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Analysis of volatile compounds of eucalyptus honey by solid phase extraction followed by gas chromatography coupled to mass spectrometry

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Abstract Solid-phase extraction (SPE) followed by gas chromatography coupled to mass spectrometry has been used for the analysis of volatile compounds of eucalyptus honey. Different amounts of honey and dichloromethane were employed to optimize solid-phase extraction conditions. The best result was obtained with 20 g of honey eluted with 60 mL of dichloromethane. This method presented a good precision for volatile compounds usually found in honeys and allowed the quantification of 35 volatile compounds (terpenes and derivatives, furan and pyran compounds, ketones, benzene compounds, acids, and norisoprenoids) of eucalyptus honey.

Keywords Eucalyptus honey · Volatile compounds · Solid-phase extraction.

Introduction

Unifloral honeys possess distinctive flavors, mainly derived from their nectar source. A large number of organic compounds have been described in different types of honey [1–8]. Some of these compounds have been described as characteristic of the floral source, whereas other compounds, like some alcohols, branched aldehydes, and furan derivatives, may be related to microbial purity or processing and storage conditions of the honey [9].

The identification and quantification of volatile compounds from a complex mixture such as honey is difficult. Australian eucalyptus honeys subjected to simple extraction with organic solvents, have been authenticated on the basis of their 8,9-dehydrotheaspyrone and 3-oxo- α -ionone

contents [7]. Continuous liquid–liquid extraction with diethyl ether was used for the extraction of polar phenolic and acidic substances [1, 10–12] and to determine linalool derivatives in New Zealand honeys [2].

Bicchi et al. [12] proposed a two-step protocol including preliminary acetone extraction followed by simultaneous steam distillation and solvent extraction [13]. This method was optimized using dichloromethane extraction under an inert atmosphere to isolate volatile compounds from lavender and eucalyptus honeys [4, 5].

Heather honeys (*Calluna vulgaris* and *Erica arborea*s) analyzed by simultaneous steam distillation–solvent extraction with dichloromethane as the extracting solvent, were distinguished by their 3-oxo- α -ionone and dehydrovomifoliol contents [14]. The aromatic profiles of French and Portuguese lavender honeys were differentiated by the same technique [15].

Serra Bonvehi [16] also used preliminary simultaneous steam distillation–solvent extraction under reduce pressure followed by solvent extraction to evaluate the aroma profiles of fresh and processed European honeys.

Techniques based on the study of the headspace, have been used for the analysis of honey volatile components and for the characterization of honeys from different floral sources [9, 17–23]. Solid-phase microextraction (SPME) technique has been considered to be a rapid and solvent free method for the extraction of volatile organic compounds in unifloral honeys [24].

Solid-phase extraction (SPE) offers the advantage of eliminating, by washing with water, some interfering substances such as sugars and acids thus making it possible to obtain the honey volatile fraction without the need of applying heat [6, 15, 25–27]. However optimization of several parameters is necessary before applying this technique.

In the present work solid-phase extraction followed by gas chromatography–mass spectrometry (GC–MS) was used to extract and identify volatile compounds from Eucalyptus honey. Extraction conditions were optimized in order to obtain the highest yields of volatile substances. Precision of the method and the extraction efficiency was also evaluated.

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Materials and methods

Study of the efficiency of extraction and method precision

Five model solutions each of 11 volatile compounds, with different physicochemical characteristics, were prepared in a sugar matrix containing 448 g/L fructose and 350 g/L glucose thus similar to honey.

The concentrations of the volatile compounds used were 300 $\mu\text{g/L}$ for 1,8-cineol, linalool, and α -terpineol; 500 $\mu\text{g/L}$ for ethyl octanoate, nonanal, furfural, benzaldehyde, hexanoic acid, and α -ionone; and 1000 $\mu\text{g/L}$ for benzyl alcohol and 2-phenylethanol.

