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Characterization of Markers of Botanical Origin and Other Compounds Extracted from Unifloral Honeys

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ABSTRACT: The possibility of tracing the botanical and geographical origin of products such as honey has become more important because of market globalization. As a consequence, numerous analytical methods have been applied to the determination of honey authenticity. The scope of the present work is to chromatographically purify and characterize 23 compounds from organic extracts of unifloral (chestnut, linden, orange, acacia, eucalyptus, honeydew) and polyfloral honeys. Of these compounds, 17 were identified as specific markers and were used for botanical discrimination in a previous study based on multivariate statistical analysis of proton nuclear magnetic resonance (^{1}H NMR) data. Together with the botanical markers, 6 other substances were isolated and characterized using NMR and mass spectrometry. These phytochemicals belong to several classes, that is, terpenes, organic acids, flavonoids, and others. For the first time, a diacylglyceryl ether and 5 other compounds present in different types of honey were identified and characterized.

KEYWORDS: *honey, botanical origin, marker compounds, NMR*

INTRODUCTION

Honey is the natural product obtained by honey bees (*Apis mellifera* L.) from the nectar of flowers or from secretions of other living parts of plants or excretions of sucker insects. Honey is mainly a supersaturated sugar solution, with >95% of its dry mass consisting of sugars, although different valuable nutrients such as vitamins, minerals, enzymes, flavoring organic compounds, free amino acids, and numerous volatile compounds are present as minor components.[1] The nature, amount, and combination of the various components endow each honey with individual organoleptic character and nutraceutical properties. These compounds represent a fingerprint of a specific honey and therefore could be used to differentiate honeys of different botanical and also geographic origins. The main parameters of honey quality, which also influence its price, derive from its botanical origin.[2] The knowledge of which minor components are present in different unifloral honeys may also substantiate (or dispute) health claims attached to some of these products.

The interest in honey has recently grown not only because of the possible therapeutic effects that accompany its nutritional value but also because legislations tend to protect its geographic and botanical origin. A metabolomic approach has been widely applied in food science to guarantee authenticity and is gaining importance to determine the origin of unifloral honeys. Various methods of minor constituents analysis are employed,[3,4] including gas chromatography−mass spectrometry (GC-MS),[5] liquid chromatography−mass spectrometry (LC-MS),[6] capillary electrophoresis−time-of-flight mass spectrometry (CE-TOF-MS),[7] MALDI-TOF MS,[8] FTIR[9] and nuclear magnetic resonance (NMR) spectroscopy.[10,11] In recent years, the use of high magnetic fields and the greater sensitivity and spectroscopic resolution that they bring have stimulated interest in 1D and 2D NMR spectroscopy as a routine method for the analysis of complex mixtures[12] such as honey.[10,11,13−15]

NMR has several advantages compared to other techniques: sample preparation is simple and easy, which minimizes the change in the chemical composition and the loss of metabolites during sample preparation; also, many components, such as flavonoids, sugars, terpenes, and organic acids, are detected simultaneously. NMR is also a very powerful technique for the determination of the chemical structure of organic molecules. In the present context, NMR can provide the identity of reliable marker compounds, a crucial step not only to characterize a certain type of honey but also to expose possible adulterations.

In this paper, the isolation and identification of several compounds present in chloroform extracts of honeys of different floral origins are described. Among all of the compounds that can be found in honey, most of the ones considered in this work were selected because they are responsible for statistical discrimination of different botanical origins. In a previously proposed NMR-based metabolomic approach that used O2PLS-DA multivariate data analysis,[11] the proton resonances of these compounds have allowed the classification of different unifloral honeys, although some of them were not characterized. Some compounds included in this study are not specific markers, but they are nevertheless intriguing. A few of these compounds were identified for the first time in honey produced by *A. mellifera*.

MATERIALS AND METHODS

Chemicals. Deuterated chloroform, $CDCl_3$ (99.96% d, H_2O <0.005%), was from Euriso-Top, Gif sur Yvette, France; acetonitrile (CH_3CN), methanol (MeOH), and standard compounds (chrysin, 97% pure; pinocembrin, 95% pure; kynurenic acid, 98% pure;

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(±)-abscisic acid, 99% pure; linalool, 97% pure; neurotensin mass spec standard, angiotensin mass spec standard, and bradykinin mass spec standard) were purchased from Sigma-Aldrich (Milan, Italy).

