of 2 ml was optimum.

3.1.1.2. Solid-phase extraction

In this procedure, the mass of sample, the washing and the elution volumes were optimized. A sample amount of 2.5 g or lower was adequate The inclusion of a washing step with water made the chromatograms cleaner. The elution of the analytes from the cartridge was complete with 0.5 ml of methanol.

3.1.1.3. Distillation

In an effort to obtain cleaner chromatograms, the possibility of isolating the essential oil components by gentle distillation in the presence of water, then trapping the vapors on a cartridge, followed by elution with methanol, was investigated. Several approaches were tested and, finally, we achieved good results using either the flask of a Kjeldahl system [19] or better, using a PTFE vessel, in both cases with electrical heating. In this procedure the effect of the temperature and the added volume of water on recovery were studied. As result, we selected a water volume of 25 ml and a distillation time of 20 min. Table 2 shows the recoveries and precision achieved in the analysis of honey samples spiked with essential oil components at different levels. Recoveries by the three methods were higher than 95% for the four concentrations assayed. The precision values were quite similar, albeit slightly higher for solvent extraction. There was a greater matrix effect in solvent extraction, even more apparent at the lowest spiking levels.

Fig. 2 shows the chromatograms obtained after applying the three procedures on a spiked honey sample. All procedures gave well resolved peaks and permitted the determination of the essential oil components considered. The cleanest chromatogram was obtained using distillation, but this procedure required a longer time than the others.

3.1.2. Beeswax samples

We tried to apply the solvent extraction procedure to beeswax by mixing a few grams of beeswax with different solvents, along with water, with and without sodium chloride or sodium sulfate. In general, persistent emulsions appeared making the phase separation step very difficult. For this reason, we also tried to apply the procedure based on distillation. A mass of 0.5 g of beeswax and 30 ml of water were enough to obtain reproducible results. As can be seen in Fig. 3, the chromatogram obtained is very clean. It

Table 2

Recovery percentages and precision (RSD) obtained in the analysis of essential oil components in spiked honey samples according to the extraction procedure used ($n=5$)

	Concentration spiked (μg/ml) (honey)				
	0.2	0.8	3.1	12.5	50
Solvent extraction					
Eucalyptol	120.5(7.5)	111.2(5.5)	111.6(4.8)	113.9(2.4)	112.4(2.0)
Camphor	106.7(3.5)	105.2(4.8)	104.9(4.6)	107.3(2.2)	111.8(2.2)
Menthol	115.8(4.3)	113.1(5.8)	119.7(4.9)	117.1(2.9)	108.0(2.2)
Thymol	117.8(5.5)	116.3(5.4)	113.5(1.6)	111.6(1.4)	109.6(2.1)
Distillation					
Eucalyptol	108.3(1.8)	102.0(3.1)	101.5(1.7)	101.7(1.5)	101.2(0.6)
Camphor	107.8(1.6)	105.5(2.0)	104.2(2.4)	100.4(0.5)	101.3(0.7)
Menthol	108.4(2.8)	104.3(3.4)	103.6(2.2)	100.9(1.1)	98.6(0.3)
Thymol	109.1(2.3)	106.5(1.4)	104.2(2.2)	103.8(1.6)	100.6(0.4)
Solid-phase extraction					
Eucalyptol	108.4(2.8)	102.1(1.6)	97.6(1.3)	99.7(1.4)	100.8(0.4)
Camphor	106.3(3.8)	101.5(0.7)	101.8(1.4)	98.4(0.2)	100.6(0.4)
Menthol	107.4(1.6)	104.0(1.8)	101.1(2.4)	100.6(2.3)	99.4(0.3)
Thymol	105.1(3.3)	103.2(3.2)	103.8(3.4)	96.8(2.1)	98.6(0.7)

RSD values (%) in parentheses.

Fig. 2. Chromatograms obtained from a multifloral spiked honey sample according the procedure used to isolate the essential oil component. (a) Solvent extraction; (b) solid-phase extraction; (c) distillation.