The cartridges used (Bond Elut, Varian) contained 1 g of polypropylene-divinylbenzene and had been previously conditioned first with 25 mL of methanol followed by 25 mL of distilled water at flow rates of 2 mL/min.

Volumes of 100 mL of different model solutions with 50 μL of 2-pentanol (1 g/L) as internal standard were passed through different cartridges. Then, sugars were washed out with 100 mL of distilled water. Volatile compounds of interest were eluted with 100 mL of dichloromethane at a flow rate of 2 mL/min. The organic phases collected were concentrated in a Vigreux column. In all cases, the extracts obtained were further concentrated to 200 μL under nitrogen and analyzed by GC-MS.

Solid-phase extraction optimization

Honey amount optimization

Samples of 5, 10, 20, and 30 g of commercial unifloral eucalyptus honey were separately dissolved in distilled water (1:5 W/V) with 2-pentanol (1 g/L) as internal standard (1:1 W/V), and passed through conditioned Bond Elut cartridges.

Then 100 mL of distilled water were passed through each cartridge to wash out sugars and other hydrophilic substances. The volatile compounds of interest were eluted with dichloromethane (1:5 W/V) at a flow rate of 2 mL/min. The organic phases collected were concentrated to 200 μL and analyzed by GC-MS.

Dichloromethane volume optimization

Samples of 20 g of commercial eucalyptus honey dissolved in 100 mL of distilled water were similarly passed through conditioned cartridges and their volatile fractions eluted with 20, 40, 60, and 100 mL of dichloromethane. The volatile fractions were again concentrated prior to GC-MS analysis.

Analysis of volatile compounds from eucalyptus honey

Eucalyptus honey from the North of Spain, where the main Eucalyptus specie is "*Eucalyptus globulus*", was acquired

at the market. Twenty (20) grams of honey were dissolved in 100 mL of distilled water and 20 μL of 2-pentanol (1 g/L) added as internal standard. This solution was passed through a conditioned Bond Elut cartridge, and then washed with 100 mL of distilled water. The volatile fraction was eluted with 60 mL of dichloromethane. The whole procedure, extraction and analysis, was carried out in duplicate.

Chromatographic conditions

The extracts were analyzed using a Hewlett Packard G 1800 B GCD System with a mass detector (Hewlett-Packard, Palo Alto, CA, USA). Two microliters of the extract were injected in the splitless mode (0.6 min) in a BP-21 capillary column (50 m \times 0.32 mm \times 0.32 μm of film thickness). Oven temperature programme was 60 $^{\circ}\text{C}$ (3 min)—2 $^{\circ}\text{C}/\text{min}$ —200 $^{\circ}\text{C}$ (30 min). Carrier gas was helium (0.8 mL/min). Injector and transfer line temperatures were 250 $^{\circ}\text{C}$ and 280 $^{\circ}\text{C}$, respectively. Mass detector conditions were: electronic impact (EI) mode at 70 eV.

Peak identifications were performed comparing their GC retention indices and mass spectra with those of authentic standards from Sigma-Aldrich. The tentative identification of compounds for which it was not possible to find reference volatiles was carried out by comparison of their mass spectra with spectral data from the Wiley G 1035 A library.

Results and discussion

Solid-phase extraction optimization

Recovery and precision data for some of the most representative volatile compounds in honey are shown in Table 1. Results indicate that solid phase extraction using Varian cartridges provides good extraction yield results with recovery percentages approaching 100% for most of the compounds analyzed. Similar percentages, ranging between 97 and 105% were reported in wine for compounds including benzaldehyde, linalool, and

Table 1 Percentage of recovery and relative standard deviation (%) of the volatile compounds in a sugar model solution extracted by SPE

Compounds	Mean recovery (%) ($n = 5$)	RSD (%)
1,8-cineol	98.06	0.86
nonanal	57.03	5.57
ethyl octanoate	96.65	1.04
furfural	65.69	1.00
benzaldehyde	94.97	3.84
linalool	99.43	0.64
α -terpineol	102.05	4.26
hexanoic acid	56.48	3.36
α -ionone	96.99	4.28
benzyl alcohol	94.64	3.54
2-phenylethanol	98.19	1.30