Honey Samples. Honey samples of seven different floral origins were obtained either from Rigoni S.p.A. or from trusted Veneto apiaries: 75 acacia, 60 chestnut, 62 linden, 40 orange, 32 eucalyptus, and 36 honeydew. The botanical origin of each sample was certified by the provider and ascertained through sensorial analysis and, in several cases, by melissopalinologic analysis. The NMR-based metabolomic analysis previously described confirmed the declared origin.[10,11]

Sample Preparation. A liquid/liquid extraction was carried out as previously reported with slight modifications.[10] The necessary amount of honey (50−100 g) was obtained by combining different samples of the same floral origin. Specifically, each honey sample was divided in many Teflon tubes, each containing 12 g of honey, 10 mL of water, and 10 mL of chloroform. After 10 min of mechanical stirring at room temperature, the tubes were centrifuged at 10000 rpm for 15 min. The lower chloroform phases were pooled, and the solvent was evaporated under a gentle stream of nitrogen. The solid residues were purified using the optimized procedure, described below.

The extracts were initially treated with an acetonitrile/water = 95:5 solution to precipitate waxes. Compound **23** also precipitates in these conditions. The solutions were dried and purified through a 25 cm × 2 cm silica gel column, equilibrated in chloroform. For acacia honey, a step gradient elution of petroleum ether/ethyl acetate from 0:5 to 5:0 (E:A) was used. For the other honeys, a step gradient elution from 0 to 5% MeOH in $CHCl_3$ was employed. The fractions were evaporated under a stream of nitrogen to eliminate the mobile phase, redissolved in 600 μL of $CDCl_3$, and placed in an NMR tube for the analysis.

NMR Measurements. A 600 MHz Bruker (Bruker, Rheinstetten, Germany) Avance spectrometer was used for one- and two-dimensional spectra. In a typical 1D experiment, 256 transients were collected as 64K points with a spectral width of 14 ppm and a relaxation time of 2 s, at 298 K, using a modified double-pulsed field gradient spin echoes sequence[16] when necessary. Two-dimensional homo- and heteronuclear correlation NMR spectra (DQF-COSY, TOCSY, NOESY, HMQC, and HMBC) were collected. The experimental conditions were as follows: for TOCSY, spectral width (SW) = 10 ppm in both dimensions, data points (DP) in the F2 dimension = 2K, relaxation delay (RD) = 2 s, number of scans (NS) = 16, number of experiments (NE) = 400−512, spin-lock time = 100−120 ms; for COSY, SW = 10 ppm, DP = 2K, RD = 2 s, NE = 400−512; for NOESY, SW = 10 ppm, RD = 2 s, NS = 128−256, NE = 400−512, mixing time = 1.2 s; for $^{1}H-^{13}C$ HMQC, SW = 10 ppm (^{1}H), SW = 220 ppm (^{13}C), DP = 1K, NS = 128−256; for $^{1}H-^{13}C$ HMBC, SW = 10 ppm (^{1}H), SW = 220 ppm (^{13}C), DP = 1K, NS = 600−800.

Mass Spectrometry. Molecular masses were determined on a Mariner (PerSeptive Biosystems, Foster City, CA, USA) quadrupole mass spectrometer equipped with an electrospray ion source. The instrument was operated in the positive ESI(+) ion mode at a probe tip voltage of 4.3 kV. The samples were introduced into the mass spectrometer ion source directly through a Rheodyne injector with a 20 μL sample loop. The mobile phase flow (10 μL/min of 50:50 v/v CH_3CN/H_2O, 0.1% HCOOH) was delivered by a Harvard Apparatus P11 to the vaporization nozzle of the electrospray ion source (140 °C), and nitrogen was employed as both a drying and nebulizing gas.

The spectrometer was calibrated using a mixture of neurotensin, angiotensin, and bradykinin, at a concentration of 1 pmol/μL, as external standard.

The ESI-MS analysis of the diacylglyceryl ether **23** was performed by dissolving the compound in chloroform/methanol (1:1) containing 0.2 mM ammonium acetate.[17]

RESULTS AND DISCUSSION

The chloroform extracts of the analyzed unifloral honeys (*Citrus sinensis* honey, *Tilia cordata* honey, *Robinia acacia* L. honey, *Castanea sativa* honey, *Eucalyptus* spp. honey, and honeydew honey) were subjected to silica gel column chromatography to afford and isolate the following compounds (Figure 1): 4-(1-hydroxy-1-methylethyl)cyclohexa-1,3-dienecarboxylic acid methyl ester (**1**), 4-(1-hydroxy-1-methylethyl)-cyclohexa-1,3-dienecarboxylic acid (**2**), 4-(1-methylethenyl)-cyclohexa-1,3-dienecarboxylic acid (**3**), and 4-(1-hydroxy-1-methylethyl)benzoic acid (**4**) from linden honey; deoxyvasicinone (**6**), the γ-lactam derivative of 3-(2′-pyrrolidinyl)-kynurenic acid, γ-LACT-3-PKA (**7**), 2-quinolone (**8**), and 4-quinolone (**9**) from chestnut honey; caffeine (**14**) and (*E*)-2,6-dimethylocta-2,7-diene-1,6-diol (8-hydroxylinalool) (**15**) from orange honey; and dehydrovomifoliol (**16**) and 3-oxo-α-ionone (**17**) from eucalyptus honey. (*E*)-2,6-Dimethyl-3,7-octadiene-2,6-diol (**5**) is a terpene that derives from linalool and is present mainly in linden honey; however, the molecule was also found in chestnut honey. This result can be explained by the overlapping flowering periods of the two plants, which can result in cross-contamination. Pinocembrin (**10**), chrysin (**11**), alpinone (**12**) and (*Z*,*E*)-abscisic acid (**13**) were found mainly in acacia honey, but they are present in almost every kind of honey. Also, other compounds are present in most honeys because they derive from bee metabolism ((*E*)-2-decenedioic acid (**18**), (*E*)-2-nonenedioic acid (**19**), decanedioic acid (**20**), nonanedioic acid (**21**)) or are ubiquitous in plants (methyl syringate (**22**)). Compound **23** was isolated from honeydew, where it is relatively more abundant, but it is also present in honey.

