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Occurrence of 2-hydroxy-5-methyl-3-hexanone and 3-hydroxy-5-methyl-2-hexanone as indicators of botanic origin in eucalyptus honeys

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Abstract

SPME GC–MS analysis of the volatile fraction from 22 Spanish eucalyptus honeys revealed two components which were present in all samples. From their mass spectra they were tentatively assigned as 2-hydroxy-5-methyl-3-hexanone and 3-hydroxy-5-methyl-2-hexanone. As no commercial standards were available for these compounds, they were enzymatically synthesized, respectively, from isovaleraldehyde and pyruvic acid, and from α-ketoisocaproic acid and acetaldehyde, using reactions catalyzed by yeast pyruvate decarboxylase. The prepared products presented retention indices and mass spectra identical to those of the compounds found in eucalyptus honeys. Since these compounds were not found in 165 other honey samples from 19 different floral origins, they appear to be good markers for eucalyptus honey.

Keywords: Eucalyptus honey; Markers 2-hydroxy-5-methyl-3-hexanone; 3-Hydroxy-5-methyl-2-hexanone; GC–MS; Volatiles

1. Introduction

Botanical origin of honey is mainly responsible for its characteristic flavour, which contributes to the quality of the product. The traditional technique for its determination is pollen analysis, but it is time-consuming and requires a well trained expert. As an alternative, several types of parameters have been assayed; physicochemical (Anklam, 1998), flavonoids (Martos et al., 2000), amino-acid profile (Nozal, Bernal, Toribio, Diego, & Ruiz, 2004), sugar profile (Cotte, Casabianca, Chardon, L'heritier, & Grenier-Loustalot, 2004) mineral composition (Hernández, Fraga, Jiménez, Jimenez, & Arias, 2005) and others (Mateo & Bosch-Reig, 1998).

The volatile fraction of honey is related to the nectar source. For this reason, the determination of the honey botanical origin, on the basis of its volatile composition, has attracted increased attention in recent years (Bouseta, Collin, & Dufour, 1992). Several volatile compounds have been proposed as markers of the floral origin of honeys. Isophorone and other 3,5,5-trimethylcyclohexene derivatives have been proposed as possible markers of heather honey (Guyot, Scheirman, & Collin, 1999; Hausler & Montag, 1991) while methyl anthranilate has been indicated as a marker of citrus honeys (Bonvehi & Coll, 1995; Ferreres, Giner, & Tomás-Barberán, 1994; White, 1966). For eucalyptus honey, several compounds have been reported as indicators: dimethyl sulfide, diketones (2,3-butanedione, 2,3-pentanedione) and hydroxyketones (acetoin, 3-hydroxy-2-pentanone and 2-hydroxy-3-pentanone) have been proposed by different authors (Bouseta et al., 1992; Graddon, Morrison, & Smith, 1979; Radovic et al., 2001). However, the presence of dimethyl sulfide, 2,3-butanedione and acetoin in honeys from other origins (Bianchi, Careri, & Musci, 2005; Soria, Gonzalez, de Lorenzo, Martínez-Castro, & Sanz, 2004) reduces their utility as markers for eucalyptus. 2,3-Pentanedione, 3-hydroxy-2-pentanone and

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2-hydroxy-3-pentanone can not be considered as reliable markers since these compounds have been not found in some eucalyptus honeys.

During recent years we have studied the volatile composition of different types of Spanish honeys (Soria et al., 2004; Soria, Gonzalez, de Lorenzo, Martinez-Castro, & Sanz, 2005) and, more recently, four types of Spanish unifloral honeys, including eucalyptus (22 eucalyptus honeys among 112 total samples) (De la Fuente, Martinez-Castro, & Sanz, 2005). Two unidentified compounds which seemed to be characteristic of eucalyptus honeys appeared in the corresponding GC–MS chromatograms. Now, enzymatic synthesis has allowed the identification of both compounds from GC–MS data, and their usefulness as qualitative markers of eucalyptus honeys has been evaluated.