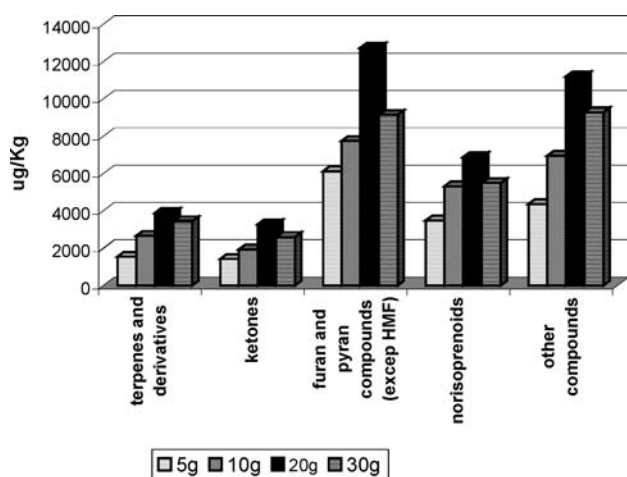


Fig. 1 Total concentrations ($\mu\text{g/Kg}$) of compounds of each chemical group identified in the solid-phase extraction (SPE) eucalyptus honey extracts

α -terpineol using Amberlite XAD-2 [28]. Nonanal, furfural, and hexanoic acid displayed lower affinity for the resin used. Relative standard deviations ranged between 0.64 and 5.57%, so this extraction method can be considered sufficiently precise for quantitative analysis.

Extraction yields for given groups of compounds using increasing amounts of honey are shown in Fig. 1. A progressive increase of the extracted volatile compound concentrations with increasing honey amounts was noted. However, in all cases a decrease was recorded for the volatile compounds extracted from the sample containing 30 g of honey with respect to the immediately preceding sample, perhaps due to saturation of resin active points. The optimum sample amount was thus taken as 20 g of honey diluted in 100 mL of distilled water.

Parallel tests were performed eluting volatile compounds from 20 g of eucalyptus honey with 20, 40, 60, and 100 mL of dichloromethane. The best yields were obtained using 60 and 100 mL, with similar results in both cases. Elution with 20 and 40 mL of dichloromethane proved insufficient for desorption of all the compounds. As a result, conditions for optimum extraction yields were taken as 20 g of honey dissolved in distilled water (1:5 W/V) and 60 mL of dichloromethane for volatile compound elution.

Volatile compounds of eucalyptus honey

Chromatographic analysis of the extracts obtained by solid-phase extraction enabled the identification of 35 volatile compounds in eucalyptus honey. The chromatogram of the extracts obtained under optimum conditions described above is shown in Fig. 2. Concentrations ($\mu\text{g/Kg}$) and relative standard deviations for the 35 compounds identified are shown in Table 2.

The volatile compounds were classified into five groups: terpenes and derivatives, ketones, furan and pyran compounds, norisoprenoids, and other compounds.

The terpenoid compounds, 1,8-cineol and *p*-cymen-8-ol, found here have also been quantified in Italian eucalyptus honey analyzed using SPME [20], and are reportedly among the major volatile compounds in most of the Eucalyptus species, especially in *E. globulus* [29].

Camphor was one of the most abundant terpenic compounds in the samples studied. It is not exclusive to eucalyptus honeys, also having been detected in rosemary honey analyzed using liquid-liquid extraction, simultaneous distillation-extraction (SDE), and solid-phase extraction (SPE) techniques [27] and in orange, chestnut, and wild flower honeys [20].