Compounds **2** and **3** were identified directly in the organic honey extract because of their strong and isolated signals, whereas methyl syringate (**22**) was detected by spiking the extract with the standard compound. The other molecules were isolated in silica gel column fractions with the following conditions: **5** (1% MeOH), **6** (3% MeOH), **7** (2.5% MeOH), **8** and **9** (0.5% MeOH), **14** (0.5% MeOH) **15** (1.25% MeOH), and **16** and **17** (0.25% MeOH). From acacia honey, **10** (E:A = 4:1), both **11** and **12** (E:A = 3:2). After the last gradient solution, all of the columns were always washed with acetonitrile/water = 95:5, obtaining compound **13** from acacia honey, compounds **1** and **4** from linden honey, and compounds **18**−**21** from all of the botanical origins. Compound **23** was purified through a silica gel column with petroleum ether/ethyl acetate = 3:1 as an isocratic eluent.

All of the compounds were identified and characterized by 1D and 2D NMR and by ESI-MS, using the procedure described under Materials and Methods; the assignments of the isolated molecules **1** and **4**−**9** are reported in Table 1; the assignments of compounds **12**, **13**, **16**−**23** are reported in Table 2, whereas the assignments of compounds **2**, **3**, **10**, **11**, **14**, and **15** have been published previously.[11]

Deoxyvasicinone (**6**), for example, was identified by ESI-MS (*m*/*z* 187 $[M + H]^+$) and by 1D and 2D NMR spectra (Figure 2). The TOCSY experiment shows a spin system that includes protons H-5, H-6, H-,7 and H-8 and a second one that consists of H-1′, H-2′, and H-3′. The carbonyl carbon (C-4) shows a cross peak with H-5 in the HMBC experiment, which demonstrates the connection between these parts of the molecules. Both H-1′ and H-2′ have a cross peak with C-2.

Linden Honey. Compounds **1**−**4** are monoterpenoid acids characterizing *T. cordata* honey. The structure of markers **2** and **3**, which are the most abundant ones, was determined with the aid of literature data.[18] Compound **4** is possibly an oxidation product of compound **2**. The linalool derivative (*E*)-2,6-dimethyl-3,7-octadiene-2,6-diol (**5**) is also characteristic of