2. Materials and methods

2.1. Samples

Twenty two samples of artisanal eucalyptus honeys obtained from several Spanish regions were selected. They were mainly from *Eucalyptus globulus* and *Eucalyptus camaldulensis* which are the most abundant *Eucalyptus* species in Spain.

2.2. Pollen analysis

The melissopalynological method used in this work was proposed by the International Commission for Bee Botany (ICBB) (Louveaux, Maurizio, & Vorwohl, 1978). The fractions were analyzed without acetolysis. Four hundred grains of pollen were identified, following the procedure of Montero and Tormo (1990).

2.3. SPME

Two grams of honey and 1 ml of milli-Q water were homogenized in a 5 ml vial, ultrasonicated for 5 min and placed in a bath at 60° for 15 min to equilibrate the gas and liquid phases. After that, a Carbowax/PDMS fiber from Supelco (Bellefonte, PA) was introduced into the vial and exposed for 30 min to vial headspace. Details are given elsewhere (De la Fuente et al., 2005).

2.4. GC–MS analysis

Analyses were carried out on an Agilent 6890 (Palo Alto, CA) gas chromatograph coupled to an Agilent 5973 quadrupole mass spectrometer detector (Palo Alto, CA). Thermal desorption of the SPME fibre was carried out at 280° in a high pressure microseal injector (Merlin, Bellefonte, PA) during 2 min. Splitless mode was used, with an split ratio of 30:1. A Carbowax 20 M capillary column (50 m × 0.2 mm × 0.2 μm) from Supelco (Bellefonte, PA) was used. Carrier gas was helium at 1 ml/min flow rate. Oven temperature was held at 40°C for 2 min, then increased at 4°/min up to 190° and held there for 30 min. Details are given elsewhere (De la Fuente et al., 2005).

Linear retention indices (RI) were calculated using mixtures from *n*-nonane to *n*-pentacosane dissolved in *n*-heptane (Van den Dool & Kratz, 1963).

2.5. Synthesis of 2-hydroxy-5-methyl-3-hexanone and 3-hydroxy-5-methyl-2-hexanone

Isovaleraldehyde and diethyl ether were acquired from Scharlau Chemie (Barcelona, Spain), while the rest of reagents were from Sigma Chemical Co., St. Louis, MO.

2-Hydroxy-5-methyl-3-hexanone and 3-hydroxy-5-methyl-2-hexanone were prepared, following a method for synthesis of acyloins developed by Neuser et al. (2000). One milligram of pyruvate decarboxylase from baker's yeast with an activity of 5 units was mixed with 2 mM thiamine diphosphate and 20 mM $MgSO_4$ in 0.1 M sodium citrate buffer (pH 6.0) in two tubes, to a final volume of 4 ml. Three hundred micromole of acetaldehyde and an equimolar amount of α-ketoisocaproic acid sodium salt were added to the first tube in order to synthesize the acyloin 2-hydroxy-5-methyl-3-hexanone. Three hundred micromole of sodium pyruvate and 300 μmol of isovaleraldehyde were added to the second tube for the synthesis of

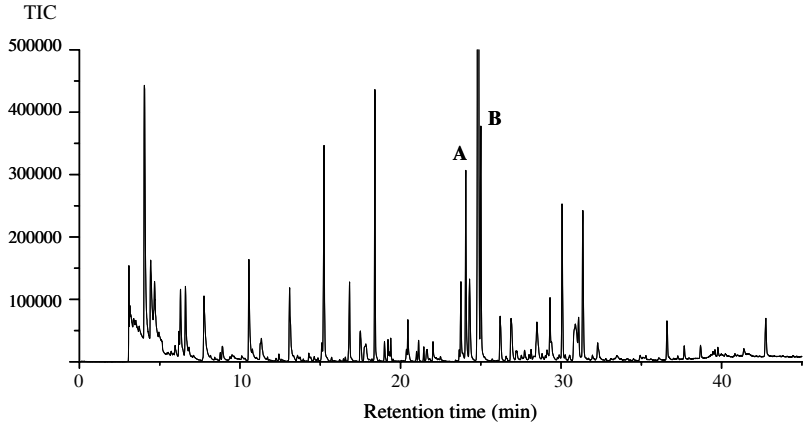

Fig. 1. Volatile profile obtained by SPME GC–MS of a typical eucalyptus honey.