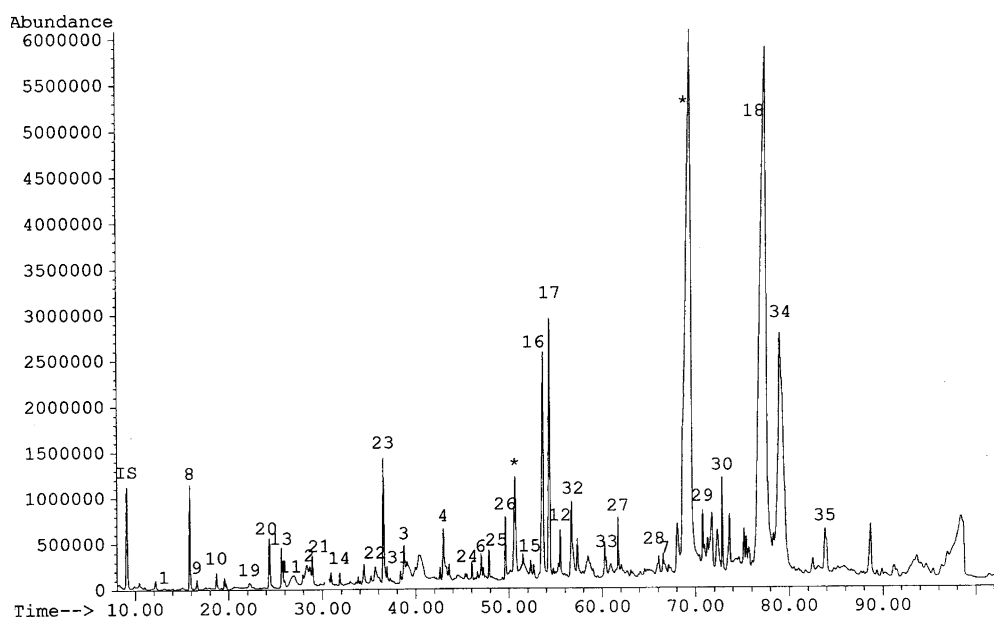


Fig. 2 Total-ion chromatogram of the SPE extract of eucalyptus honey. IS: Internal Standard; * represents resin impurities

Table 2 Concentrations ($\mu\text{g}/\text{Kg}$) and relative standard deviation (%) of the volatile compounds identified in the SPE extract of eucalyptus honey

Peak	Compounds	Mean concentration ($n = 2$)	RSD (%)
Terpenes and derivatives			
1	1,8-cineol	447.6	3.4
2	camphor	995.8	0.8
3	isoborneol	66.5	4.9
4	2,7-dimethyl-4,5-octanediol	1296.8	2.0
5	p-cymen-8-ol	179.1	1.4
6	2-hydroxycineol	481.0	1.6
7	(<i>Z</i>)-2,6-dimethyl-2,7-octadien-1,6-diol	410.2	18.8
Ketones			
8	3-hydroxy-2-butanone	1500.6	1.2
9	1-hydroxy-2-propanone	163.2	16.5
10	3-hydroxy-2-pentanone	242.9	3.4
11	5-hydroxy-2,7-dimethyl-4-octanone	443.3	10.5
Furan and pyran compounds			
12	pantolactone	924.0	6.0
13	furfural	530.8	0.8
14	5-methylfurfural	204.0	11.0
15	maltol	528.0	3.3
16	6-methyl-2-methoxypyrazine	6869.1	9.0
17	methyl furoate	4610.1	7.8
18	hydroxymethylfurfural	30787.9	12.2
Other compounds			
19	nonanal	202.7	4.7
20	isobutyl-2-methyl butanoate	982.3	0.8
21	benzaldehyde	606.7	0.4
22	phenylacetaldehyde	633.0	5.3
23	2-methyl butanoic acid + furfuryl alcohol	2216.3	5.9
24	hexanoic acid	353.1	8.9
25	benzyl alcohol	454.7	0.4
26	2-phenylethanol	1089.8	4.7
27	nonanoic acid	836.5	1.8
28	decanoic acid	398.9	14.5
29	3,4,5-trimethyl phenol	1367.0	13.8
30	benzoic acid	2057.4	6.4
Norisoprenoids			
31	oxoisophorone	213.7	7.4
32	norisoprenoid (108/150/135/121) + octanoic acid	1973.5	15.6
33	4-hydroxy-isophorone	348.9	1.5
34	3-oxo- α -ionone	2490.4	8.7
35	3-oxo- α -ionol	1857.4	3.4

Although 2-hydroxycineol is reported as an aroma component in Japanese Haze honey [6], it is characteristically found in higher concentrations in eucalyptus honey.