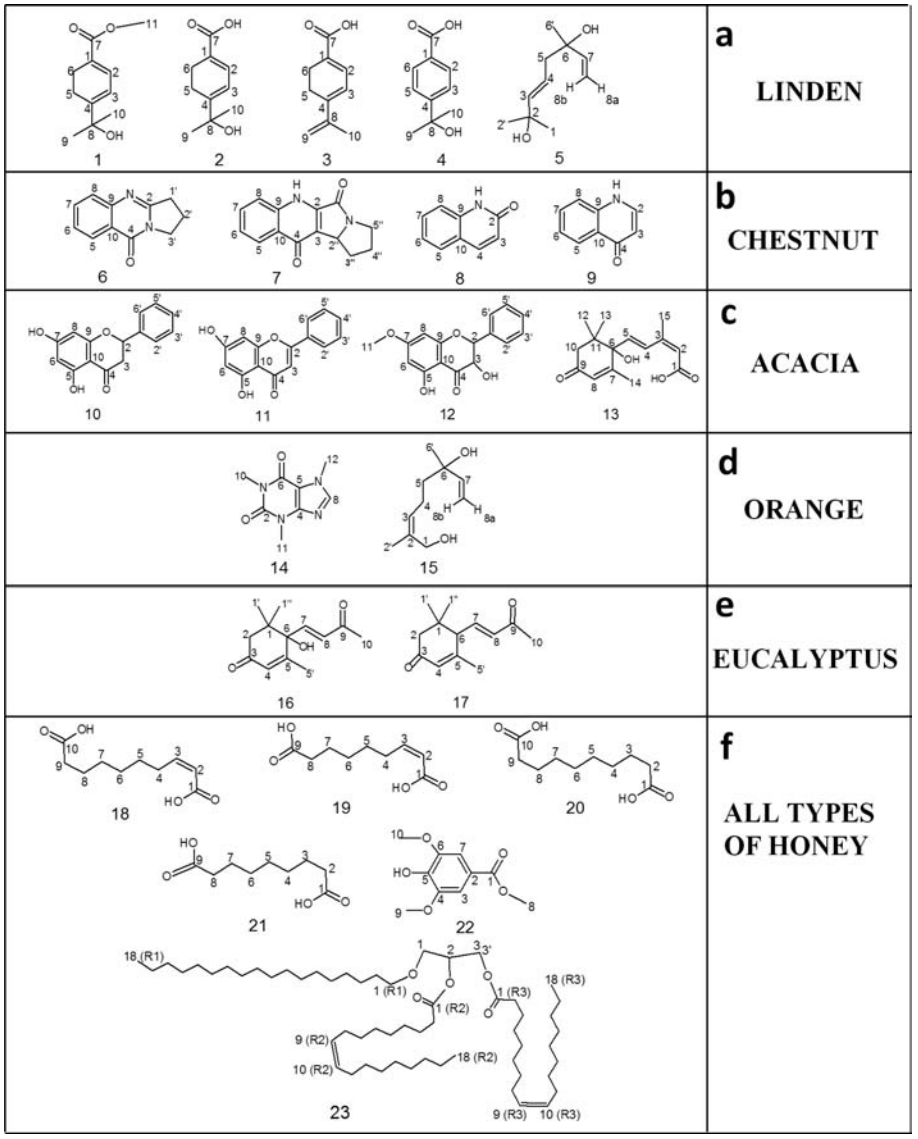

Figure 1. Structures of compounds **1**–**23**.

linden honey, although it is present in smaller quantities than the other markers.

Chestnut Honey. Compounds **6**–**9** are typical molecules of chestnut honey; their chemical shift values are reported in Table 1. Deoxyvasicinone (**6**) is a quinazolinone fused with a pyrrolidine ring: it had never been found in honey before, and it has been demonstrated to have antimicrobial, anti-inflammatory, and antidepressant activities.[19] Compound **7** has been previously indicated as γ-LACT-3-PKA, the typical marker of chestnut honey.[6] The ^{1}H NMR spectra of compounds **6** and **7**, obtained from the isolated fractions of chestnut honey, are reported in Figure 2. Compounds **8** and **9** are structural isomers with the molecular formula C_9H_7NO. In the literature, compound **8** and its derivatives are endorsed with antibacterial, anticancer, antiviral, and antioxidative activities,[20] and compound **9** and its derivatives show antibacterial activity.[21]

Acacia Honey. Among the natural antioxidants present in food, flavonoids represent one of the most important classes.[22,23] Compounds **10** and **11** (pinocembrin and chrysin) are flavonoids that can be regarded as characteristic of *R. acacia* L. honey because of their higher concentration with respect to other types of honeys.[11] Agüero et al.[24] demonstrated that pinocembrin (**10**) is moderately active against some types of fungi. The assignment of compounds **10** and **11** has been published.[11] Another flavonoid was isolated from acacia honey and identified as alpinone (**12**): the assignments are reported in Table 2. This is the first time that this compound has been found in honey: previously, its presence in propolis[25] and in the extracts of *I. tenuifolia*[26] was reported. The plant hormone abscisic acid (**13**) is important in seed maturation and germination and also in adaptation to abiotic environmental stresses; its content rises during exposure of a plant to drought.[27] In the plant, it is produced from the C_{40} carotenoid zeaxanthin through the intermediate xanthoxin.[28,29] The assignment of abscisic acid is reported in Table 2.

Orange Honey. Compounds **14** and **15** are typical of orange honey and have been previously assigned.[11] A NOESY cross peak between protons 1 and 3 confirms the *E* configuration for the double bond C2–C3 in compound **15**. This compound derives from ω-hydroxylation of linalool.[30] Polashock et al.[31] reported antifungal and antimicrobial

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Table 1. ^{1}H and ^{13}C NMR Data of Compounds 1, 4, and 5–9 ($CDCl_3$)[a]