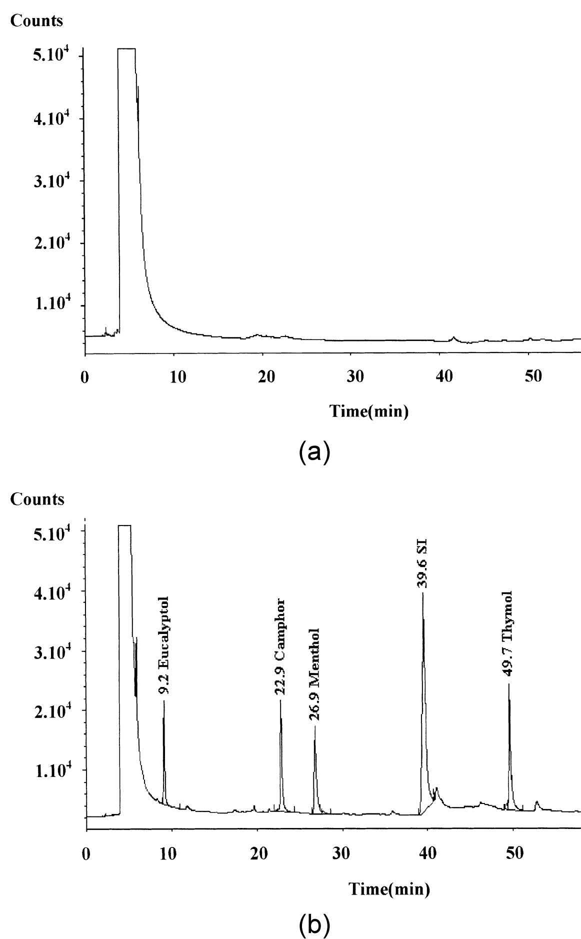

Fig. 3. Chromatograms obtained by applying the distillation procedure to a sample of pure beeswax (a) and to the same sample spiked with the essential oil components (b).

is interesting to notice that with larger amounts of beeswax, larger volumes of water added or temperatures higher than 70 °C, the distillation produced other substances that were also trapped in the cartridge which, when eluted, gave more complex chromatograms. In these chromatograms, the determination of the essential oil components was not as easy as under the optimized conditions.

The recoveries and precision data obtained for spiked samples are summarized in Table 3. The results indicate that the methods are adequate for all the concentrations assayed. Therefore, we recommend that this procedure be used for the analysis of beeswax samples.

Table 3
Recovery percentages and precision (RSD) obtained in the analysis of essential oil components in spiked beeswax samples using the distillation procedure ($n=5$)

	Concentration spiked (μg/ml) (beeswax)				
	0.2	0.8	3.1	12.5	50
Eucalyptol	103.4(4.7)	102.6(2.6)	102.2(1.9)	98.7(1.6)	94.1(1.7)
Camphor	104.1(2.5)	102.7(1.6)	102.6(1.7)	100.2(1.6)	99.7(1.7)
Menthol	102.4(2.6)	101.2(1.6)	102.8(1.6)	101.1(1.8)	98.4(1.9)
Thymol	102.5(1.9)	102.3(1.6)	101.8(1.7)	101.1(1.8)	99.4(1.6)

RSD values in (%) parentheses.

3.2. Analytical characteristics

The average analytical characteristics of the proposed methods for honey and beeswax, after isolation of the essential oil components and their determination by GC–FID, are summarized in Table 4.

Calibration graphs were obtained using samples free of the essential oil components (honey and beeswax) that were spiked with known amounts of the compounds of interest in a range of concentrations from 0.1 to 100 μg/ml and the internal standard, geraniol, at a concentration of 10 μg/ml, always applying the procedures described above. The coefficients of correlation (r^2) of the linear fitting were always higher than 0.9990, for the four compounds studied, for the three methods of analyzing honey samples and for the distillation procedure applied to beeswax samples. The detection and quantitation limits were calculated experimentally at signal-to-noise ratios of 3 and 10, respectively.

In all cases, a matrix-standard calibration had to be performed, in conjunction with the recommended sample preparation procedure, to correctly quantify the target analytes avoiding the matrix effect.

3.3. Validation of the methods

The accuracy of the analytical results was tested by comparing the analyte contents obtained by standard calibration and standard additions. The results obtained were not significantly different. It was concluded that the determination of these four compounds in honey and beeswax could be carried out directly by the standard calibration method.

The reproducibility of methods was obtained by applying them to five aliquots of the same spiked sample (honey and beeswax) at two concentration levels and injecting them five times. The results are also shown in Table 4, indicating that the proposed methods are reproducible.

In conclusion, the procedures (solvent extraction, distillation, and solid-phase extraction) for honey and distillation for beeswax can be used to isolate the compounds.