3-hydroxy-5-methyl-2-hexanone. Both experiments were carried out in duplicate.

Tubes were incubated at 24 °C during 48 h. Reaction mixtures were extracted with diethyl ether (2 × 2 ml) and the extracts were directly analyzed by GC–MS.

3. Results and discussion

3.1. Identification

Fig. 1 shows a characteristic volatile profile of a eucalyptus honey sample. Peaks A and B, more clearly shown in the expanded view (Fig. 2a), presented RI values of 1449 and 1475, respectively.

Although their mass spectra were not included in the available MS library (Mc Lafferty & Stauffe, 1989) the compounds were tentatively identified as 2-hydroxy-5-methyl-3-hexanone (peak A) and 3-hydroxy-5-methyl-2-hexanone (peak B) on the basis of their fragmentation patterns.

These compounds have not previously been reported in honeys and standards were not available. However, 2-hydroxy-5-methyl-3-hexanone and 3-hydroxy-5-methyl-3-hexanone, as well as other acyloins had been previously synthesized by Neuser et al. (2000a) using enzymatic synthesis from an oxo acid with acetaldehyde or an aldehyde with pyruvic acid, and catalyzed by pyruvate decarboxylase. (Neuser et al., 2000a; Neuser, Zorn, & Berger, 2000b). The reaction of acetaldehyde and α-ketoisocaproic acid produced 2-hydroxy-5-methyl-3-hexanone as the major acyloin, and 3-hydroxy-5-methyl-2-hexanone was the main component when the reaction was carried out with isovaleraldehyde and pyruvic acid (Fig. 3). The mass fragments reported by Neuser et al. (2000a) for these compounds corresponded with those found for peaks A and B in eucalyptus honeys.

Syntheses of both 2-hydroxy-5-methyl-3-hexanone and 3-hydroxy-5-methyl-2-hexanone were carried out in our laboratory in order to confirm our tentative identification. Following reaction 1 in Fig. 3, a mixture of 80% of the first acyloin and 20% of the second was obtained. These compounds were also obtained (22.5% of the first and 77.5% of the second) from reaction 2. Neuser et al. (2000a) attribute the formation of the secondary acyloins to a keto-enol equilibrium, possibly caused by the enzyme.

Mass spectra obtained for the synthesized compounds (Table 1) were similar to those published by Neuser et al. (2000a) and identical to those registered for peaks A and B in our eucalyptus honeys. Retention indices of 2-hydroxy-5-methyl-3-hexanone and 3-hydroxy-5-methyl-2-hexanone (RI in Table 1) also agreed with experimental retention data of the peaks A and B found in eucalyptus honeys, confirming the previous tentative identification.

3.2. Presence of acyloins A and B in honey samples

Table 2 shows the concentration data (relative to total amount of volatile components) of 2-hydroxy-5-methyl-3-hexanone (compound A) and 3-hydroxy-5-methyl-2-hexanone (compound B) in the 22 honey samples containing eucalyptus pollen. Peaks 1, 2 and 3 correspond to linalool oxide (*cis*-), acetic acid and furfural, respectively. Compounds A and B were present in the analyzed honeys in concentrations between 0.25% and 8.06% for compound A and 0.30% and 9.93% for compound B. A low correlation was obtained between pollen percentage and relative composition of compounds A and B. This result was expected, since both pollen percentage and relative volatile composition depend markedly on the characteristics of other plants which can contribute, in different partial