	1		4		5		6		7		8		9	
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	/	154.6	/	125.0	1.33	29.3	/	/	9.74[b]	/	nd	/	nd	/
2	7.18 dt (5.8, 1.7)	136.8	8.08 d (8.1)	130.1	/	71.2	/	159.8	/	nd	/	162.5	7.95 d (6.6)	139.5
3	6.18 dt (5.8, 1.7)	115.8	7.62 d (8.1)	124.4	5.73 dt (15.7, 1.1)	143.4	/	/	/	126.3[b]	6.68 d (9.5)	121.8	6.3 d (6.6)	109
					5.64 ddd (15.7, 7.9, 6.6)	121.9								
4	/	125.3	/	155.3	2.25 dd (7.9, 1.1)	44.9	/	nd	/	175.1	7.78 d (9.5)	141.0	/	180.1
5	2.47 t (9.6)	21.6	7.62 d (8.6)	130.1	2.30 dd (6.6, 1.1)	44.9	8.3 dd (8.0, 1.1)	126.2	8.44 dd (8.2, 0.8)[b]	126.4[b]	7.58 dd (8.1, 1.2)	127.9	8.07 dd (7.8, 1.3)	130.7
6	2.32 t (9.6)	23.6	8.08 d (8.6)	124.4	/	73.0	7.66 d (8.1)	127.0	7.69 t (7.7)	132.8	7.24 td (8.1, 1.2)	122.4	7.59 td	121.6
7	/	170.9	nd	170.1	5.93 dd (17.3, 10.8)	144.5	7.46 t (7.5)	125.9	7.42 t (7.7)	124.2	7.52 td (8.4, 1.2)	130.5	7.65 td	132.4
8	/	73.2	/	73.0	5.07 dd (10.8, 1.1)	112.2	7.74 td (7.0, 1.4)	134.2	7.57 dd (8.2, 0.8)[b]	118.4[b]	7.17 d (8.4)	115.1	7.89 dd	115.8
					5.22 dd (17.3,1.1)	112.2								
9	1.42 s	28.1	1.61 s	31.0			/	149.2	/	127.5	/	137.8	/	144.8
10	1.42 s	28.1	1.61 s	31.0			/	120.9	/	139.8	/	120.2	/	129.9
11	3.76 s	63.4												
1′							3.2 t (7.9)	32.9						
2′					1.33	29.3	2.3 t (7.6)	19.8	/	167.0				
3′							4.2 t (7.2)	46.4						
6′					1.29	27.4								
2″									4.80 dd (10.9, 5.8)	63.0				
3″a									1.26 m	29.3				
3″b									2.63 dt (5.6, 0.5)	29.3				
4″a									2.42 m	29.3				
4″b									2.45 m	29.3				
5″a									3.52 m	41.5				
5″b									3.66 m	41.5				

[a] J values are in parentheses and reported in hertz; chemical shifts are given in parts per million. [b] Correction of the assignment reported in previous articles.[10,11]

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Table 2. ^{1}H and ^{13}C NMR Data of Compounds 12, 13, and 16−23 ($CDCl_3$)[a]

	12		13		16		17		22			23	
position	δ_H	δ_c	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	position	δ_H	δ_C
1	/	/	/	169.0	/	42.5	/	nd	/	121.0	1	3.56 dd (5.4, 2.8)	68.2
2	5.04 d (11.9)	83.1	5.80 s	117.4	2.51 d (17.2)	49.8	2.51 d (17.2)	nd	7.34 s	106.5	2	5.21 m	69.5
					2.34 d (17.2)	49.8	2.34 d (17.2)	nd			3	4.18 dd (11.9, 6.4)	61.8
3	4.57 d (11.9)	72.4	/	163.7	/	196.8	/	nd	/	147.5	3′	4.35 dd (11.9, 3.7)	61.8
4	/	198.0	7.82 d (16.1)	127.7	5.97 s	128.2	6.01 s	126.1	/	139.9	1 (R1)	3.45 m	71.4
5	11.25 s (−OH)	162.8	6.20 d (16.1)	136.1	/	160.8	/	nd	/	147.5	2 (R1)	1.56 m	29.8
6	6.08 d (2.2)	94.4	/	79.7	/	79.3	2.74 d (9.6)	55.0	7.34 s	106.5	3 (R1)	1.3 m	22.5
7	/	163.7	/	152.2	6.47 d (15.9)	130.3	6.68 dd (15.7, 9.6)	143.5	3.96 s	56.3	2a (R3, R2)	2.33 dt (15.1, 7.4)	34.5
8	6.11 d (2.2)	95.4	5.95 s	126.9	6.84 d (15.9)	145.0	6.2 d (15.7)	133.2	3.96 s	56.3	2b (R3, R2)	2.30 dt (15.1, 7.4)	34.5
9	/	169.0	/	197.8	/	198.0	/	nd	3.91 s	51.8	3 (R2, R3)	1.64 m	25.2
10	/	101.0	2.48 d (17.2)	49.4	2.31 s	28.4	2.31 s	34.0	/	167.3	4−7, 12−17 (R2, R3)	1.31 m	29.1
			2.32 d (17.2)	49.4							9, 10 (R2, R3)	5.36 m	129.9
11	3.81 s	55.6	/	4l.9							8, 11 (R2, R3)	2.04 b	27.8
12			1.12s	22.6							18 (Rl, R2, R3)	0.91 t (7.2)	14.0
13			1.03 s	24.0									
14			1.93 s	18.6									
15			2.05 s	20.9									
1′	/	138.9			1.03 s	23.8	1.03 s	nd					
1″					1.11s	24.7	1.11 s	nd					
2′,6′	7.44 m	128.7											
3′	7.46 m	125.8											
4′	7.42 m	128.2											
5′	7.46 m	125.8			1.89 s	18.8	1.92 s	23.0					