Polyoxygenated terpenes such as (*Z*)-2,6-dimethyl-2,7-octadien-1,6-diol and 2,6-dimethyl-3,7-octadien-2,6-diol have been also detected in orange honeys [7, 20, 30, 31].

3-Hydroxy-2-butanone (acetoin) and other hydroxyketones are characteristic of eucalyptus honeys [5, 7, 9, 16, 20, 21] and could be considered as floral markers.

Certain volatile compounds, especially furan derivatives (furfural, methyl furoate), may be formed by heating or during prolonged storage of honey. Hydrox-

ymethylfurfural (HMF) is used as a marker of excessive heating, and maximum limits are regulated by law.

Some compounds, including maltol (3-hydroxy-2-methyl-4H-pyran-4-one), 2-methoxy-6-methyl pyrazine, and pantolactone, identified in eucalyptus extracts, may be produced by Maillard reactions [7, 27] and are indicative of loss of freshness, which could negatively influence the sensory properties of a honey [32–35].

Benzene derivatives, including benzaldehyde, 2-phenylethanol, benzyl alcohol, and phenylacetaldehyde, have a honey-like aroma [6, 36] and are widely reported in studies characterizing unifloral honeys.

Organic acids are also common in honeys of different sources. The most typical and also detected in the eucalyptus honey were hexanoic, nonanoic, and decanoic acids [20, 37].

Five norisoprenoids were identified in eucalyptus honey extracts, at concentrations ranging between 214 and 2490 ppb. The solid phase extraction technique was found suitable for the analysis of this type of compounds.

High levels of norisoprenoids found in Australian heather honeys [11] and in Sardinian strawberry-tree honey, of which isophorone (3,5,5-trimethyl-2-cyclohexen-1-one) is the major component [25, 39], confirm their potential significance for determining the floral origin of honey.

Australian eucalyptus honeys have been authenticated on the basis of their 3-oxo- α -ionone and 3-oxo- α -ionol content, the latter being produced by reduction of the former [7, 37, 38]. In this study, 3-oxo- α -ionol was found at a mean concentration similar to those reported in Australian honeys.

The most abundant norisoprenoid detected was 3-oxo- α -ionone. Dehydrovomifoliol has been proposed as a possible marker for European heather honeys [14], while vomifoliol is reported as one of the most abundant volatile compounds in Australian eucalyptus honeys [7].

Finally, the norisoprenoids 2,2,6-trimethyl-2-cyclohexen-1,4-dione (oxoisophorone) and 4-hydroxy-isophorone were present in smaller amounts than the rest, in the honey extracts. The latter has been identified as an aroma component in Spanish eucalyptus, rosemary, and lavender honeys analyzed using SPME [21] and could confer tobacco, tea, or smoke aromas.

Conclusions

Solid-phase extraction is a good method to isolate volatile compounds in honeys. This method avoids heating treatment of the sample and presents good precision for quantification of volatile compounds usually found in honeys. The best operating conditions were a sample size of 20 g of honey dissolved in distilled water (1:5 W/V) and eluted with 60 mL of dichloromethane.

Solid-phase extraction technique followed by gas chromatography-mass spectrometry enabled efficient characterization of the volatile profile of eucalyptus honeys. Hydroxyketones (especially 3-hydroxy-2-butanone) and norisoprenoids (3-oxo- α -ionone, 3-oxo- α -ionol, oxoisophorone, and 4-hydroxy-isophorone), were the most characteristic volatile compounds in eucalyptus honey.

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