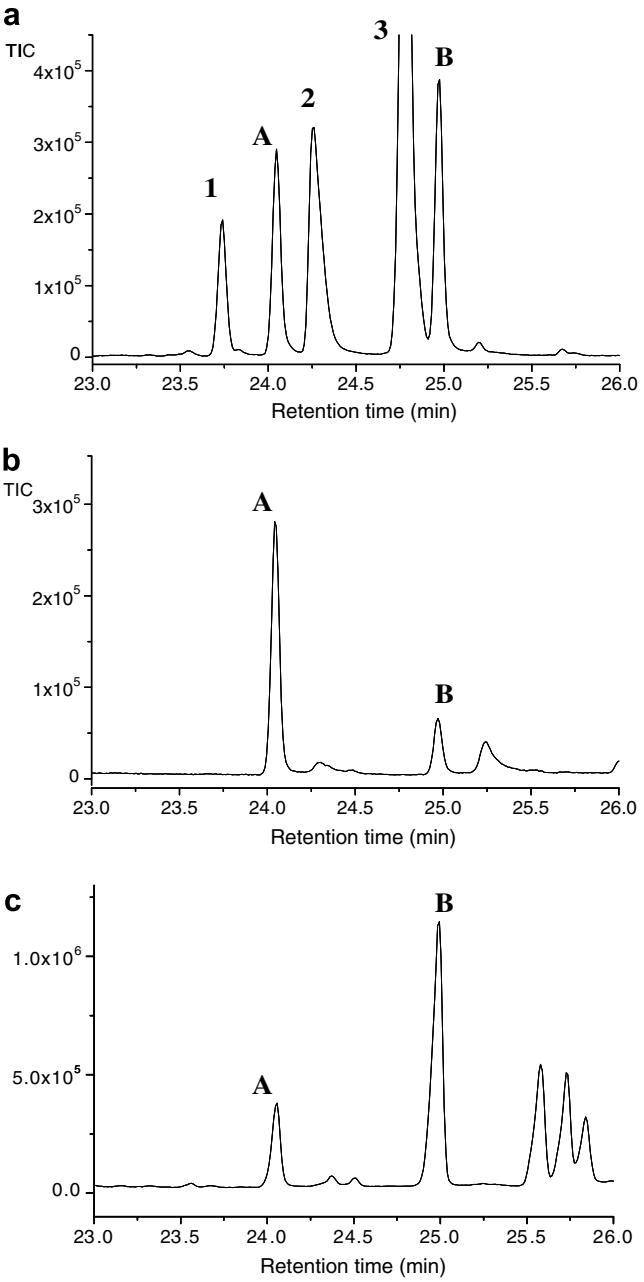

Fig. 2. Expanded view (peaks A and B) of a TIC profile for eucalyptus honey sample (a), for the enzymatic reaction products of acetaldehyde and α-ketoisocaproic acid (b), and of isovaleraldehyde and pyruvic acid (c).

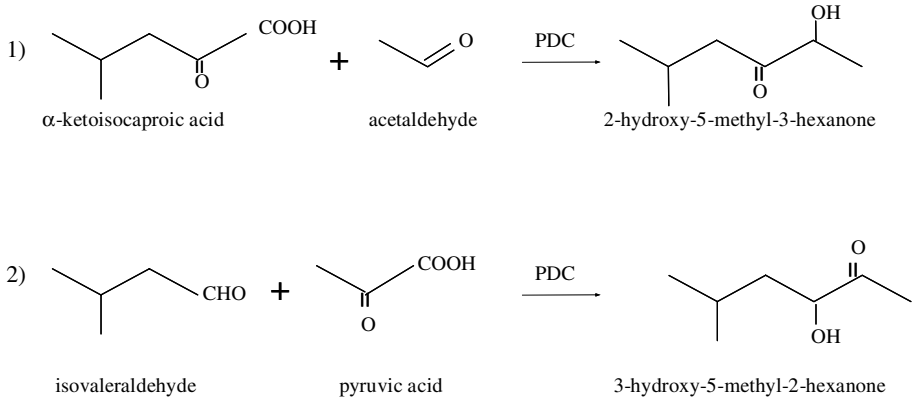

Fig. 3. Enzymatic formation of (1) 2-hydroxy-5-methyl-3-hexanone and (2) 3-hydroxy-5-methyl-2-hexanone.

Table 1
Characterization of acyloins A and B

Compound	RI	Mass fragments: *m/z* (% intensity)
2-Hydroxy-5-methyl-3-hexanone	1449	130 (0.4), 85 (61), 57 (100), 45 (91), 43 (39)
3-Hydroxy-5-methyl-2-hexanone	1475	130 (0.3), 87 (24), 69 (59), 45 (41), 43 (100)

amounts, to pollen and nectar in every eucalyptus honey. However, all the samples containing eucalyptus pollen presented these two compounds.