	18		19		20		21	
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	/	170.4	/	170.4	/	178.0	/	178.0
2	5.85 dt (15.6)	120.1	5.85 dt (15.6)	120.1	2.37 m	33.2	2.37 m	33.2
3	7.07 dt (15.6)	151.7	7.07 dt (15.6)	151.7	1.66 m	24.4	1.66 m	24.4
4	2.26 m	31.8	2.26 m	31.8	1.38 m	29.1	1.38 m	29.1
5	1.51 m	27.4	1.51 m	27.4	1.51 m	27.4	1.51 m	27.4
6	1.38 m	29.1	1.38 m	29.1	1.51 m	27.4	1.38 m	29.1
7	1.37 m	29.3	1.66 m	24.4	1.38 m	29.1	1.66 m	24.4
8	1.66 m	24.4	2.37 m	33.2	1.66 m	24.4	2.37 m	33.2
9	2.37 m	33.2	/	178.0	2.37 m	33.2	/	178.0
10	/	178.0			/	178.0		

[a] J values are in parentheses and reported in hertz; chemical shifts are given in parts per million.

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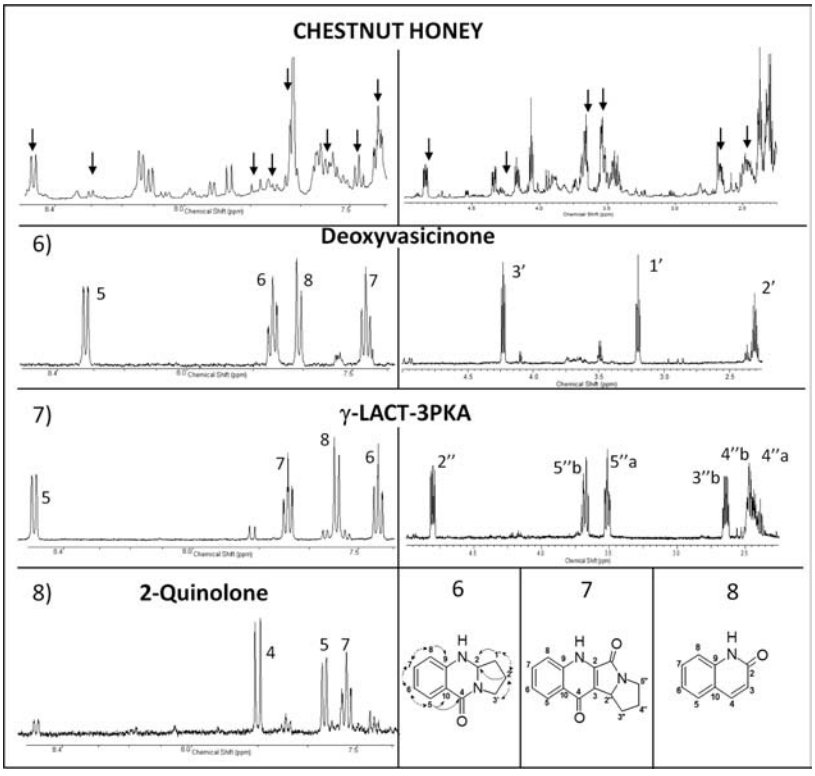

Figure 2. [1]H NMR spectra of the isolated markers of chestnut honey deoxyvasicinone (**6**), γ-LACT-PKA (**7**), and 2-quinolone (**8**). The HMBC (→) and TOCSY(--->) correlations for compound **6** are shown on the molecular structure.