3.4. Application of the methods

The results obtained by applying the procedure of solvent extraction to unifloral honey samples are

Table 4
Analytical characteristics of the GC–FID method

	Eucalyptol	Camphor	Menthol	Thymol
Linearity range (μg/ml)	0.1–100	0.2–100	0.1–100	0.1–100
Linear regression (r^2)	0.999	0.999	0.999	0.999
Sensitivity (μg/ml)	0.032	0.031	0.023	0.030
Detection limit (μg/ml)	0.05	0.05	0.05	0.05
Quantitation limit (μg/ml)	0.1	0.15	0.1	0.1
RSD (%, $n=8$, 50 μg/ml) inter-day	2.4	2.2	2.3	1.4
RSD (%, $n=8$, 50 μg/ml) intra-day	3.1	2.8	3.2	2.3

Table 5
Average concentration of essential oil components found in the honey samples analyzed after applying solvent extraction

Botanical origin	Concentration (μg/g)		
	Eucalyptol	Menthol	Thymol
Multifloral			0.17
Biercol (*Calluna vulgaris*)			0.36
Heather (*Erica* spp.)		0.18	0.12
Cantueso (*Lavandula stoechas*)		0.18	
Rosemary (*Rosmarinus officinalis)*		0.23	
Thyme (*Thymus vulgaris*)	0.05	0.20	0.27
Esparceta (*Onobrycis sativa* lam)		0.27	
Lavender (*Lavandula latifolia*)		0.13	
Evergreen oak (*Quercus ilex*)			0.17

summarized in Table 5. Five samples of each honey of every botanical origin were analyzed. Camphor did not appear in any of the samples, eucalyptol was found in some thyme samples, whereas menthol and thymol appeared more frequently. Nevertheless, the variation within honey samples of the same botanical origin is very high, so these data can be used only as a guide.

The results obtained when Apilife VAR was applied are summarized in Table 6. Great disparity among beehives can be observed, just as in the case of powered thymol. Thymol residues appeared in all samples, eucalyptol residues were only found in three beehives, while menthol and camphor were not detected. After this experiment we decided that the sample should be taken after the last treatment, because the atmosphere inside the beehive could be preserved better this way.

The data obtained after treatment with powered thymol are shown in Table 7. There was a residual quantity of thymol 7 days after the last treatment. The differences found in the thymol content can be attributed to the diverse activity of the bees, the various locations within the apiary, and also to the different extents of ventilation inside the hives. Taking these facts into account, we suggest the use

Table 6
Concentration of essential oil components found in honey from beehives treated with Apilife VAR

Beehive (number and sampling)[a]	Concentration (mg/kg)	
	Eucalyptol	Thymol
7-A		0.84
7-B		2.11
7-C		1.12
8-A	0.19	0.50
8-B		1.92
8-C	0.18	0.79
10-A		0.52
10-B		1.10
10-C		1.00
11-A		1.68
11-B	0.24	2.65
11-C	0.16	2.32
31-A		0.89
31-B		1.05
31-C		1.04
37-A	0.22	0.62
37-B	0.20	1.20
37-C	0.18	0.88
43-A		0.98
43-B		1.11
43-C		0.92
44-A		1.35
44-B		1.27
44-C		1.22

[a] A, sampling after 7 days; B, sampling after 14 days; C, sampling after 21 days.

Table 7
Thymol found in honey, 35 days after start of the treatment, from beehives treated four times with 8 g of powered thymol

Beehive (number)	Thymol (mg/kg)
1	0.07
5	0.09
6	0.09
13	0.07
14	0.05
15	0.07
21	0.05
25	0.09
29	0.05
33	0.08
41	0.05
42	0.05
43	0.06

of an average value of at least ten beehives, placed in different sites within the apiary.

Samples taken in the beehives used as controls did not have essential oil components in quantities above the detection limits.

4. Conclusions

Extraction with methylene chloride, distillation, and solid-phase extraction on ODS cartridges permits the reliable and precise determination of traces of thymol, menthol, eucalyptol and camphor in honey. Distillation and trapping on ODS cartridges is the best method for determining these analytes in beeswax samples.

Acknowledgements

Financial support of this project (MEC-FEDER 1FD97-0460) by the Spanish Ministry of Education is gratefully acknowledged. M.J.G. thanks Laboratorios Calier S.A. (Barcelona,Spain) for a fellowship.

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