These compounds were not detected in 161 honey samples of 19 different floral origins rosemary, heather, citrus, (De la Fuente et al., 2005), honeydew, Rosaceae, dandelion, (Soria et al., 2004), avocado, medlar tree, Teide broom, *Echium wildpretii,* Quercus sp., strawberry tree, fir, *Anthyllis cytisoides*, savory, Agave sp, willow, lavender and multiflower (unpublished results) whose volatiles were analyzed by the same procedure. Only two samples, labelled as heather and citrus, respectively, presented these compounds: however, melissopalynologic analyses revealed the presence of minor amounts of eucalyptus pollen in both samples.

Table 2
Concentrations (relative to total amount of volatile components) of 2-hydroxy-5-methyl-3-hexanone (compound A) and 3-hydroxy-5-methyl-2-hexanone (compound B) in 22 honey samples containing eucalyptus pollen

Sample	% Compound A	% Compound B	% Eucalyptus pollen
H1	1.54	1.25	68
H2	0.58	0.88	25
H3	0.87	0.59	44
H4	0.40	1.10	38
H5	2.33	4.77	21
H6	5.92	9.93	70
H7	4.31	8.47	19
H8	3.29	4.00	87
H9	0.31	0.40	71
H10	0.76	0.96	56
H11	5.93	7.64	42
H12	4.82	7.85	21
H13	1.12	1.49	54
H14	1.42	1.63	22
H15	6.45	7.51	83
H16	2.40	3.85	73
H17	5.69	7.20	57
H18	0.25	0.30	13
H19	5.19	8.90	46
H20	4.28	6.03	40
H21	1.75	1.78	65
H22	8.06	9.57	34

Other authors have reported two unknown peaks in eucalyptus honeys, which could be assigned to the compounds identified in this work. Radovic et al. (2001) reported the volatile composition of 7 acacia, 9 chestnut, 3 eucalyptus, 8 heather, 2 lavender, 4 lime, 4 rape, 2 rosemary and 4 sunflower honeys from different countries; two unidentified compounds, with mass spectra and retention data similar to those of the hydroxyketones reported above, appeared in the three eucalyptus samples and were absent in the rest of samples. Piasenzotto, Gracco, and Conte (2003) also found two unidentified compounds only in eucalyptus honeys among 40 samples analyzed from different origins; these compounds, with RI = 1446 and 1482 and mass fragments (57, 45, 51, 85, 74) and (43, 69, 87, 41, 74), seem to correspond to 2-hydroxy-5-methyl-3-hexanone and 3-hydroxy-5-methyl-2-hexanone, respectively. D'arcy, Rintoul, Rowland, and Blackman (1997) reported the composition of ethyl acetate extracts of Blue Gum (*Eucalyptus leucoxylon*) and Yellow Box (*Eucalyptus melliodora*) honeys. These Australian honeys present low average concentrations (below 1 mg/kg) of two unidentified compounds, with mass spectral data similar to those found in our work for the two hydroxyketones.

The presence of these compounds in a honey sample appears to be a good indicator of eucalyptus origin, since 2-hydroxy-5-methyl-3-hexanone and 3-hydroxy-5-methyl-2-hexanone are detected by SPME, followed by GC–MS in all the eucalyptus samples analyzed while they do not

appear in the rest, allowing recognition of eucalyptus honeys from volatile qualitative data. To the best of our knowledge, both compounds are the honey volatile components most specific as eucalyptus markers. More eucalyptus honey samples need to be studied from other countries, and particularly from Australia, before the proposed hydroxyketones can be confirmed as good indicators of honeys of eucalyptus origin. Also, further research might be necessary to determine the minimum amount for considering a eucalyptus honey as monofloral, since this value would depend on the volatile composition of plants contributing partially to this honey. Additional work is being carried out in order to explain the origin of these compounds in eucalyptus honeys.

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