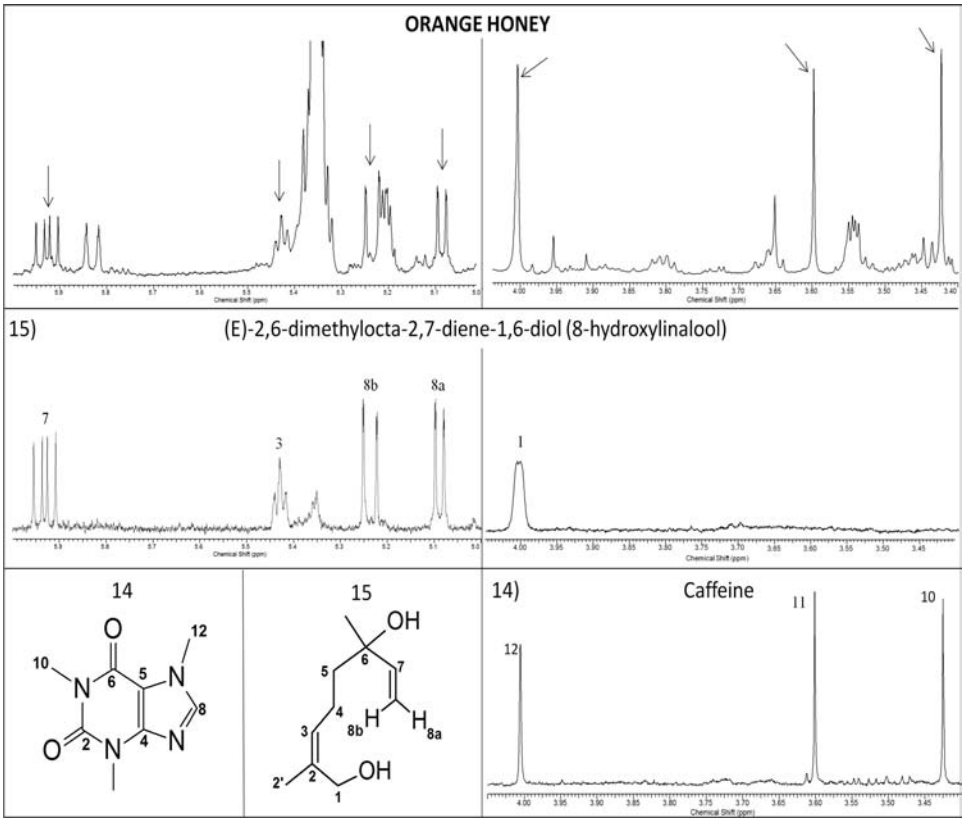

Figure 3. [1]H NMR spectra of the isolated markers of orange honey: caffeine (**14**) and (*E*)-2,6-dimethylocta-2,7-diene-1,6-diol (**15**).

activities of **15**, which was found to accumulate in ripening fruits.[32] See Figure 3 for the [1]H NMR spectra of compounds **14** and **15** isolated from orange honey.

Eucalyptus Honey. Norisoprenoids **16** and **17** are typical of eucalyptus honey and derive from cleavage of the carotenoid chain that can occur at different double bonds. Dehydrovomifoliol

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(**16**) probably arises from the degradation of abscisic acid. On the other hand, 3-oxo-α-ionone (**17**) is generated by cleavage of the C_9-C_{10} double bond of C_{40} carotenoids and has already been found in sulla honey.[33] See Table 2 for all of its assignments.

Compounds Isolated in All of the Honeys. Honeybee larvae are fed by "nurse" bees with a jelly-like secretion (royal jelly) of the mandibular and the hypopharyngeal glands.[34] Compounds **18**−**21** are characteristic of larval food of the honeybee: although they mainly derive from royal jelly, some traces can be found also in honey (Table 2). These compounds were identified as free fatty diacids, because of the absence of ester signals in the ^{1}H NMR spectrum and of carbons of carboxylic esters in the HMBC spectrum. The ESI-MS spectrum confirmed the presence of all four of these compounds in the same fraction.

These diacids are of special interest because they have long been recognized as part of the pheromone system of the honeybee *A. mellifera*.[35−37] Queen and worker honeybees produce functionalized 8-, 9-, and 10-carbon fatty acids in their mandibular glands: these compounds regulate the queen's and worker's activities in the hive. The honeybee queen produces pheromones such as 9-hydroxy-(*E*)-2-decenoic acid (9-HDA), 9-keto-(*E*)-2-decenoic acid (ODA), and other acids functionalized at the penultimate (ω-1) position.[38] Worker bees secrete acids functionalized at the last (ω) position, such as 10-hydroxy-(*E*)-2-decenoic acid (10-HDA), its saturated counterpart, and the corresponding diacids (C10:1 DA and C10:0 DA).[39] The worker's mandibular gland secretions are believed to function primarily as a preservative additive to the larval food.[40]

It has been previously shown that whereas 10-HDA is the main acid in royal jelly, C10:1 DA represents the principal aliphatic acid in honey.[41] As expected, we found only diacids functionalized at the ω-position in honey, because worker bees play the main role in the production of honey. Diacids with 9 carbon atoms were also found, confirming the work by Tan et al.,[35] who found compound **21**, even if only in traces, and by Lercker et al.,[36] who found **19** and **21** in an amount comparable to diacids with 10 carbon atoms.

Methyl syringate (**22**) is a general aromatic constituent in plants. It was previously found in many types of honeys and also in grapevines, leaf, and root.[42] Jermnak et al. described its potential as an aflatoxin production inhibitor.[43]

Compound **23** is a diacylglyceryl ether that contains two oleic acid chains in positions 2 and 3 and a saturated hydrocarbon C18 chain in position 1. The chemical shifts, the TOCSY connectivities, and the patterns of *J*-couplings of protons H-1, H-2, H-3, and H-3′ are consistent with the glycerol skeleton of a 2,3-diester (see Table 2). This is confirmed by the correlations found in the HMBC spectrum (Figure 4) between H-3, H-3′, and carbonyl C-1(R3) (173.73 ppm) and between H-2 and carbonyl C-1(R2) (173.33 ppm). Also, H-2(R2) correlates with both C-1(R2) and C-3(R2) (Figure 4). The presence of a correlation between H-1 and C-1(R1) (71.4 ppm) is indicative of an ether oxygen in position 1. This finding is further supported by the cross peaks in the NOESY spectrum between H-1 and H-1(R1) that confirms an ether chain $-OCH_2R$ linked in position 1.

In addition, the presence of a multiplet at 5.36 ppm for the olefinic protons (H-9,10(R2,R3)), the TOCSY correlation with the allylic protons resonating at 2.04 ppm (H-8,11(R2,R3)), and the absence of signals from bisallylic protons in the spectrum indicate that the fatty acid chains in positions 2 and 3

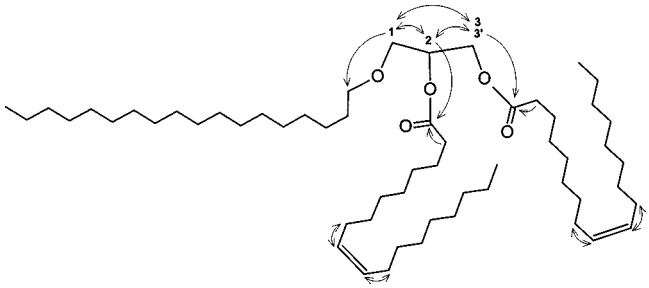

Figure 4. Compound **23** with all of the detected HMBC correlations (H → C).

are monounsaturated. This conclusion is corroborated by the following peaks in the mass spectrum: m/z 873.8 $[M + H]^+$, m/z 890.9 $[M + NH_4]^+$, and m/z 591.5. The latter peak results from the loss of one of the fatty acids in position 3 or 2, yielding a monoacylglyceryl ether ion. The fact that a unique $[M - RCO_2]^+$ ion was found demonstrates that the same fatty acid, an oleic acid chain, is present in these positions. This finding and the value of the molecular weight derived from the mass spectrum led us to conclude that a saturated C18 chain occupies position 1.

To confirm the proposed structure, a transmethylation reaction was performed. To a solution of the pure molecule in 500 μL of CH_3OH was added 20 μL of H_2SO_4. The mixture obtained was placed in a water bath at 80 °C for about 60 min, and then 800 μL of *n*-hexane was added. The two phases were separated. The *n*-hexane fraction was dried under a nitrogen stream and characterized by 1D NMR and GC-MS. The results (not shown) indicate the presence of only methyl oleate.

The presence of this molecule in honey has never been reported in the literature. Therefore, a biosynthetic pathway that might explain its formation is not yet available. Interestingly, this type of molecule has been found only in the muscles and liver of a giant squid and certain species of sharks. Diacylglyceryl ethers are fundamental molecules for these species as a short-term energy resource, and they may also be important in imparting buoyancy regulation.[44]

In conclusion, the work presented here is a significant step toward the characterization of the organic components of unifloral honeys. The compounds described here were selected on the basis of a previous multivariate statistical analysis of the NMR signals of chloroform extracts.[10,11]

The present results clearly show how the use of NMR in the study of complex mixtures, in conjunction with appropriate data analysis, can not only lead to the differentiation of food products of different origin but can extend to the possibility of elucidating the structure of single components in such mixtures. In the case of unifloral honey, the identification of markers of each botanical origin can lead to the formation of a database in which each type of honey can be associated with one or more specific molecule. The present work contributes in this direction, although many unifloral honeys were not considered here, and no consideration regarding concentration of analytes was possible.

Much of this information is already present in the literature, and it could all be put together in a database of markers of botanical origin, which can be used to classify honeys of different sources. Such a database could be used for different purposes. In many cases, the markers identified may be quantified directly using analytical techniques different from the ones used here. This may provide an alternative method to

melissopalinological analysis to control botanical claims and protect consumers. Also, many of the various components are endowed with specific nutraceutical properties and enhance the value of the honey in which they are present. A marker database could be used as a reference for health-related studies to derive reliable information about the efficacy of honey in therapy and prevention.

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Notes

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